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**Grapes, Wines and By-Products as Potential Sources
of Antioxidants**

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3. Abstract

Several grape varieties and red wines was found to contain large concentration of phenolic compounds which work as antioxidant in-vitro and in-vivo. Wastes from wine production contain antioxidants in large amounts, between 2-6% on dry material basis. Red wines but also white wines were found to prevent lipid peroxidation of turkey muscle tissues stored at 5°C. The antioxidant reaction of flavonoids found in red wines against lipid peroxidation were found to depend on the structure of the molecule. Red wine flavonoids containing an orthodihydroxy structure around the B ring were found highly active against LDL and membrane lipid peroxidation. The antioxidant activity of red wine polyphenols were also found to be dependent on the catalyzers used. In the presence of H₂O₂-activated myoglobin, the inhibition efficiency was malvidin 3-glucoside > catechin > malvidin > resveratol. However in the presence of an iron redox cycle catalyzer, the order of effectiveness was resveratol > malvidin 3-glucoside = malvidin > catechin. Differences in protein binding were found to affect antioxidant activity in inhibiting LDL oxidation. A model protein such as BSA, was investigated on the antioxidant activity of phenolic compounds, grape extracts, and red wines in a lecithin-liposome model system. Ferulic acid followed by malvidin and rutin were the most efficient in inhibiting both lipid and protein oxidation.

Catechin, a flavanol found in red-wines in relatively high concentration was found to inhibit myoglobin catalyzed linoleate membrane lipid peroxidation at a relatively very low concentration. This effect was studied by the determination of the by-products generated from linoleate during oxidation.

The study showed that hydroperoxides are catalytically broken down, not to an alcohol but most probably to a non-radical adduct. The ability of wine-phenolics to reduce iron and form complexes with metals were also demonstrated. Low concentration of wine phenolics were found to inhibit lipoxygenase type II activity.

An attempt to understand the bioavailability in humans of anthocyanins from red wine showed that two anthocyanins from red wine were found unchanged in human urine. Other anthocyanins seems to undergo molecular modification. In hypercholesterolemic hamsters, aortic lipid deposition was significantly less in animals fed diets supplemented with either catechin or vitamin E. The rate of LDL accumulation in the carotid arteries was also significantly lower in the catechin and vitamin E animal groups. These results suggested a novel mechanism by which wine phenolics are associated with decreased risk of coronary heart diseases. This study proves in part our hypothesis that the "French Paradox" could be explained by the action of the antioxidant effects of phenolic compounds found at high concentration in red wines. The results of this study argue that it is in the interest of public health to increase the consumption of dietary plant flavonoids. Our results and these from others, show that the consumption of red wine or plant driven polyphenolics can changes the antioxidant tone of animal and human plasma and its isolated components towards oxidative reactions. However, we need more research to better understand bioavailability and the mechanism of how polyphenolics affect health and disease.

4. Objectives of the original research proposal

1. Evaluation of the effectiveness and utilization of crude antioxidant extracts from grapes and by-products in foods.
2. Determination of the specific active components in grapes and assess their structure-potency relationship as antioxidants in food systems.
 - a. Determination of the potential of these compounds as antioxidants in biological systems. These objectives will involve a series of systematic studies viz.
 - b. Quantification of the antioxidant capacity of grapes, fresh and aged wines, grape must and pomace, using a sensitive model system relative to known food grade antioxidants such as tocopherol and BHT.

- c. Characterization of the principal compounds with antioxidant properties to clarify the mechanism of antioxidant action.
- d. Evaluation of the biological activity of these antioxidants from grapes and wine on a number of sensitive biochemical reactions ex-vivo.

5. Introduction

Lipid peroxidation is a major problem in foods and in living systems. Its regulation, control and inhibition in these systems are important for normal food quality and health.

5.1 Lipid peroxidation in food systems

The challenge to the food scientist is to develop food products with maximal storage and shelf life. This can be achieved by preventing microbial spoilage and minimizing oxidation, the former being the most significant pathway of food deterioration.

Oxygen is a vital component of several exergonic reactions which are essential for life. Because oxygen and oxygen activated compounds can be toxic to cells, oxidative reactions are tightly controlled in biological systems. However, under pathophysiological conditions, or with deficiencies of antioxidants or after slaughtering or harvesting of biological food materials, normal control mechanism may be perturbed with resulting generation of active oxygen species and rampant peroxidation may ensue (Kanner et al., 1987; Hsieh and Kinsella, 1989; Auroma and Halliwell, 1991). Oxidative damage can therefore be a consequence of insufficient antioxidant potential or excessive stress. This can cause disease in vivo and reduce the quality and safety of many foods.

Lipid peroxidation is in most instances a free radical chain reaction that can be described in terms of initiation, propagation and termination processes. Unsaturated fatty acids PUFA oxidation by direct interaction with triplet oxygen ($^3\text{O}_2$) is spin-forbidden. This barrier can be overcome by changing the electron spin state of the molecules during reactions with free radicals of high

oxidation potential such as hydroxyl radical ($\text{HO}\bullet$) or perhydroxyl radical ($\text{HO}_2\bullet$). The change of the electron spin state of oxygen from triplet to singlet could also initiate peroxidation of PUFA molecules. We discovered that for H_2O_2 -activated metmyoglobin and methmyoglobin, a ferryl radical, $\text{P}^+-\text{Fe}^{\text{IV}}=\text{O}$, could initiate the oxidation of membranal lipids and several other molecules of biological (Kanner and Harel, 1985). Lipoxygenases and cyclooxygenases in plant and animals catalyze the production of hydroperoxides and bioactive hydroperoxides (Smith and Lands, 1972; Hamberg and Samuelsson, 1974; Kanner et al., 1987; Halliwell and Gutteridge, 1990).

Lipid peroxidation can occur in foods from the handling of the raw material through the processing, storage and distribution to the consumer. Oxidative rancidity is often the decisive factor stored foods, even when the fat content is very low. Decomposition of lipid hydroperoxide creates a wide range of free radicals, carbonyl compounds, hydrocarbons, furans and other materials that contribute to quality deterioration of foods. These compounds affect the flavor, color, texture and nutritive value of many food products (Addis and Park, 1989; Kubow, 1992). Some of these materials are also implicated in biological oxidation (Esterbauer et al., 1988; Frankel, 1991).

The revival of the study of lipid peroxidation in the last 15 years can be attributed in large part to the accumulating evidence that PUFA free radicals and reactive oxygen species participate in tissue injuries and in many diseases (Halliwell, 1987; Max, 1987, Ames, 1993).

5.2 The control of lipid peroxidation by antioxidants

In view of the potential adverse effects of oxygen and its reactive species, it is important that a number of antioxidant defense systems be present in living cells to protect against oxidative damage. Antioxidants are divided mainly into two classes: a) Preventive antioxidants, which reduce the rate of initiation of active oxygen species, such as superoxide dismutase (SOD), catalase and thiol-specific antioxidants glutathione peroxidase and metal ion-binding and storage

proteins which remove or reduce superoxide, hydrogen peroxide and hydroperoxides, respectively; b) Primary antioxidants or chain-breaking antioxidants, such as α -tocopherol and flavonoids, and other synthetic phenolic compounds (BHA, BHT, TBHQ). Such antioxidants are complemented in vivo and in vitro by ascorbic acid (Tappel, 1980; Kanner et al., 1987; Loliger, 1991).

5.3 Primary antioxidants in foods

α -Tocopherol (vitamin E) is a fat soluble molecule that concentrates in the hydrophobic interior of biological membranes. Burton et al. (1983) found that α -tocopherol interacts with the peroxy radical in apolar solvents at a rate constantly higher than BHT. However in water emulsion and in foods its antioxidative effects were found to be lower than synthetic antioxidants (Porter, 1980). α -Tocopherol is found in high concentration in several vegetable oils and in paprika fruits (Kanner et al., 1979). Observation that foods prepared with various spices and herbs for flavor enhancement seem to have longer keeping time led to examination of these and the extracts as antioxidants for foods (Dugan, 1980). Chipault et al. (1956) evaluated the antioxidant properties of many plants and found that rosemary and sage were the most effective extracts. Similar results were published by others (Chang et al., 1977). More recently we found several sage varieties with significant higher antioxidative activities than rosemary (Kanner et al., 1990; Atzmon, 1990). A food antioxidant extract from rosemary is in the market (Loliger, 1991). Antioxidants are also found in various vegetables (Pratt, 1965), soybeans (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).

Typical classes of antioxidants are derivatives of flavonoids, catechins phenolic acids, tocopherols and polyfunctional organic acids (Dugan, 1980). Many flavonoids are strong free radical scavengers and metal chelators (Letan, 1966

a,b; Hudson and Lewis, 1983; Bors and Saran, 1987; Frankel, 1989; Yuting et al., 1990; Liu et al., 1992). This area was recently reviewed by Schuler (1990), Pratt and Hudson (1990), Namiki (1990) Loliger (1991) and Bland (1995).

5.4 Lipid peroxidation in vivo

Under physiological conditions, endogenous oxidants are produced at a relatively high rate, resulting in oxidative damage to DNA, proteins and lipids. The damage produced by endogenously generated oxygen radical may be major factors contributing to aging, cancer, heart diseases and atherosclerosis (Ames, 1983; MacGregor et al., 1985; Halliwell, 1987; Fraga et al., 1991; Lijinsky, 1991; Steinberg et al., 1992, Heineke, 1997).

5.5 Eicosanoids, cyclooxygenase (CO) and lipoxygenase (LO)

Polyunsaturated fatty acids located in membranes are precursors to signal molecules termed eicosanoids. Fatty acids are released following cellular stress and are oxidized enzymatically by free radical mechanisms (Kanner et al., 1987). The enzymatic products are used as signal molecules for coordinated cellular responses to specific stress states (Kineslla and Lokesh, 1990). The concentration of these oxidized lipids is critical to their signaling excess or unbalanced severation of lipid peroxidation which can promote a variety of diseased including atheroscelrosis (Steinberg et al., 1989; Yla et al., 1990), thrombosis (Huang et al., 1988; Nicosia and Patrono, 1989), cancer (Danon et al., 1986) and immune dysfunction (Klasing, 1988; Nicosia and Patrono, 1989). The ability of antioxidants to modulate the eicosanoids to a less peroxidized balance is predicted to reduce coronary heart disease mortality and cancer. This argues for research into antioxidants from plant sources as modulators of cellular signaling and reduction in many of the common chronic diseases (Jang et al., 1997).

Lekotrienes, products of lipoxygenase action on arachidonic acid are signal compounds for the immune system. Again, while balanced production is

necessary for successful recruitment and activation of immune cells to the site of infection, unbalanced or excessive production of these compounds promotes inflammation and autoimmune diseases. Antioxidants reduce 5 lipoxygenase activity (Chan et al., 1989) and have been proposed to attenuate their over-reaction in inflammatory tissues (Atroschi et al., 1989). Lipoxygenase together with phospholipase A2 was found to oxidize LDL in vitro (Sparow et al., 1988; Parthasarathy et al., 1989).

Both 15-lipoxygenase mRNA and protein have been detected in human atherosclerotic lesions (Yia-Harttuala et al., 1987), raising the possibility that the enzyme promotes LDL oxidation in-vivo. When LDL is first incubated with lipoxygenase and the oxygenated products in the lipid extract are then subjected to analysis by chiral phase high-pressure liquid chromatography, the stereospecific isomer 13 hydroxy 9,11 octadecadienoic acid is the major product generated. This stereospecific isomer was found in lipids extracted from human atherosclerotic lesions, (Folcik et al., 1996).

5.6 Coronary heart disease

The prevailing opinion, that atherosclerosis is simply an accumulation of cholesterol in arteries, has been shown to be erroneous. Therefore, this hypothesis has become less accepted and has been replaced by two competing hypotheses, i.e. a) response to injury (Ross, 1986); b) dietary lipid peroxidation products (Smith and Johnson, 1989; Addis and Park, 1989; Addis and Warner, 1991) which includes in vivo (LDL) oxidation and modification of low-density lipoproteins (LDL) (Steinberg et al., 1989; Esterbauer et al., 1990). Both hypotheses deal in terms of initiation of oxidative processes, which may be regulated and protected by antioxidants (Wilson, 1976; Addis and Park, 1989; Parthasarathy et al., 1990; Warner, 1990). Circulating LDL are one of the fundamental targets of deleterious oxidation in vivo causing the accumulation of atherogenic lipoproteins in vivo. This is a critical assay for the biological

value of an endogenous antioxidant in protecting the LDL from oxidation (Steinberg et al., 1992, Xu et al., 1998).

We have developed several assays for evaluating the time course of human LDL oxidation and the potency of antioxidants in preventing this oxidation (Frankel et al., 1993; Kanner et al., 1994). It is interesting to note the inverse relation of plasma levels of antioxidant vitamins, to the incidence of ischemic heart disease (Gey et al., 1987). The so-called French Paradox indicate that some regions of France have lower heart disease rate, despite consumption of fat at comparable levels to the U.S. which are correlated to increased risk of heart attack (Renaud and De Lorgeril, 1992). Red wine may protect the French from heart attacks via inhibition of LDL oxidation and inhibition of platelet aggregation (Frankel et al., 1993; Kinsella et al., 1993; Kanner et al., 1994).

5.7 Primary antioxidants in vivo and ex vivo

Cell membranes and plasma lipoproteins contain α -tocopherol which is the most effective, and it appears to be the most important lipid-soluble chain breaking antioxidant in vivo in humans (Tappel, 1982; Burton et al., 1983; McCay, 1985; Esterbauer et al., 1989). Low plasma concentration of α -tocopherol and vitamin C seem to correlate with an increased incidence of myocardial infarction (Gey et al., 1987), perhaps because of the important role played by lipid and LDL peroxidation in atherosclerosis (Steinberg et al., 1992). Most recently ascorbic acid was found to have a protective effect against endogenous oxidative DNA damage in human sperm in vivo (Fraga et al., 1991).

Because of the important biological effects of the products of lipoxygenase and cyclooxygenase, there has been considerable interest in the effects of antioxidants on these enzymes. α -Tocopherol was found to affect the activity of both enzymes in vivo (Carpenter, 1981). Other experiments also show such an effect in vitro (Reddanna et al., 1985).

Another protective factor not yet explored, might be the intake of plant antioxidants from food material such as phenolic acid derivatives and flavonoids. These plant compounds are regularly consumed by humans through the diet containing fruits and vegetables. Flavonoids and other plant phenolics have been reported to have anti-inflammatory, anti-allergic, antiviral activities (Hausteen, 1983; Middleton, 1984), inhibiting tumor promotion (Xiao et al., 1988; Hirano et al., 1989). Landolfi et al., (1984) reported that bioflavonoids from plant sources can modify platelet arachidonic acid metabolism. They demonstrated anti-aggregatory activity of flavone, chrysin, apigenin and phloretin in blood platelet systems. Van-Wauwe and Gossenc (1983) showed that these were effective in controlling CO and LO products in arthritic tissue (i.e. anti-inflammatory action). Many phenolic compounds found in plants, especially flavonoids are powerful inhibitors of both lipoxygenase and cyclooxygenase in vitro and ex vivo (Lands and Hanel, 1982; Moroney et al., 1988; Hsieh et al., 1988). In a recent study, some compounds were found to be selective as LO inhibitor, some to CO, some inhibit both and some were poorly active against either enzyme (Laughton et al., 1991). These selectivities suggest that different structure-dependent mechanisms determine the two enzymes by the flavonoids and promoted further studies.

Dried herbs of some *Artemisia* species have been used for the treatment of inflammation, and blood diseases in traditional Asian medicine. "Caffetanins" have been found in those plants which are antioxidants and also very active inhibitors of thromboxanes from activated platelets. Several coumarins were potent inhibitors of platelet lipoxygenase (Kimura et al., 1985).

The peroxidizability of LDL depends upon fatty acid composition and on the presence of chain-breaking antioxidants. The most important of these is α -tocopherol but lycopene and β -carotene are also effective (Esterbauer et al., 1989). Much interest was raised when it was found that a cholesterol-lowering drug, probucol, also had an antiatherogenic effect in hyperlipidemic rabbits which was greater than would have been expected from its cholesterol-lowering

action (Carew et al., 1987). Carew et al. (1987) suggested that inhibition of lipid peroxidation is the major mechanism of action of probucol. Probucol is indeed a powerful chain breaking inhibitor of lipid peroxidation (Auroma et al., 1991). Several flavonoids inhibit LDL oxidation in-vitro (DeWhalley et al., 1990; Mangiopane et al., 1992; Negre-Salvayre and Salvayre, 1992). However, the potential use of phenolic acid derivatives and flavonoids in human is highly dependent on their metabolism in vivo. Several of these compounds such as (+)-catechin, BHT, BHA and probucol, which is closely related to BHT, were found to be absorbed and transported by the bloodstream (Das, 1971; Griffith, 1982; Middleton, 1984; Carew et al., 1987). Most recently other studies demonstrated absorption of several flavonoids in animal and human (Ameer et al., 1996; Cova et al., 1992; Fuhr et al., 1995; Hollman et al., 1995; Nieder et al., 1991; Paganga et al., 1997; King et al., 1998; Manach et al., 1998). However, direct evidence does not exist regarding the absorption of wine flavonoids in human. Several indirect data about the possible in vivo effects of red wine and grapes have been published (Kondo et al., 1994; Maxwell et al., 1994; Serafini et al., 1994; Fuhrman et al., 1995; Whitehead et al., 1995; Hayek et al., 1997; Day et al., 1997; Demrow et al., 1995; Nigdikar et al., 1998).

5.8 Phenolic compounds in grapes and wine

The grape berry has three main parts - skin, pulp and seeds - in which polyphenols concentration may be very different. Most of them are located in the skin, seeds and conductive tissues. The amount of these compounds are dependent mostly on the variety but also on light irradiation, temperature and horticultural treatments (Singleton, 1982; Macheix, 1991). The amount of phenolics in wine depends on the cultivar, the extraction and wine making techniques and finally on numerous chemical reactions during aging. Grapes and especially red varieties contain relatively large amounts of phenolic compounds, including catechins, flavanols, anthocyanins and procyanidins. Much work has been carried out on the study of phenolics in grapes and wine

and a very complete review was published by Singleton and Esau (1969) and Macheix et al., (1991). The total phenolics in red wine attain a level of 1000-1800 mg/ml, mostly flavonoids, while white wine contains only 150-300 mg/l. Besides the extensive work on antioxidants from many plants, the possible antioxidant effects of grape phenolic compounds, has not been systematically studied (Brasseur et al., 1986). (Further new information can be find in our publications).

6. Materials and Methods

The materials and methods used in this project are described in publications attached to this report.

Several of the methods used but not published are presented bellow:

6.1 Total phenolics

The concentration of wine phenolics was estimated by analyzing for total phenol by the Folin-Ciocalteu and expressed in methods described by us (Kanner et al, 1994) mg/L or molar equivalent (μM^*) of catechin (MW 290.3) or gallic acid (MW 188.1), a naturally occurring polyphenol in wines.

6.2 Fractionation of phenolic compounds from red and white wines

Two samples of wine, Petite Sirah (red) and Chardonnay (white) were used for fractionation of the wine phenolics. The method employed was developed by Oszmianski et al (Am. J. Enol. Vitic. 39, 259, 1988) using C_{18} sep-pak cartridges (Merck).

Fraction 1 - (eluted by methanol) contained phenolic acids such as caffeoyl tartarate and coumaroyl tartarate.

Fraction 2 - (eluted by aqueous pH 2) contained caffeic acid and gallic acid.

Fraction 3 - (eluted by 16% acetonitrile pH 2) contained mainly procyanidin B_1 , catechin, procyanidin B_2 , epicatechin and anthocyanin monoglucosides.

Fraction 4 - (eluted by ethyl acetate) contained flavonols such quercetin and quercetin monoglucoside.

Fraction 5 - (eluted by methanol) contained polymeric anthocyanins and procyanidins.

6.3 HPLC separation of phenolic compounds from wines

Red (Petite Sirah) and white (Chardonnay) wine were separated by HPLC. In order to ensure a good separation, the method of Lammela-Raventos and Waterhouse (Am. J. Enol. Vitic. 45, 1-5, 1994) was employed. More information of the separation was also described in our report, Lapidot et al (1998).

6.4 Lipid peroxidation

Several methods were employed see -

Kanner et al, 1994

Frankel et al, 1995

Lapidot et al, 1998a

Teissedre et al, 1996

Lapidot et al, 1998b

Yi et al, 1997

Teresa Satue-Gracia et al, 1997

Lipoxygenase was determined as described previously by Kanner et al (1991).

Microsomal lipid peroxidation and inhibition was evaluated as described previously by Kanner et al (1985).

6.5 HPLC analysis of the products of linoleic acid oxidation by lipoxigenase (Soybean) and myoglobin.

The products of the reaction between soybean lipoxigenase and myoglobin with linoleic acid were formed in our model system at pH 7.0. The reaction mixture after 5 min incubation at 25°C was acidified to pH 2.0 with dilute HCl and extracted twice with 25 ml of diethyl ether. The extract was dried with sodium sulfate, evaporated under vacuum, and redissolved in 3 ml of hexane for HPLC. Hydroxy linoleic acid compounds were prepared by reduction of the hydroperoxides with 100 mg sodium borohydride for 3 min at room

temperature prior to acidification of the reaction mixture. HPLC analysis was carried out according to Wu and Robinson (1995).

The HPLC system used (LC-10A, Shimadzu, Germany) consisted of an auto injector, a photodiode array detector (SPΔ-M 10 Arp) and a software system which controlled all the equipment and carried out data integration and processing (Class-vP, Shimadzu). The injection volume was 20 µl using a normal phase Eurosphere Si column (250 x 4.6) and eluted at 0.7 ml/min with hexane/2 - propanol/ acetic acid (98:2:0.05 v/v). The fraction were detected at 235 nm for hydroperoxides and hydroxy compounds and at 270 nm for the keto compounds.

6.6 Hydroxyl radical generation

Hydroxyl radical generation was determined by benzoate hydroxylation. The addition of hydroxyl radicals to benzoate at pH 7.0, produces the 2-monohydroxy benzoate, which was separated by HPLC from RP-18 column by 0.05 µ acetate buffer, pH 4.5 and acetonitrile (v/v 8:2) and detected fluorometrically using excitation at 300 nm and emission at 410 nm. The amount of monohydroxy benzoate produced was calculated from a salicylate standard curve, (Kanner et al, 1991).

6.7 Superoxide Anion Scavenging activity

Scavenging of superoxide anion was determined using a method described by Fridovich (1985). In principle $O_2^{\cdot -}$ is generated by xanthine oxidase at a rate of 1-1.5 µM $O_2^{\cdot -}$ / min. Cytochrome C, 10 µM, is thus reduced by $\Delta A_{550nm}/min = 0.025$. The same amount of malvidin bleached at a rate of 0.01 A 580 nm/min,. The bleaching was inhibited by 0.1 nM of SOD.

7. Results

7.1 Cultivar evaluation for antioxidants

Our previous paper (Kanner et al, 1994) showed that a good correlation was obtained between total phenol determined by the Folin-Ciocalteu method and

antioxidant activity as determined by linolate oxidation catalyzed by myoglobin (Fig. 26). Such a correlation was also found by Frankel group (Frankel et al, 1995; Meyer et al, 1997; Yi et al, 1997 and Frankel et al, 1998) at Davis for the presence of phenolic compounds expressed as gallic acid equivalents (mg/L) and antioxidant activity, determined in systems containing LDL or liposomes oxidized by Cu ions.

Several red and white wines produced in Israel were selected for determination of antioxidant activity (Table 1). The total phenolic content of the white wines ranged from 229 to 242 mg/L as catechin equivalents (CE). The concentration of the phenolic compounds in the red wines ranges between 1480 to 4700 mg/L CE. Californian wines evaluated for antioxidants properties showed that the phenolic compounds in white wines ranged from 165 to 331 mg/L and those of red wines from 1800 mg/L to 4059 μ g/L (Frankel et al, 1995). The phenolic compounds are concentrated within the skin and large amounts remain in the precipitate after fermentation. The grape wastes contained 19 to 64 g/kg CE (Table 2) on a dry weight basis. It is possible to extract the flavonoids and flavonols more quickly from the seeds by crushing the seeds before extraction (Meyer et al, 1997; Yi et al, 1997), however this process did increase the antioxidant activities significantly (Yi et al, 1997).

7.2 Evaluation of antioxidants in crude extracts

Five varieties of red wine grapes were tested for their phenolic content and antioxidant activities in two systems: lecithin liposomes and microsomal membranes.

The content of the phenolic compounds (mg/kg) of raw grapes varied from 1490 mg/kg (Grenash) to 4300-4700 mg/kg (Argaman). The last variety is a new Israeli variety containing large amount of anthocyanins (Table 3). The same varieties were tested for phenolic compounds content and activities found in the peel. The phenolic content in the peel of these varieties is noted in Table 4. The lowest amounts were found in Grenash (2,100 mg/kg peel) and the

highest amount in the Argaman (11,083 mg/kg peel). A similar determination was performed in Davis, California with selected grape varieties (Meyer et al, 1997; Yi et al, 1997).

Fourteen varieties of table grapes and wine grapes grown in the fields of the University of California at Davis were extracted with 60% aqueous-methanol and the extracts were tested for their potency as natural antioxidants in the lecithin liposome and the LDL oxidation systems. The grape extracts were analyzed for their phenolic components by HPLC. The total phenolic contents of the grape extracts varied from 176 to 1236 mg/L gallic acid equivalents (GAE). Extracts of red wine grape varieties contained higher concentrations of phenolics than other varieties. HPLC analyses showed that anthocyanins were the most abundant phenolic compounds in extracts of red grapes and flavonols were most abundant in extracts of green grapes.

7.3 Separation of phenolic compounds from red and white wines by HPLC

Red (Petite Sirah) and white wines (Chardonnay) were Separated by HPLC. In order to ensure a good separation, the method of Lammela-Raventos and Waterhouse (1994) was employed.

The compounds were detected by UV-visible spectra of each chromatographic peak at 280 and 520 and nm (Figure 1,2). Standard compounds were injected for retention time with: gallic acid, caffeic acid, catechin coumaric acid, expicatechin, quercetin, rutin and malvidin-3-glucoside. Figure 1 shows the separation of Petite Sirah and Figure 2 the separation of chardonnay (Attenuated x 3). In Chardonnay there were gallic acid, trans caffeoyl-tartarate, cis and trans coumaoryl tartarate, catechin, procyanidin B₂,epicatechin and a flavonol glucoside. The compounds determined in red-wine are given in Table 5.

7.4 Fractionation and antioxidant activity of phenolic compounds from red and white wine

Two samples of wines, Petite Sirah (red) and Chardonnay (white) were used for fractionation into 5 fractions (Table 6). The method of fractionation employed was that developed by Oszmianski et al. (Am. J. Enol. Vitic. 39, 259, 1988) using C18 Sep-pak Cartridges (Merck).

Fraction 1 - (eluted by methanol) contained phenolic acids: cis-trans caffeoyl tartrate, cis p-coumaroyl tartrate.

Fraction 2 - (eluted by aqueous pH 2) contained mainly caffeic and gallic acid.

Fraction 3 - (eluted by 16% acetonitrile pH 2) contained mainly procyanidin B₁, catechin, procyanidin B₂, epicatechin and anthocyanin monoglucosides.

Fraction 4 - (eluted by ethyl acetate) contained flavonols such quercetin and quercetin monoglucoside.

Fraction 5 - (eluted by methanol) contained mostly polymeric anthocyanins and procyanidins.

Table 6 shows the amount of phenolics in each fraction and their antioxidative effects. The amount of total phenolics determined in the red wine was almost 8.5 higher than those in white wine. But practically the same ratio was found between the total antioxidative activity of red and white wine.

The antioxidant unit index shows that the red wine was relatively more active than the white wine, and contained almost 10 times more total antioxidants.

The most active fractions in red wine were 2, 4 and 5. However, the most antioxidant units was found in fraction 3.

The antioxidant activities of 20 California wines which inhibited the oxidation of human LDL in vitro were determined (Frankel et al., 1995). This antioxidant activity was related to the principal phenolic compounds analyzed by HPLC and GC-MS. Based on total phenol contents the relative inhibition of LDL oxidation varied from 46 to 100% with red wines and from 3 to 6% with white wines. Correlation of antioxidant activity with the level of phenolic components decreased in the order: gallic acid, catechin, myricetin, quercetin, caffeic acid, rutin, epicatechin, cyanidin, and malvidin-3-glucoside.

Antioxidant evaluations in the liposome systems with a 20 μ M GAE concentration of total phenols (Yi et al., 1997) showed that the grape extracts inhibited formation of conjugated diene hydroperoxides by 25 to 68% and hexanal formation by 49% to 98%. Extracts of greenish red table grape varieties Red Globe and Emperor, and white wine grape varieties Chardonnay and Sauvignon Blanc gave the highest antioxidant activities. The relative percent inhibition of conjugated dienes and hexanal correlated with total phenols. On this basis the red grape were found to varieties have more antioxidant activity than the green varieties.

Antioxidant evaluations in the human LDL system (Meyer et al., 1997) showed that at 10 μ M GAE concentration, the grape extracts inhibited LDL oxidation by 22% to 60% and by 62% to 91% at 20 μ M GAE concentration. The antioxidant activities of grape extracts against LDL oxidation were comparable to those found for wines. The relative LDL antioxidant activity correlated with the concentrations of total phenols, anthocyanins and flavonols. Extracts from crushed seeds contained higher levels of flavan-3-ols and showed high correlations with antioxidant activities with flavan-3-ols and hydroxybenzoates. The antioxidant activity of commercial grape juices was also evaluated with our LDL oxidation assay (Frankel et al., 1998). When standardized to a total phenolic concentration of 10 μ M GAE, samples of commercial grape juices inhibited LDL oxidation from 62 to 75%. White grape juices inhibited LDL oxidation by 72% on the average, Concord purple grape juice by 67%, and grape juice blends (mixture of white and Concord grape juice) by 63%. Vitamin C had no significant effect on the antioxidant activity of the grape juices tested. The antioxidant activity of Concord juice samples was related to their anthocyanin levels, while that of the white grape juices was related to their levels of flavan-3-ols and hydroxycinnamates, as determined by HPLC. Based on the same total phenolic concentration, the antioxidant activity of grape juices toward LDL oxidation was comparable to that of several California red wine. However, based on their undiluted total phenolic concentration, the

Concord and blends of grape juices had activities comparable to that of the red wines, while the white grape juices were less active.

7.5 Anthocyanins, separation, identification and activity

The extracts of 6 different red wine grapes were also tested by HPLC for their anthocyanin content. In the varieties, the anthocyanins were separated into 13 different peaks except Pinot-Noir. These peaks were identified as anthocyanidin-3-glucosides described in table 7. The differences in pigment amounts between the varieties determined by HPLC and detected at 530 nm are described in table 8 and fig 3. The lowest amount of anthocyanins were found in Grenash and the highest in Argaman, which contained almost 10 fold more.

In order to make comparison between the amounts of anthocyanins and other phenolic compounds, the polyphenolic compounds were separated by HPLC and detected at 280 nm (Fig. 4). The extinction coefficient of most of the phenolics at 280 nm is the same.

The results are presented in table 9. From the results it is possible to differentiate three groups of varieties - Group I, Pinot-Noir and Grenash in which the ratio of the phenols to the anthocyanins was about 2 (50%). Group II, Merlot and Carignan in which the ratio was 1.4 (~ 70% anthocyanins) and Group III Argaman and Cab. Sauvignon in which the ratio was 1.2-1.1 (~ 85% anthocyanins).

High concentrations of anthocyanins in red-grape wine varieties were found also at Davis (Mayer et al, 1997; Yi et al, 1997). The amount found at Davis was about 98-80% for all the phenolic compounds. Commercial grape juices contained only 50-60%.

However, in red wine its concentration is ~ 10%-20% for all the phenolic compounds, and Frankel et al (1995).

As anthocyanins are one of the main classes of flavonoids in red wines, they appear to contribute significantly to their powerful antioxidant properties. However, during digestion they may reach higher pH's, forming the carbinol

pseudo-base, quinoidal-base or the chalcone. We evaluated the antioxidant activity of these compounds in several metals system and compared it with other antioxidants, such catechin or resveratrol.

The pseudo-base and quinoidal-base malvidin-3 glucoside significantly inhibited the peroxidation of linoleate by myoglobin. Both compounds were found to work better than catechin, a well known antioxidant. In a membrane lipid peroxidation system, the effectiveness of the antioxidant was dependent on the cattle. In the presence of H_2O_2 activated myoglobin, the inhibition efficiency of the antioxidant was: malvidin-3-glucoside > catechin > malvidin > resveratrol. However, in the presence of an iron redox cycle catalyzer the order of effectiveness was: resveratrol > malvidin-3-glucoside = malvidin > catechin. The pH-transformed forms of the anthocyanins remained as an effective antioxidant in these systems, and their I_{50} 's were between 0.5 and 6.2 μM (Lapidot et al, 1998).

In other related research, the antioxidant activity of several pure anthocyanins was tested in vitro on both the human LDL oxidation assay and on the lecithin liposome system (Saute'-Gracia et al., 1997). In the LDL system, the effect of copper catalyst concentration influenced the trends of antioxidant activity. When LDL oxidation was catalyzed with 10 μM copper, the antioxidant activity of anthocyanins decreased in the order: malvidin, delphinidin, cyanidin, and pelargonin. When LDL oxidation was catalyzed with 80 μM copper, the activity decreased in the order: delphinidin, cyanidin, malvidin and pelargonin. In the liposome system oxidized with 3 μM copper, delphinidin were prooxidants. We showed that the antioxidant activity of anthocyanins and other phenolic compounds is greatly affected by the system used as substrate and the conditions used to catalyze oxidation.

7.6 Antioxidant mode of action

Evaluation of several flavonoids found in grapes and wines as antioxidants

Several phenolic compounds which represent the four classes found in wine are known antioxidants synthesized by plants. These compounds are: caffeic acid (phenolic acid), catechin (flavanol), quercetin (flavonol), malvidin (anthocyanin) and α -tocopherol, (a natural antioxidants). Linoleate oxidation by myoglobin oxidation were determined by measuring the accumulation of conjugated dienes at 234 nm and β -carotene oxidation at 460 nm.

The results from both methods showed that catechin was the most active antioxidant among the phenolic compounds analyzed.

The other antioxidants were effective in the decreasing order, catechin > caffeic acid > quercetin > malvidin > α -tocopherol, Fig. 5,6.

The index of antioxidant units (ANU) were: Catechin = 1; caffeic acid = 0.6; quercetin = 0.23; malvidin = 0.1 and α -tocopherol = 0.08.

Employing the above index the order of prevention of the β -carotene system, catalyzed by ferrous iron (Fe-ascorbic acid), the index of antioxidant units calculated were: catechin = 1; caffeic acid = 1.0 and malvidin = 0.22 (Fig. 7).

It should be noted that some of the antioxidants tested by the group in Israel were also tested by a different method at Davis using LDL oxidation by Cu^{++} as an index of oxidation.

The antioxidant activities were grouped almost in the same order, however, the activities of the antioxidants were totally lower than those found by in-vitro methods.

The influence of chemical structure on antioxidant activity of five phenolic compounds having a similar ortho-dihydroxy moiety was evaluated by in-vitro LDL oxidation assay system (Meyer et al., 1998a). The order of antioxidant activity was catechin > cyanidin $\approx$ caffeic acid > quercetin > ellagic acid. The antioxidant activities were tested statistically on different combinations of two or three phenolic compounds. All the antioxidant effects were additive except for the combinations that included ellagic acid with catechin, where ellagic acid exerted an antagonistic antioxidant effect. This antagonistic interaction may be

due to hydrogen bonding between carbonyls in ellagic acid and ortho-dihydroxyl groups in catechin.

The antioxidant activity towards LDL oxidation of para-coumaric, ferulic, caffeic and their hydroxycinnamoyl tartaric and quinic acid derivatives, typically present in fruits, were also investigated (Meyer et al., 1998b). The ortho-dihydroxy compounds caffeic, caftaric, chlorogenic and neochlorogenic acids had the highest antioxidant activities and inhibited LDL oxidation from 86-97% at μM . Ferulic and fertaric acid had lower antioxidant activity and thus primarily related to the hydroxylation and methylation pattern. The esterification to tartaric acid slightly enhanced the antioxidant activity of para-coumaric and ferulic acid, but esterification of caffeic acid (as in chlorogenic and neochlorogenic acid) had no effect or slightly decreased antioxidant activity. It is suggested that for the less active hydroxycinnamic acids para-coumaric and ferulic acid esterification to tartaric acid may enhance their ability to sterically block initiation of LDL oxidation via increased binding to apolipoprotein B in the LDL molecule.

7.7 Antioxidant mode of action of wine-flavonoids

The mode of action of many antioxidants depends on their capability to act as electron donors, electron acceptors, decomposers of peroxide and hydroperoxides, metal activators and deactivators and UV absorbers. Some of these activities were determined by us with several phenolic compounds that represent a part of the flavonoids found in red wines.

Catechin, a flavonoid found in red-wines in relatively high concentration was found by us to inhibit myoglobin catalyzed linoleate or membrane lipid peroxidation in relatively very low concentration, (0.25-0.5 μM). We have conducted a study in order to better understand the antioxidant activity of catechin in a system catalyzed by myoglobin. Figure 8 shows that the lag time induced by catechin in prevention of β -carotene oxidation in the system of linoleate peroxidation increases with the addition of catechin. However after

an addition of a critical amount, the lag time increased 30 fold more than expected. This effect was studied by evaluation of the by-products generated from linoleate with hemoglobin alone and in the presence of catechin. In order to evaluate some of the oxidized lipid peroxidized compounds, linoleic acid was oxidized by lipoxygenase Type I and the well known by-products were separated by HPLC, and used as standards. The hydroperoxides (Fig. 9) were reduced by NaBH_4 to obtain the reduced hydroxy compounds (Fig. 10). HPLC separation produced 10 major compounds identified as, ketones, hydroperoxides and hydroxy fatty acids as shown in table 10.

Myoglobin enhanced lipid peroxidation and the formation of 13-cis-trans hydroperoxide, 13 cis-cis hydroperoxide, 9 cis-trans hydroperoxide, 13 cis-cis hydroxy of linoleic acid. In the presence of 10 μM of catechin the level of 13 cis-trans hydroperoxide and 9 cis-trans hydroperoxide decreased significantly below the level of the control (Fig. 11).

The level of oxidized ketocompounds remained low, similar to the control (Fig. 12). The results demonstrated, not only inhibition of hydroperoxide accumulation but a decrease of those compounds, which indicated that the coupled - myoglobin-catechin decomposes hydroperoxides. Our results also showed that the couple reaction did not produce significant amounts of hydroxy compounds.

7.8 Metal chelation by wine flavonoids

The capability of polyphenolic compounds to donate electrons was demonstrated by their reaction with cytochrome-C, metmyoglobin or ferric iron (Figs. 13-15) and with some of the metal flavonoids by chelation and complexation. It was found for example that catechin could chelate iron at a ratio of six molecules of the flavonoid to 1 atom of iron (Fig 16). The interaction could lead to reduction of the metal which could interact with oxygen producing a "Fenton" reaction which generate hydroxyl radicals. Such a reaction was demonstrated when ferric ions were incubated at pH 7.0 with

ascorbic acid, myricetin, quercetin or pseudo-base malvidin-3-glucoside but not with catechin, or quinoidal-base malvidin-3-glucoside (Figs. 17-18).

Malvidin and delphinidin were tested as copper chelators at acidic or alkali pH. At pH 10, the addition of copper to delphinidin solution produced an immediate shift to a higher wavelength (maximum from 532 to 586) and a change in the shape of the spectrum (Satue-Gracia et al. 1997). Malvidin solution only showed a decrease in the absorbance due to its apparent oxidation at high pH. Delphinidin and other anthocyanins except malvidin were found to act as prooxidants and antioxidants. This activity was very much affected by the concentration of the metal, or the ratio of the metal to flavonoid.

7.9 Scavenging of superoxide by anthocyanins

Most recently it was demonstrated that cyanidin, an anthocyanin, has the capability of scavenging O_2^- at a high rate. We determined the scavenging effect on O_2^- by qb-malvidin and found it to be half of that of cyanidin (Fig.19).

7.10 The antioxidant potency of ortho-dihydroxy flavonoids

Flavonoids with catechol like B ring structure were found to be highly active antioxidants against membrane lipid peroxidation. The influence of the catechol like structure on the antioxidant activity against LDL oxidation by catechin, quercetin, cyanidin, caffeic acid and ellagic acid was evaluated. The order of antioxidant activity was catechin > cyanidin > caffeic acid > quercetin > ellagic acid (Mayer et al 1998).

7.11 Complexation of flavonoids by proteins and solubility

Our research on the structural effects of polyphenolic compounds demonstrated that the ranking of their antioxidant activity is strongly dependent on the test system and on the substrate to be protected by the antioxidant. The differences in antioxidant activities that we observed can also be ascribed to other factors, including differences in solubilities and partitioning between the aqueous and

lipid phases in the LDL system. We have previously shown that the physicochemical properties of antioxidants affect their antioxidant efficacy in complex, multiphase system (Frankel et al., 1994). We also suggested that structural features conferring differences in protein binding may affect the antioxidant activity of flavonoids in inhibiting oxidation of LDL (Teissedre et al., 1996).

To verify possible interactions between flavonoids and proteins, the effect of a model protein such as bovine serum albumin (BSA) was investigated on the antioxidant activity of phenolic compounds, grape extracts, and red wines in the lecithin-liposome oxidation system (Heinonen et al., 1998). Lipid oxidation was measured by the formation of conjugated diene hydroperoxides, and the production of hexanal. Protein oxidation was measured fluorometrically by the decrease of tryptophan and lysine emission and the formation of carbonyl-protein fluorescence. In the absence of BSA, the antioxidant activity of phenolic compounds (5 μ M), on the basis of hydroperoxide formation, decreased in the order, ferulic acid, epicatechin, catechin, rutin, malvidin, caffeic acid, quercetin and propyl gallate; and on the basis of hexanal formation, decreased in the order, ferulic acid, epicatechin, catechin, malvidin, caffeic acid, quercetin, rutin and propyl gallate. Gallic acid and delphinidin were prooxidants on the basis of both hydroxyperoxide and hexanal formation. In the presence of 20% BSA, the liposome oxidation was much slower. Ferulic acid followed by malvidin and rutin were the most efficient in inhibiting both lipid and protein oxidation. Two grape extracts and two red wines inhibited formation of both hydroperoxides and hexanal more efficiently in the absence of BSA. In the presence of BSA, the two red wines were more active antioxidants than the grape extracts in inhibiting lipid oxidation, but they showed no difference in inhibiting protein carbonyls. The relative antioxidant potency of phenolic compounds in liposomes is mediated by the presence of protein.

7.12 Determination of the potential of phenolic compounds in wine as antioxidants in biological systems

A good dietary antioxidant is not only one of low redox potential or high free radical scavenging properties, but one of high bioavailability.

Anthocyanins are one of the main class of flavonoids in red-wines and they appear to significantly contribute powerful antioxidant properties to them. The bioavailability of red wine anthocyanins as detected in human urine, was determined (Lapidot et al., 1998).

7.13 Lipoxygenase: Lipoxygenase in animal utilize arachidonic acid, the same substrate as for cyclooxygenase, to generate a single stereospecific hydroperoxide as the primary product. In plants lipoxygenase could oxidize an unsaturated fatty acid with a 1-4 pentadiene structure. Soybean 1 lipoxygenase converted linoleic acid to the 13- hydroperoxide formation (German, 1994).

It was found by us that lipoxygenase reactivity is stimulated by low concentration of wine phenolics and inhibited at high concentration. Such effects were found for the activation and inhibition of cyclooxygenase. In addition we found that low concentration which does not inhibit conjugated diene accumulation, inhibit significantly β -carotene oxidation in a couple model system of β -carotene and linoleate (Kanner, 1994, unpublished) (Fig. 20). German (1996) showed the same results with 12-lipoxygenase.

7.14 Hamster model system: on a hypercholesterolemic diet was used to test dietary antioxidants for their possible intervention in the development of atherosclerosis (Xu et al., 1998; Rein et al., 1998). Three groups of hamsters were fed for 30 weeks a hypercholesterolemic diet that contained either 3 IU vitamin E (low E), 3 IU vitamin E and 200 mg catechin (low E + cat) or 30 IU vitamin E (high E) per kg body weight. Fatty streaks were measured by staining aorta arch sections with oil red O and quantified by a photomicroscopy color scanning technique. Aortic lipid deposition was significantly less in animals fed

diets supplemented with either catechin or vitamin E than those fed a basal low antioxidant-containing diet.

We also examined the effect of dietary vitamin E and catechin on the accumulation of fluorescently-labeled LDL in the carotid arteries from these animals were perfused with fluorescently-labeled LDL and the rate of LDL accumulation determined by quantitative fluorescent microscopy. The rate of LDL accumulation in the carotid arteries was significantly greater in the low E group than in either the low E + catechin or high E group. The rates of ex vivo LDL accumulation in the low E + catechin and high E groups were not statistically different from each other.

The rate of LDL efflux from the artery wall reflects indirectly the affinity of LDL binding to arterial wall components. Therefore, vitamin E and catechin primarily modulate LDL flux through the artery wall by reducing endothelial layer permeability rather than by affecting binding. These results suggest a novel mechanism by which dietary vitamin E and plant flavonoids, such as catechin, are associated with decreased risk of coronary heart disease. The consumption of dietary flavonoids derived from fruits and vegetables may thus have an important impact in reducing mortality from coronary heart diseases.

The effect of red wine, alcohol and selected flavonoids was investigated on vascular smooth muscle cells. The effects on endothelial-dependent relaxation response were investigated by determining the extent of contraction of aortic rings from rabbits caused by noradrenalin (Cishek et al. 1997). Red wine and polymeric phenols evoked a dose-dependent relaxation in aortic rings. Ethanol had no effect. Endothelium-dependent relaxation in response to acetylcholine was attenuated in tissues incubated with red wine and polymeric phenols. Although catechin, epicatechin and quercetin are potent antioxidant in inhibiting oxidation of LDL, these compounds did not evoke an endothelium-dependent relaxation on rabbit aortic rings. Therefore, it is suggested that the effect of polymeric phenols could be mediated by an alternate mechanism.

Endothelium-dependent relaxation on rat aorta may be the result of an increase in nitric oxide (NO) production. Phenolic polymers could influence NO production and release from intracellular stores, preserve or stabilize NO, and stimulate NO synthase activity. This new area of flavonoid research may thus have important clinical implications.

7.15 Evaluation of the effectiveness and utilization of crude antioxidants from grapes and wines in foods

The effectiveness of crude extracts from grapes and red and white wines to inhibit lipid peroxidation was determined in a muscle tissue system. Muscle tissues from red turkey meat were cut into small pieces (1x1x0.5 cm) and dipped in a solution of 12% ethanol, grape extract or wines for 2 min. After the short dipping the muscle pieces were transferred to petri dishes and stored at 4°C.

Turkey red muscles stored at 4°C after dipping in acetate buffer pH 5.0 showed a rate of oxidation of ~ 0.2 A/10 days. Ethanol (12%) significantly increased the rate of oxidation, which was inhibited totally by dipping the red muscle in red-wine or white wine. The same results were obtained when heart muscle or liver tissue were dipped in ethanol. The prooxidant effect of ethanol was inhibited by dipping the tissues for 2 min in red wine (Petite Sirah) (Figs. 21-25).

8. Discussions

Currently, there is considerable interest in elucidating the role of dietary antioxidants in human nutrition and medicine. Flavonoids and phenolic compounds have gained much interest as natural antioxidants (Frankel et al 1993, Hertog et al 1993, Kinsella et al 1993, Kanner et al 1994). They represent important group of bioactive compounds in foods which may prevent the development of many diseases including atherosclerosis and cancer (Kumpulainen and Salonen 1996, Formica et al 1995). Wines and especially red wines contain very large concentrations of phenolic compound which work as antioxidants (Kanner et al, 1994; Frankel et al, 1995; Vinson and Hontz, 1995; Soleas et al, 1995). Several wine grape varieties such Cabernet Sauvignon, Petite Sirah and Argaman are among the best varieties to produce red wines with a high content of phenolic compounds and antioxidant activity. White wines contain 10 fold lower amount of phenolic compounds and relatively lower total antioxidants. During wine production not all the phenolic compounds are extracted from the berries to the wine and the wastes contain between 2-6% phenolics on the basis of dry material. The enormous amount of polyphenolics, should be utilized in the future as antioxidants in foods and nutraceuticals. Some of these products are already now in the market. The antioxidant effects of wines, red and white were investigated on lipid peroxidation of red muscle, heart and liver tissues.

Ethanol was found to increase significantly the oxidation of the tissues in-situ during incubation at 4°C. Red wine but also white wine evoked a very significant protection and prevented lipid peroxidation which was enhanced by ethanol. The concentration of the phenolics compounds on catechin equivalent basis are in the range of 10 mM in red wines and 1 mM at white wine, high enough to provide a significant protection on lipid peroxidation in-situ muscle, or liver tissues.

We found a good correlation between total phenolics in grape berries or wines and total antioxidant activity. These results are not a surprise as both methods

work to identify total reducing compounds. Folin Ciocalteu method used to determine total phenolic compounds, is based on the capability of reducing compounds to reduce molybdenum and tungsten to a pigmented stable reduced complex (Keith et al, 1958).

Grape berries and wines, both red and white contain various phenolics from different classes of benzoic acids, hydroxy-cinnamic acids, flavans, flavonols and anthocyanidins. When some representative from those classes were tested as antioxidants, a large differences in them activities was found. In our model system the following order was found: catechin > caffeic acid > quercetin > malvidin > α -tocopherol. At Davis several more compounds were determined, but the order was almost the same; catechin > myricetin = epicatechin = rutin > gallic acid > quercetin > cyanidin. The influence of chemical structure on antioxidant activity of five phenolic compounds having a similar ortho-dihydroxy moiety was evaluated with the in vitro LDL oxidation assay system (Mayer et al, 1998 a). The order was catechin > cyanidin = caffeic acid > quercetin > ellagic acid. These results are in general agreement with previous findings (Nardini et al, 1995; Teissedre et al, 1996, Van Acker et al, 1996; Jogensen and Skibsted, 1996). However, in contrast with our earlier report (Teissedre et al, 1996) it was found that cyanidin, an anthocyanidin is more active than quercetin.

The antioxidant activity toward LDL oxidation of several hydroxycinnamic acids found in wines and grapes, were also investigated (Mayer et al, 1998b). The ortho-dihydroxy compounds were also found to have the highest antioxidant activities. It was suggested that esterification of the less active compounds such coumaric acid to tartaric acid may enhance their ability to sterically block LDL oxidation via increased binding to apolipoprotein B-100 in the LDL particle.

The radical scavenging capacity of flavonoids was demonstrated with superoxide, hydroxyl and peroxy radicals (Jovanovic et al, 1994). The rate constant reactivity with hydroxyl radical is high, but this result is not surprising

due to the generally high reactivity with aromatic compounds. However, for O_2^- , the rate constant is relatively low. Most recently it was demonstrated that cyanidin-3 sophorose, an anthocyanin, has the capability of scavenging O_2^- at a rate of $2.2 \cdot 10^5 M^{-1} S^{-1}$ at pH 7.0. That of the aglycon (anthocyanidin) is 10 fold higher ($1.9 \cdot 10^6 M^{-1} S^{-1}$) (Yamasaki et al, 1996). Our results show that quinoidal base of malvidin scavenge O_2^- at a rate of $10^5 M^{-1} S^{-1}$, almost 10 fold lower than cyanidin.

Of great interest is the reactivity of flavonoids and phenolic compounds with peroxy radical. This rate was found to be in the range of 10^8 to $10^7 M^{-1} S^{-1}$ (Bors et al, 1990; Bors et al, 1994). The reactivity of the flavonoids with free radicals was found to depend on the structure and several groups which are allocated around the heterocyclic and aromatic B ring (Pryor et al, 1993; Tsuda et al, 1996; Lapidot et al, 1998). On the basis of the results collected from several studies, it is possible to summarize that flavonoids such as the flavanones (eriodictol, taxifolin) the flavone (luteolin) the flavan (catechin) and flavonol (quercetin and myricetin) containing an ortho dihydroxy structure like catechol are highly active against membrane lipid peroxidation, or as electron donors to a ferryl system (van Acker et al, 1996; Rice-Evans, 1996; Jorgensen et al, 1998). These results emphasize the fact that hydroxyl groups around the B ring increases antioxidative activity. The 2,3-double bond which is necessary for maintaining a conjugated double bond along the entire molecule appears to be of great importance. Galandin a flavonol (OH substitution at 3,5,7, of heterocyclic and A ring position) which does not contain hydroxyls around the B ring, was found to work as a good antioxidant against enzymatic and non enzymatic lipid peroxidation van (Acker et al, 1996). The 2,3 double bond in conjugation with carbinol at position 4 and hydroxyl at position 3 on the heterocyclic ring greatly increases antioxidant activity of the flavonoid. The presence of the carbonyl with heterocyclic ring and the 2-3 double bond could participate in radical stabilization that also increases antioxidant activity. This structure increases the capability of the molecule to donate electrons and to

reduce ferryl and to work as a scavenger of free radicals (van Acker et al, 1996; Rice-Evans, 1996; Bors et al, 1990; Bors et al, 1994). Data from some studies have shown that it is possible to predict the antioxidant effect by the redox potential of the flavonoid (van Acker et al, 1996; Cao et al, 1997; Jorgensen et al, 1998). However, the solubility and steric effects may be affected by the structure of the molecule (glucosides or other adducts) which could increase or decrease its activity. Addition of a 3-hydroxy group to luteolin to yield quercetin increases the reactivity for reduction of ferryl myoglobin. However, glycoside formation at this position like rutin, dramatically lowers the reactivity (Jorgensen et al, 1998). In contrast with these results our results have shown that malvidin 3-glucoside works almost 3 fold better than malvidin in inhibiting membranal lipid peroxidation catalyzed by ferryl myoglobin (Lapidot et al, 1998). The effectiveness of an antioxidant could be affected by the substrate in which this antioxidant is found such as molecules in emulsion, membranes, organelles, low density lipoprotein etc., and of course by the catalyst which catalyzes the reaction such as: free metals, hemoproteins, H_2O_2 -activated hemoprotein, $HO^\cdot$, NO_x , $HOCl$ organic hydroperoxide and others (Lapidot et al, 1998). The antioxidant activity of anthocyanins and other polyphenolics is strongly affected by the system and catalyzers in use. In a membrane system catalyzed by H_2O_2 -activated myoglobin, malvidin 3-glucoside was found to be the most effective antioxidant and resveratrol the least, whilst in the system catalyzed by iron redox cycle, the most effective antioxidant was resveratrol > malvidin 3-glucoside = malvidin > catechin (Lapidot et al, 1998). Our results and those published most recently by Satue-Gracia et al (Satue-Gracia et al, 1997) have demonstrated that the use of radicals assays which are not relevant to oxidizable substrates or catalyzers (Rice-Evans et al, 1994; Wang et al, 1997). They may not provide data which are relevant to the mechanism of action of these antioxidants in biological systems.

Reaction of free radicals with flavonoids or other phenolic compounds leads to the generation of a semiquinone radical by the following reaction:

The stability of the phenoxyl radical is very important (Bors et al, 1990; Ruiz et al, 1996). This could greatly affect its capability of interacting with a second free radical by the following termination reaction

Indeed, an effective antioxidant has been shown to react in a 1:2 stoichiometry; one antioxidant molecule interacts with two free radicals (Bors et al, 1990; Ruiz et al, 1996; Boozer et al, 1955; Winterle et al, 1984).

Rate constants for hydrogen abstraction by the phenoxyl radical have been measured in non aqueous media (Foti et al, 1994). The results have demonstrated that the reaction between a phenoxyl radical (reaction 3) with other phenolic compounds is ~100-300 fold higher than the reaction of a peroxy radical (reaction 4) with phenolic compounds.

This could explain the possible reduction of active oxidized antioxidants in a mixture, an effect which produces synergism. It is also known that phenoxyl radical partially interacts with a second phenoxyl radical producing a dimer, similar to a termination reaction.

An attempt to find synergism between phenolic compounds was done by Meyer et al (1988) without success. It seems that a such synergism could exist only between very weak and strong electron donor phenolics.

If the reduction potential of the phenoxyl radical is high enough it could propagate rather than interrupt chain reaction. A series of articles by Stocker group has shown that increasing the amount of α -tocopherol in LDL increased the rate of LDL oxidation by several catalyzers including H_2O_2 -activated peroxidase (Witting et al, 1997). It was also reported that coumaric acid could enhance oxidation of α -tocopherol (Laranjinha et al, 1995). We have also

found in a microemulsion of linoleate peroxidized by myoglobin (results not shown), that coumaric acid enhances lipid peroxidation probably by the following reaction:

A part of the semiquinone radicals, especially those formed from dihydroxy phenols like catechol, could interact with oxygen producing quinones and superoxide radicals (Kanner et al, 1987). Such a reaction could decrease the antioxidant effects of the phenolic compounds, or produce a prooxidant effect.

Due to this possible effect it is recommended to enhance the reactivity of phenolic antioxidants by the addition of SOD and catalase into the system.

Considerable use has been made of the ability of phenolic compounds to form complexes with metals. Some of these complexes are natural pigments of plants. Metal chelation could lead to: a) activation of the metal as a catalyst of oxidation, b) the chelation could deactivate the metal to oxidation and c) the chelation could lead to a complex which works as a hydroperoxide decomposer (Kanner et al, 1987; Osawa, 1982; Harel, 1995).

The possible interaction of catechin with complexed iron was demonstrated by its reaction with ferric cytochrome-C. During this interaction catechin donate electrons reducing cytochrome-C to the ferrous state. This reaction was also demonstrated with metmyoglobin which was reduced to oxymyoglobin by quercetin. Chelation of iron by catechin and quercetin was demonstrated by changing absorption and formation of a chromophor.

The redox chemistry of iron is itself directly affected by coordination ligands. The effects of these ligands on the electronic structure and the reactivity of iron is critical for understanding the possible antioxidant role of polyphenols in biological systems (Kanner et al, 1987).

Chelation to some flavonoids or phenolics compounds could affect the redox potential of iron and its solubility in water/lipid phases.

The hydroxyl and carbonyl groups around the heterocyclic and B ring can chelate metal, such Fe, Cu, Mn, Ca Mg or Al.(Ribe-reau-Gayon, 1972; Thompson et al, 1976). The groups which contain oxygen with 2 pairs of non-bonded electrons are important for complexation of the metals. Several flavonoids were found to produce relatively stable complexes with iron and copper, among the flavonols: fisetin, quercetin, kaempferol and rutin, among the flavanones: taxifolin, naringenin and the flavanol, catechin. For chelation of iron, the hydroxyls at position 3'4' are important and more over, 3-OH appear to be more important than 5-OH. The structural feature of a carbonyl at the 4 position is necessary for the metal chelation of many flavonoids. Several authors shown that the 4 carbonyl and 3 or 5 hydroxyl groups act cooperatively to chelate metals ions (Thompson et al, 1976; Puppo, 1992; Morel et al, 1993). One could assume that the antioxidant effect of flavonoids depends not only on their radical scavenger capability but also on their metal chelation effect. This effect could delocalize the metal from membranes or other targets for oxidation, sterically preventing the interaction between the metal with the biological target or prevent interaction with oxygen or hydroperoxides or work as a hydroperoxide decomposer. Metal ions chelated by phenolics compounds could form compounds of iron in its reduced form which catalytically breaks down hydroperoxides and by a site specific mechanism scavenge the free radicals to an alcohol or a non-radical product (Spiteller et al, 1998; Kanner 1998).

An attempt to understand the strong antioxidant effect of catechin in the presence of myoglobin, was done by us, using fractionation of secondary oxidized products from linoleic acid by HPLC. This study showed that hydroperoxides are catalytically broken down, but not to an alcohol. We assume that the non-radical products are adducts between alkoxyl and phenoxyl

radicals, generated site specifically to the iron-heme, by the following reactions:

Polyphenol-protein complexation depends on the characteristics of the protein, pH and the structure of the phenolic compound such as molecular size, conformational structure and water solubility.

The interaction of polyphenols and protein is thought to take place in two successive phases, one by hydrogen bonding and the other by hydrophobic interaction between protein sites and aromatic rings (Spencer et al, 1988; Haslam et al, 1988). Phenolic compounds could interact with serum albumin (Bartholom et al, 1996; Maralidhara et al, 1995). This complexation should be taken into consideration when polyphenolic compound are extracted from blood during evaluation of the bioavailability of those compounds. Furthermore, in this substances could be specific targets such enzymes, endothelial cells, apoproteins, LDL, receptors, cytokines, etc. The effect of bovine serum albumin on the antioxidant activity of several phenolic compounds, were determined by Heinonen et al, 1998. Vinson et al, 1994, have used the LDL to bond polyphenolic antioxidant, as a method which may be used to predict a good antioxidant activity in-vivo.

The antioxidative effect of many polyphenols are not only dependent on their specific structure, and binding capability to macromolecules but also on their solubility. This effect was recently determined by several of us (Schwarz et al, 1996; Frankel et al, 1994; Hopia et al, 1997) and was found to depend on a interfacial phenomean. Flavonoids are also known to interact specifically with phospholipids producing reversible complexes (Huang et al, 1992; Bombardelli et al, 1991).

Of great importance is the reaction between phenolic compounds and metal enzymes such as peroxidases, cyclooxygenases and lipoxygenases. In the

presence of cyclooxygenase, phenol or phenolic compounds stimulate enzyme reaction and prostaglandin generation at relatively small concentrations. However, at high concentration they inhibit prostaglandin production (Smith et al, 1971, Dewhirst, 1980; Lands, 1982).

Lipoxygenases in animals utilize arachidonic acid, the same substrate such as cyclooxygenase, to generate a single stereospecific hydroperoxide as the primary product. In plants lipoxygenase could oxidize an unsaturated fatty acid with a 1-4 pentadiene structure. Studies on purified soybean enzyme indicate that the native inactive form contains a high spin ferrous state in the active center. Binding and reaction with a lipid hydroperoxides results in oxidation of the iron to a high spin ferric state and activation of the enzyme,

Increased production of leukotrienes, has been implicated in several human diseases. There has been considerable interest in the development of lipoxygenase inhibitors for therapeutic use (Lewis et al, 1990; Cashman, 1985). Lipoxygenase and 15-lipoxygenase mRNA have been detected in human atherosclerotic lesion (Yla-Herttuala et al, 1987), raising the possibility that the enzyme promotes LDL oxidation in-vivo (Heinecke, 1997). It was found by us, that similar to cyclooxygenase, lipoxygenase reactivity is stimulated by low concentration of phenolics and inhibited at high concentration. Small concentration of wine phenolics were found to inhibit co-oxidation of β -carotene in the presence of linoleic acid, however, not the production of conjugated dienes (Kanner, 1998). German (1996) showed that wine phenolics inhibit both plant and animal lipoxygenases at high concentration. However, at low concentration they increase lipogxygenase end products. The increase in end products formation after a long incubation is consistent with the hypothesis that the enzyme is stabilized against self inactivation by leak of free radicals which can oxidize the protein. We believe that a high concentration of flavonoids or

wine phenolics inhibits lipoxygenase mostly by scavenging the enzyme free radical complex or by reducing the active ferric form of the enzyme to the native ferrous inactive form.

By down regulating cyclooxygenase and lipoxygenase, phenolic compounds may reduce thrombosis and inflammatory reactions involved in immune suppressions. These physiological events tend to increase chronic diseases such as heart diseases and cancer. Three possible mechanisms for protective role of plant flavonoids include: a) inhibiting LDL oxidation, a process implicated in atherosclerosis. They may protect cholesterol from forming cytotoxic compounds and prevent atherosclerosis, b) interference with the immune response by reducing monocytes from vessel walls, and c) interference with platelet aggregation involved in the clotting process and preventing thrombosis. This ability of wine phenolics to partially inhibit the lipoxygenase class of enzymes suggests that the magnitude of oxidant generated during oxidation would be lowered. Our results confirm that wine phenolics may protect vital biological targets from the consequences of oxidant damage. Since lipoxygenase is itself a target of oxidant damage, wine phenolics can protect the lipoxygenases and presumably other sensitive target from excessive oxidation.

A hamster model on a hypercholesterolemic diet was used to test dietary antioxidants for their possible intervention in the development of atherosclerosis (Xu et al. 1998; Rein et al 1998). Aortic lipid deposition was significantly less in animals fed diets supplemented with either catechin or vitamin E than those fed basal low antioxidant containing diet.

We also examined the effect of dietary vitamin E and catechin on the accumulation of fluorescently-labeled LDL in the carotid arteries of the above hamster model (Pearson et al., 1997). Individual carotid arteries from these animals were perfused with fluorescently-labeled LDL and the rate of LDL accumulation determined by quantitative fluorescence microscopy. The rate of LDL accumulation in the carotid arteries was significantly greater in the low E

group than in either the low E+catechin or high E group. The rates of ex vivo LDL accumulation in the low E+cat and high E groups were not statistically different from each other. The rate of LDL efflux from the artery wall reflects indirectly the affinity of LDL binding to arterial wall components. Therefore, vitamin E and catechin primarily modulate LDL flux through the artery wall by reducing endothelial layer permeability rather than by affecting binding. These results suggest a novel mechanism by which dietary vitamin E and plant flavonoids, such as catechin, are associated with decreased risk of coronary heart disease. The consumption of dietary flavonoids derived from fruits and vegetables may thus have an important impact in reducing mortality from coronary heart diseases.

The effect of red wine, alcohol and selected flavonoids was investigated on vascular smooth muscle cells. The effects on endothelial-dependent relaxation response were investigated by determining the extent of contraction of aortic rings from rabbits caused by noradrenaline (Cishek et al. 1997). Red wine and polymeric phenols evoked a dose-dependent relaxation in aortic rings. Ethanol had no effect. Endothelium-dependent relaxation in response to acetylcholine was attenuated in tissues incubated with red wine and polymeric phenols. Although catechin, epicatechin and quercetin are potent antioxidants in inhibiting oxidation of LDL, these compounds did not evoke endothelium-dependent relaxation on rabbit aortic rings. Therefore, it is suggested that the effect of polymeric phenols could be mediated by an alternate mechanism. Endothelium-dependent relaxation on rat aorta may be the result of an increase in nitrous oxide (NO) production. Phenolic polymers could influence NO production and release from intracellular stores, preserve or stabilize NO, and stimulate NO synthase activity. This new area of flavonoid research may thus have important clinical implications.

Taking in to consideration the many factors which affect the capability of phenolic compounds and flavonoids to act as antioxidants, thus could easily explain the great difference in their activity found by many researchers. Thus, it

must be emphasized that it is necessary to determine as much as possible the antioxidant activity in a system which could simulate the in-vivo conditions or to act with these compounds in in-vivo.

More important, a good dietary antioxidant is not only one of low redox potential with a high free radical scavenging capability, or a compound which could act on several in vitro or ex-vivo model system as antioxidants, but one of high bioavailability which could reach the target of oxidation in-vivo and at a critical concentration in order to provide protection.

The potential bioavailability of several anthocyanins from red wine, in human was investigated. Two of the compounds found among the wine anthocyanins were found unchanged in urine. Other anthocyanins, which seems to have undergone molecular modification were detected in the urine after incubation with HCl. The anthocyanin level in the urine reached a peak within 6 hours, from the wine drinking. Within 12 hours of the wine drinking, were found 1.5-5.1% of the ingested anthocyanins, in the urine.

These results with in humans along with our results in-vivo with hamster and other reports (Jang et al, 1997; Folcik et al, 1996; Xu et al, 1998; Maxwell et al, 1994; Whitehead et al, 1995; Kondo et al, 1994; Doy et al, 1995; Furhman et al, 1995; Serafini et al, 1998; Demorow et al, 1995) demonstrated that a part of the flavonoids from wines are absorbed in the blood stream and could provide protection.

9. Evaluations

- Evaluation was made on the activity of crude grape extracts and wines on meat muscle food oxidation. The antioxidant action on paprika powder was not investigated since it was found difficult to disperse the wine active compounds in the powder.
- The antioxidant activities of special components in grapes were tested on food models (linoleate emulsion, lecithin liposomes and muscle

membranes). The levels of antioxidants in wines, wine grapes and grape pomace, were tested.

- Mode of action of phenolic compounds was carried out with special wine components such as catechin, quercetin, malvidin etc.
- The activity of the antioxidants was tested in biological models such as: LDL, blood platelets, a hamster model for atherosclerosis and a model for relaxation of blood vessels.
- The bioavailability of wine anthocyanidins in humans was investigated.

10. Description of the Cooperation

Through out the project duration there were scientific exchanges amongst the researchers of both labs. Prof. Ed Frankel came to the Israeli Lab to work and Prof. Joseph Kanner went to Davis. Throughout the project the two principal investigators met at various scientific meetings and presented papers and posters of the results of their research. Such meetings afford the opportunity to discuss results and plan further experiments. The principal investigator from Israel and the U.S. and the other researchers in both labs cooperated fully and were in constant contact, discussing methods, necessary modification, results obtained and literature references.

The Israeli team concentrated its efforts on food and several biological systems including the development of a method to identify dietary anthocyanins in human urine and plasma. The California group investigate LDL oxidation and the effect of flavonoids and vitamin E in animal models and their effect on atherosclerosis.

11. Conclusions

1. Wines and especially red wines and several grape varieties contain large concentrations of phenolic compounds which work as antioxidants.
2. During wine production not all the phenolic compounds are extracted from the berries of the wine and the wastes contain between 2-6%

phenolics on a dry matter basis. These enormous amounts of polyphenolics should be utilized in the future as antioxidants for foods and nutraceuticals.

3. The concentration of phenolics in red wine and white wines are ~ 10 mM and 1 mM (on catechin equivalent basis); high enough to provide significant protection from lipid peroxidation of foods such as muscle tissues.
4. We found a good correlation between total phenolics determined by the Folin-Ciocalteu method and total antioxidant activity. However, when some representatives from different classes of phenolic compounds found in red wine were tested as antioxidants, large differences in their activities were found.
5. Generally, the reactivity of the flavonoids with free radicals was found to depend on the structure and the hydroxyl groups which are allocated around the heterocyclic and aromatic B ring. On the basis of our results and those collected from others, it is possible to summarize that flavonoids or phenolic acids containing an orthodihydroxy structure around B ring are highly active against LDL or membrane lipid peroxidation. The 2,3-double bond in conjugation with a carbinol at position 4 and hydroxyl at position 3 on the heterocyclic ring, greatly increases antioxidant activity of the flavonoid.
6. The antioxidant activity of polyphenolics is strongly affected by the system and catalyzers in use. This activity is dependent also on the specific interaction of polyphenols with proteins, apoproteins, phospholipids, LDL, enzymes, receptors, cytokines, etc. The antioxidant effect is also dependent on their solubility.
7. The ability of phenolic compounds to form complexes with metals is of great importance for the ability of the flavonoids to work as antioxidants. This effect delocalizes the metal from the target of oxidation, sterically preventing interaction between the metal with fatty acids, hydroperoxides

or oxygen. Metal ion chelated by phenolics could form iron in its reduced form which catalytically breaks down hydroperoxides and by a site specific mechanism scavenge the free radicals to a non-radical product.

8. Low concentrations of wine phenolics inhibit lipoxxygenase type II activity, which generate free radicals in the system. Higher concentrations of wine phenolics inhibit lipoxxygenase type I. The inhibition seems to be mostly by scavenging the enzyme free radical complex or by reducing the active ferric form to ferrous inactive form.
9. A good antioxidant is not only a compound that donates electrons but one of high bioavailability which could reach the target of oxidation in vivo and at a critical concentration to provide protection. Anthocyanins are one of the main classes of flavonoids in red wines, and they contribute significantly to the powerful antioxidant properties of wine flavonoids. Two of the comounds found among the wine anthocyanins were found unchanged in human urine. Other anthocyanins which seem to have undergone molecular modification were detected in urine after incubation with HCl. Within 12 h of the wine drinking, 1.5-5.0% of the ingested anthocyanins, were found in the urine.
10. Flavonoids and phenolic compounds represent important groups of bioactive compounds in foods but especially in red wines which may prevent the development of atherosclerosis and coronary heart diseases. Aortic lipid deposition in hypercholesterolemic hamster was significantly less in animals fed diets supplemented with either catechin or vitamin E. The rate of LDL accumulation in the carotid arteries was also significantly lower in the catechin or vitamin E animal groups. Catechin and vitamin E primarily modulate LDL flux through the artery wall by reducing endothelial layer permeability rather than by affecting binding. These results suggest a novel mechanism by which dietary vitamin E and wine flavonoids, such as catechin, are associated with a decreased risk of coronary heart disease.

11. Although only limited information is available on digestion and absorption of flavonoids, our results and these from others indicated that they may be considerably bioavailable. Given their abundance in red wines but also in other plant foods, their potent antioxidant properties and bioavailability, the results of this study show that flavonoids and phenolic compounds, contribute to the observed reverse relationship between plant food consumption and the risk of coronary heart disease.

12. References

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Table 1 Total phenols contents of Israeli wines

Wine class		Total phenolic compounds mg/l CE
<u>Red wines</u>		
Cabernet Sauvignon	(CM)	2038
Cabernet Sauvignon	(B)	1955
Merlot	(B)	1887
Petite Sirah	(CM)	2022
“Hilulim”	(CM)	2190
“Village”	(B)	1484
Zinfandel (pink)	(CM)	447
<u>White wines</u>		
Emerald Riesling	(G)	229
Chardonnay	(G)	238
Chenin Blanc	(CM)	242
“Hilulim”	(CM)	232

CE = catechin equivalent

Table 2 Total phenolics content of wine wastes

Grape	Total phenolic compound CE mg/kg dry weight
Merlot	64,000
Emerald Riesling	46,000
Sauvignon Blanc	42,000
Cabernet Sauvignon	36,000
Carignan	19,200

Table 3 Polyphenolic compounds extracted from different wine grape varieties

Variety	Method 1	Method 2
	mg/kg CE	
Argaman	4700	4300
Cabernet Sauvignon	1850	2870
Carignian	2150	2480
Merlot	1950	2300
Pinot Noir	2280	2650
Grenash	760	1490

Table 4 Polyphenol compounds extract from grape peel of different varieties

Variety	mg/kg CE
Argaman	11083
Cabernet Sauvignon	4282
Carignian	4774
Merlot	3672
Pinot Noir	4497
Grenash	2100

Table 5 HPLC separation of Petite Sirah wine

Tentative identification	Retention time	Area	Relative %	mg/L
Caffeic acid	1.29	311	1.5	35
Gallic acid	5.85	2065	10.0	250
t-caffeoyl tartarate	17.22	1189	6.0	150
c-coumaroyl tartarate	19.95	693	3.4	75
t-coumaroyl tartarate	20.37	1104	5.5	135
procyanidin B ₁	22.68	987	5.0	125
catechin	27.40	1000	5.0	125
procyanidin B ₂	29.60	1450	7.2	175
epicatechin	33.80	1100	5.5	135
anthocyanin glucoside (petunidin-3-glucoside)	36.30	1170	5.6	140
malvidin-3-glucoside	43.80	1400	7.0	175
flavonol glucoside	45.30	260	1.3	33
flavonol glucoside	48.00	278	1.4	34
malvidin-3-glucoside acetate	51.90	780	4.0	100

Table 6 - Antioxidant activity of several classes of flavonoids fractionated from red and white wines

Wine	Phenols		I ₅₀		AU	AUI
	µg/ml	µM*	µg/ml	µM*		
Chardonnay	225	775	0.7	2.3	336	0.32
Fraction						
1	60	207	1.4	4.8	43	0.15
2	26	90	1.2	4.2	21	0.17
3	52	180	1.1	3.9	46	0.18
4	32	112	0.7	2.6	160	0.28
5	10	34	0.8	2.6	43	0.28
Total	180	623			313	
Petite Sirah	1910	6586	0.7	2.3	2863	0.56
Fraction						
1	87	300	2.7	9.4	32	0.14
2	116	400	0.8	2.7	174	0.48
3	720	2482	0.9	3.3	752	0.39
4	320	1103	0.6	2.2	501	0.59
5	560	1931	0.6	2.2	877	0.59
Total	1803	6216			2337	

µM*, wine phenolics determined by Folin Ciocalteu method using catechin as standard and for calculation.

AU = Antioxidant units, total phenolics/I₅₀

AUI - Antioxidant unit index is the relation between I₅₀ of catechin to I₅₀ of each sample.

Table 7 - Anthocyanin peak identification

Peak	Compound
1	Delphinidin 3-0-glucoside
2	Cyanidin 3-0-glucoside
3	Petunidin 3-0-glucoside
4	Peonidin 3-0-glucoside
5	Malvidin 3-0-glucoside
6	Delphinidin 3-0-glucoside acetate
7	Cyanidin 3-0-glucoside acetate
8	Petunidin 3-0-glucoside acetate
9	Peonidin 3-0-glucoside acetate
10	Malvidin 3-0-glucoside acetate
11	Del 3 glucoside 6-0-coumaroyl
12	Cyan 3 glucoside 6-0-coumaroyl
13	Malvidin 3 glucoside 6-0-coumaroyl

Table 8 - Data on peak area of anthocyanins detected at 530 nm.

Peak	Merlot	Argaman	Cab. ARO	Cab. Carm	Carignan	Grenash	Pinot Noir
1	2750	772	967	1816	2241	313	1534
2	1477	9490	205	197	306	591	2193
3	2740	640	1232	2083	2972	684	2065
4	3742	20304	1421	2008	839	4792	23187
5	14354	261180	17377	33650	14773	15975	44517
6	88	1244	305	541	62	91	
7	516	1090	1765	404	226		
8	526	1404	130	266	57		
9	820	3646	429	756	741	71	
10	9848	38914	17015	29435	4912	2325	
11	2554	45508	1980	18618	5224	1075	
12	831	21940	285	3369	3425	163	
13	6256	106470	4028	8594	15541	3726	
Total	46502	512602	47139	101737	51319	29806	74496

Table 9 - Data on peak area of anthocyanin detected at 280 nm and of the total area which include other polyphenols

Peak	Time retention	Merlot	Argaman	Cab. ARO	Cab. Carm	Carignan	Grenash	Pinot Noir
1	36-40	1826	4450	593	979	583	630	468
2	39-40	1436		386	113	772	409	948
3	41-43	1856	12250	776	1312	1191	3866	480
4	43-45	2899	4450	967	1361	1176	10780	4470
5	45-48	9300	191800	10260	20984	13026		12214
6	46-52	527	6050	925	1057	835	213	
7	49-53	1541	27890	70	474	106		
8	53-55	1224	16160	135	2657	1007	703	
9	56-58	1846	30050	310	666	473	4775	
10	59-62	8528	44870	11480	19604	14116	1206	
11	63	2843	22350	1682	2356	2081	5386	
12	64	2972			1159	396		
13	65-67	8643	325510	6539	10374	7423	27988	18580
Total		45441	685930	34123	63096	43185		
Total area		65290	795533	54301	76145	66561	53191	40612
%		70	86	63	83	65	53	46

Fig.1 Fractionation of red wine, Petite Sirah by HPLC.

Fig. 2 Fractionation of white wine, Shardonay by HPLC.

Fig.3 Fractionation of grape peel anthocyanins from several varieties determined by HPLC at 530nm

Fig. 4 Fractionation of grape peel anthocyanins from several varieties determined at 280nm

Fig. 5 Myoglobin catalyzed linoleate peroxidation inhibited by several red wine flavonoids

Fig. 6 Myoglobin catalyzed β-carotene-linoleate peroxidation inhibited by several red wine flavonoids

Fig. 7 Ferrous ion catalyzed β-carotene - linoleate peroxidation inhibited by several red wine flavonoids

Fig. 8 Myoglobin β-carotene - linoleate peroxidation inhibited by catechin at several concentrations

Fig. 9 Linoleic acid oxidized compounds generated by LOX (SB Type I)

KODE= keto-octadecadienoic acid
 HP = hydroperoxy-octadecadienoic acid
 HOX = hydroxy- octadecadienoic acid
 CC = cis-cis
 CT = cis-trans

Fig. 10 Linoleic acid oxidized compounds treated by NaBH_4

Mb (2.5 μ M) Mb + Catechin
(10 μ M)

Fig. 12 Linoleic acid oxidized keto - compounds (270nm) separated by HPLC

Fig 13 Reduction of cyt-c ($30\mu\text{M}$) by catechin ($200\mu\text{M}$)

Fig 14 Reduction of metmyoglobin ($30\mu\text{M}$) by quercetin ($300\mu\text{M}$)

Fig. 15 Chelation of Fe ions by quercetin

Fig. 16 Chelation of iron by catechin

Fig. 17 Generation of hydroxyl radicals by "iron redox cycles" in the presence of ascorbic acid and several flavonoids at pH 7.0

Ascorbic acid (200μM); Ferric chloride (50μM); M=Myricetin (100μM);
Q=Quercetin (100μM); C= Catechin (100μM)

Fig. 18 Generation of hydroxyl radical by malvidin 3 - glucoside, at pH 7.0

Ascorbic acid (200μM); Ferric chloride (50μM); Malvidin 3 - Glu (100μM);
Catechin (100μM). PB=pseudo-base, QB=quinoidal-base

Fig 19 Bleaching of malvidin by O_2^- (xanthin/xanthin oxidase)

Fig 20 Stimulation and Inhibition of LOXs

Fig. 21 Inhibition of muscle lipid peroxidation by white grape extract

Fig. 22 Inhibition of muscle lipid peroxidation by white wine

Fig. 23 Inhibition of heart muscle lipid peroxidation by red wine

Fig. 24 Inhibition of heart muscle lipid peroxidation by red wine

Fig. 25 Inhibition of liver tissue peroxidation by red wine

Fig. 26 Correlation between antioxidant activity and phenolic compounds concentration in grapes and wine