ANTIOXIDANT ACTIVITIES OF GRAPE SKIN AND GRAPE SEED

POLYPHENOLICS AND POTENTIAL USE OF ANTIOXIDANTS IN FOODS AS A

FUNCTIONAL FOOD INGREDIENT

by

YUSUF YILMAZ

(Under the direction of ROMEO TOLEDO)

ABSTRACT

Grape skins and seeds, byproducts of the grape juice/wine industry can be utilized as dietary supplements or used in functional foods because of their potential health functional components. The effectiveness of different aqueous solvents in extracting phenolics from muscadine seed powder was investigated. Antioxidant capacities of byproduct grape seeds and skins from *Vitis vinifera* varieties Merlot and Chardonnay and the seeds of *Vitis rotundifolia* variety Muscadine were determined using oxygen radical absorbance capacity (ORAC) assay. The contribution of major phenolics in these byproducts to the total antioxidant capacities was also evaluated. Finally, the stability of muscadine seed extract (MSE) was determined in a puffed rice cereal bar during storage at different temperatures. Aqueous solutions containing 60% ethanol (190 proof), 60 to 70% methanol, and 50 to 75% acetone were better than any single compound solvent system in extracting phenolics from muscadine grape seed powder. Antioxidant capacities of Chardonnay, Merlot and Muscadine grape seed powders were 637.8, 344.8 and 310.8μmol TE/g d.m., respectively. Gallic acid, catechin and epicatechin concentrations on a dry basis were 68, 7, and 69mg/100g in Muscadine seeds, 10, 211, and 303mg/100g in Chardonnay seeds, and 7, 74, and 83mg/100g in Merlot seeds, respectively. Concentrations of these three compounds were lower in winery byproduct grape skins than seeds. These three major phenolic constituents of grape seeds contributed less than 17% to the antioxidant capacity measured as ORAC. Phenolic

constituents of MSE in a puffed rice cereal bar were more stable at 19°C compared to 37°C over three months. Antioxidant capacities of the food product supplemented with the MSE measured as ORAC was reduced over time with no difference attributed to storage temperature ($p > 0.05$). Moreover, MSE provided natural antioxidant activity by inhibiting lipid peroxidation. Byproducts of grape juice/wine industry contain valuable phenolic antioxidant compounds. Procyanidins other than monomers are responsible for most of the superior antioxidant capacity of grape seeds. Health conscious consumers can have the health benefits of cereal products supplemented with MSE.

INDEX WORDS: Antioxidant, antioxidant capacity, catechin, Chardonnay, epicatechin, gallic acid, galvinoxyl, TBARS, grape, grape seed, grape skin, Merlot, Muscadine, ORAC, phenolics.

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DEDICATION

This dissertation is dedicated to my parents Hüsmen and Sebile Yılmaz

on behalf of the

People of the Republic of Turkey

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CHAPTER 1

INTRODUCTION

Grapes (genus *Vitis*) and grape products have an economic value of approximately $3 billion in the US. (NASS, 2002). The berries of the *vinifera* varieties of *Vitis* are crushed and mostly used to make wine while the *rotundifolia,* because of their low sugar contents make poor wine, are often processed into juices, jams or preserves. Some processors de-seed the *rotundifolia* variety, Muscadine before further processing to improve release of juice from the seed sac. Although *vinifera* grapes can be crushed/de-seeded before processing, this is not standard industry practice. Thus, byproducts of *vinifera* grape processing include seeds and skins. The red *vinifera* varieties are crushed, fermented and pressed following fermentation so more of the polyphenolics are transferred to the wine and less is retained in the byproduct. Skins and seeds of grapes are in abundant supply and can be valuable raw materials for extraction of polyphenols and other health functional compounds. Much research has been done on vinifera grape seed and skin extracts but not on muscadine grape. Shrikhande (2000) in a review article on the health benefits of wine industry byproducts cited that there are 22 grape seed, 5 grape skin, and 7 red wine powder products commercially available as dietary supplements in the US. Thus, grape seeds and skins are winemaking byproducts with huge commercial potential.

Flavonoids have been reported to have *in vivo* and *in vitro* antioxidant activities through their ability to scavenge the radicals of hydroxyl, peroxyl, superoxide, nitric

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oxide and DPPH (2,2-diphenyl-1-picrylhydrazyl). Besides the free radical scavenging activities of the flavonoids, metal chelating properties of phenolic flavonoids have also been studied extensively. Moreover, flavonoids have been also reported to possess anticarcinogenic activity, cardiovascular disease preventing activity, and antiulcer activity. Grape skins and seeds have numerous flavonoids of a phenolic nature including gallic acid, monomeric catechins as well as dimeric catechins. Gallic acid, catechin and epicatechin were identified as the main phenolic compounds in grape seeds (Palma and Taylor, 1999). Besides those monomeric and dimeric catechins, grape skins and seeds have trimeric, oligomeric and polymeric proanthocyanidins. Therefore, in this research we studied the effectiveness of different solvents in extracting phenolics from muscadine seed powders, measured antioxidant capacities of byproduct grape seeds and skins, evaluated the contribution of major phenolics in grape byproducts to the total antioxidant capacities, and determined the stability of muscadine seed extract in an actual food during storage at different temperatures.

This dissertation is composed of six chapters including this introduction and the final chapter, summary and conclusions. In the second chapter, a broad literature review regarding related topics of the following chapters is given. This chapter mainly includes topics on grapes, grape industry byproducts, oxidation in foods, free radical species, antioxidants, flavonoids in general and those present in grape seeds, skins and wine, health benefits of flavonoid intake, and flavonoids as a functional food ingredient.

The effectiveness of different solvents in extracting phenolics from muscadine grape seed powder is presented in the third chapter. We also evaluated antioxidant

capacities of powders of skins and seeds from Chardonnay, Merlot and Muscadine grapes, obtained as byproducts of either winemaking or juice processing industry.

The fourth chapter includes a study on the analysis of the major monomeric flavanols present in seeds and skins of *Vitis vinifera* variety Merlot and Chardonnay, and seeds of *Vitis rotundifolia* variety Muscadine. The contribution of the major monomeric flavanols and phenolic acid to the total antioxidant capacity of grape seeds and skins was determined.

The primary objective of chapter five was to determine the stability of phenolic grape seed antioxidants in an actual food product stored at two different temperatures up to three months. The effect of muscadine seed extract on the inhibition of lipid oxidation in a puffed rice cereal bar was also tested.

Studies in chapter 3, 4 and 5 were summarized and general conclusions of these studies are included in the last chapter.

LITERATURE CITED

NASS. 2002. http://www.usda.gov/nass. Accessed May 26, 2002.

Shrikhande, A.J. 2000. Wine by-products with health benefits. Food Res. Int. 33:469-474.

Palma, M. and Taylor, L.R. 1999. Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. J. Chromatogr. A. 849:117-124.

CHAPTER 2

LITERATURE REVIEW

GRAPES

Grapes are taxonomically classified under the order of *Ramnales*, the family of *Vitaceae* and the genus of *Vitis* (Vine et al., 1997). The genus *Vitis* can be divided into two subgenera *Euvitis*, the true grapes with origins in Asia Minor and Europe, and the *Muscadinia*, muscadine grapes that originated in the southeastern US and Mexico. Primarily, the number of chromosomes defines the biological differences between the two subgenera. *Euvitis* species have 19 chromosomes whereas *Muscadinia* has 20.

Grapes can be consumed as fresh or processed into raisins, juices and wines. Muslim countries mostly consume grapes as table grapes, grape juice or raisins, whereas in European Mediterranean and the United States, the main use of grapes is in wine making. Merlot and Chardonnay are the two most known varieties of *Vitis vinifera* (Table 1) that are widely used to make red and white wines, respectively. Since v*inifera* grapes have higher sugar contents and lower acidity than the *lubruscana* grapes, they are unsuitable for juice production (McLellan and Acree, 1993) but are excellent raw materials for wine production.

Vitis lubruscana, *Vitis vinifera*, *Vitis rotundifolia* and French hybrids are grape varieties grown in the US (McLellan and Acree, 1993). According to USDA National Agricultural Statistics Service (NASS, 2002), approximately 90% of the grapes in the US

were produced in California in 2001. About half of those grapes were used for wine production, 35% for raisin production and the rest for table grapes. The dollar value of grape production changed little from 1999 to 2001 at around $3billion.

Table 1. Major *Vitis* varieties.

Variety	Example
Vitis vinifera	
White	Chardonnay, Sauvignon Blanc, Riesling, Semillon, Pinot Blanc
Red	Cabernet Sauvignon, Merlot, Pinot Noir, Gamay, Zinfandel, Syrah, Cabernet Franc
Vitis lubruscana	Concord, Ives
Vitis rotundifolia	
Bronz-skinned	Scuppernong
Red-skinned	Muscadine

Merlot

Merlot Noir belongs to the *Vitis vinifera* red variety. Berries of Merlot are conical, compact and medium size with blue-violet color. Merlot is very popular in France, Italy, Hungary, and Bulgaria, as well as in the Unites States, New Zealand and Australia. Merlot has a unique fruitiness, and its skin is thinner than the Cabernet Sauvignon. Tannin content of Merlot tends to be lower than Cabernet's (Robinson, 1986). Because of its early ripening, it is sensitive to spring frosts. Its production has increased markedly as a result of increased demand for Merlot wines (Robinson, 1986).

Chardonnay

Chardonnay is a white grape variety, which is 'a naturally vigorous vine' (Robinson, 1986). It has small compact cone-shaped berries with light green color. It is native to France but it is grown worldwide. Sugar content of Chardonnay is high, which increases the yield. Aroma of Chardonnay is not strong but it has a complex apple-citrus, honey-olive flavor (Vine et al., 1997).

Muscadine

Three genera *rotundifolis*, *munsonia*, and *popenoei* are included in the family *Muscadinia* and they are found in the Southeastern United States and Mexico. *Muscadinia* differ from other members of *Vitis* in their chromosome numbers and morphologies. Berries of the *Muscadinia* members are thick skinned and pulpy. Red-skinned variety of *Muscadinia* is usually called Muscadine, while the bronze-skinned is called Scuppernong. This *Vitis* variety is insect and fungal resistant, and it is the most disease resistant of all grapes cultivated in America (Anonymous, 2001). Their culture requires neither pesticide nor fungicide.

Muscadine and Scuppernong are the best-known *Vitis rotundifolia* varieties among the members of *Muscadinia*. Sugar enrichment is required during the fermentation of Muscadine to wine because of their typical low sugar contents. Muscadine vines have a more prominent musky flavor compared with the *Vinifera* vines. Berries of Muscadine vines have very thick skins.

Wine Industry Byproducts

Wine is a fermented beverage made with different varieties of grapes. For white wines, the juice is pressed out of the skin and pulp prior to fermentation. For red wine, grapes are crushed first and the whole crushed grape fermented. Therefore, in red wine most of the red pigments in grapes are retained in the wine. Grape skins and seeds are major byproducts of the winemaking industry. Recently, the wine industry has realized that there are potentially health functional compounds present in the byproduct grape skin and seeds. Therefore, much research has been done on grape seed and skin extracts. Shrikhande (2000) in a review article on the health benefits of wine industry byproducts cited that there are 22 grape seed, 5 grape skin, and 7 red wine powder products

commercially available in the US. Thus, grape seeds and skins are winemaking

byproducts with huge commercial potential.

Grape seeds and skins contain natural phenolic compounds with antioxidant

properties. Production of grape seed or skin extracts mainly involves solvent extraction

and evaporation of solvent. Procedures could be modified to give unique properties to the

final extracts by separating the monomeric and oligomeric fractions from the polymeric

fractions. Numerous patents have been issued for grape-related substances or processes.

A list of the patents assigned by the US Patent Office is given in Table 2.

Wine and Health

Moderate wine consumption has been associated with improved health. Some

epidemiological studies showed that heart disease rates in France were 66% lower than in

the US or Britain while all of these nationalities consume the same or similar amount of

dairy fat (Kolpan et al., 1996). This phenomenon has been called the 'French paradox'.

One major difference in the diets of these three nationalities is that the French consume

wine with meals while the British and Americans prefer beer or spirits. Another

difference between the French and American diets is the higher consumption of fruits,

vegetables and bread by the French. Wine contains phenolic compounds whose

antioxidant properties could reduce the risk of cardiovascular diseases. Alcohol is also

believed to possess protective effects on the heart by increasing the level of high-density

lipoproteins (HDL) cholesterol, reducing low-density lipoproteins (LDL) and inhibit

blood clotting. Wine consumption has been also associated with reduced risk of ulcers by

Table 2. Patents related to grape seed and its flavonoids

Date/ Number	Inventor(s)	Title	Claim / Explanation
1987/ 4,698,360	Masquelier, J.	Plant extract with a proanthocyanidins content as therapeutic agent having radical scavenger effect and use thereof	Plant proanthocyanidins extracted from pine bark are claimed to protect the body from harmful effects of free radicals.
1990/ 4,963,527	Bombardelli, E. and Sabadie, M.	Phospholipid complexes of extracts of *Vitis vinifera*, their preparation process and pharmaceutical and cosmetic compositions containing them.	Flavonoids of grape extracts can be reacted with phospholipids of soy, egg, bovine or porcine brains and skins. The products made with the reaction complexes are claimed to have protective effect on skin.
1990/ 4,968,438	Siderquistm, C.A., Kelly, J.A. and Mandel, F.S.	Gallic acid as an oxygen scavenger	Gallic acid is claimed to scavenge dissolved oxygen from water used for steam generation.
1996/ 5,484,594	Frangi, E., Bertani, M., Mustich, G. and Tuccini, G.	Process for preparing grapeseed extracts enriched in procyanidol oligomers.	Monomers of an extract obtained from *Vitis vinifera* could be removed by membrane filtration or solvent extraction (ethers or esters or mixtures of ethyl acetate and aromatic hydrocarbons).
2000/ 6,022,901 and 2001/ US6,211,247B1	Goodman, D.W.	Administration of resveratrol to prevent or treat restenosis[1] following coronary intervention	*Cis*, *trans* resveratrol, a mixture of them or "a pharmacologically acceptable salt, ester, amide, prodrug or analog" prevents or treats restenosis. Any of these active agents can be used to prevent "the recurrence or progression of coronary heart disease".
2000/ 6,048,903	Toppo, F.	Treatment for blood cholesterol with *trans*-resveratrol	*Trans*-resveratrol intake increases HDL while reducing LDL in blood, which also reduces the risk of hypercholesterolemia.
2000/ 6,123,977	Diamond, G.B.	Food spray containing grape seed oil	Grape seed oil addition increases the smoke point, reduces the flammability of spray and produces nutritionally better food spray with high PUFA, lack of *trans* fatty acids, low saturated fatty acids and bioflavonoid contents.
2001/ US6,245,336B1	Ray, S.D. and Bagchi, D.	Prevention and treatment of acetominophen toxicity with grape seed proanthocyanidin extract	Incorporation of grape seed proanthocyanidin extract intake with acetoaminophen is claimed to reduce the risk or protect sensitive people from toxicity.
2001/ US6,270,780B1	Carson, R.G., Patel, K., Carlomusto, M., Bosko, C.A., Pillai, S., Santhanam, U., Weinkauf, R.L., Iwata, K, and Palanker, L.R.	Cosmetic compositions containing resveratrol	Resveratrol improves the appearance of aged or damaged skin, inhibits proliferation of keratinocytes and melanin production by skin cells. It induces differentiation of keratinocytes and has skin lightening activity. Inflammation of skin caused by alpha-hydroxy acids can be eased by resveratrol.

[1]Restenosis is an accelerated form of atherosclerosis, which can be defined as the reappearance of stenosis (artery stricture) after corrective surgery.

inhibiting the growth of ulcer-causing bacterium, *Helicobacter pylori* (Saito et al., 1998).

One wine phenolic constituent, resveratrol, shows antioxidant properties and may prevent

or suppress cancer (Pace-Asciak et al., 1995).

Frankel et al. (1993) diluted red wine to get 10μmol/L total phenolics (1000 times

dilution) and tested the effect of wine phenolics on *in vitro* inhibition of oxidation of

human LDL. Diluted red wine was able to inhibit LDL peroxidation induced by copper

and the inhibition was independent of the copper concentration. Moreover, the inhibition

was significantly higher than that of α-tocopherol's. Diluted wine and 10μmol/L

quercetin equally inhibited the human LDL oxidation induced by copper.

Analysis of the data obtained from twenty-one industrialized nations (Kolpan et

al., 2001) indicated that there was an inverse relationship between wine consumption and

deaths from heart disease ($r = -0.661$, $p<0.01$) while the correlation was not statistically

significant between per capita wine consumption and overall deaths ($p>0.05$) (Figure 1).

Figure 1. Relation between alcohol consumption and heart disease deaths in
developed nations (data from Kolpan et al., 2001).
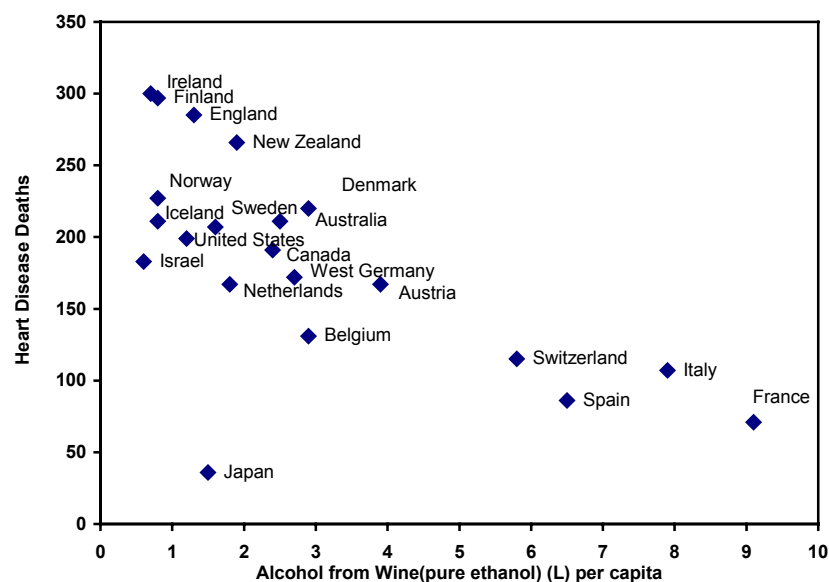
On the other hand, a positive and significant correlation (r = 0.737) was found between wine consumption and death rates from cirrhosis of the liver (p<0.001).

Ethanol metabolism produces mainly hydroxylethyl radicals and metabolism also induces the formation of cytochrome P-450 (CYP2E1), which has a high NADPH oxidase activity rate (Albano et al., 1993). This induction increases the production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) whose formation can lead to hydroxyl radical formation in the presence of iron (Albano et al., 1993). Alcohol abuse can increase oxidative stress and this may damage the liver. Therefore, moderate consumption should be the key in order to acquire beneficial effects from wine.

OXIDATION IN FOODS

During storage, lipids and fats may undergo a series of reactions with molecular oxygen. Oxidation of lipids in foods can simply be defined as their reaction with molecular oxygen, which leads to deterioration reactions and the formation of off-flavor compounds. As a result of lipid oxidation, shelf life of foods is shortened, and overall quality such as health functionality, nutritional benefits and sensory properties can be significantly impaired.

Autoxidation

Unsaturated bonds in polyunsaturated fatty acids are susceptible to reactions with oxygen. Molecular oxygen in direct contact with the food or singlet oxygen formed in the food by the action of radiation or prooxidants is necessary for the reaction to occur. Products of the oxygenation of lipids may be classified as primary, secondary and tertiary products. Rancidity is the final result of oxidative deterioration of lipids. Vegetable oils, fats and dairy products are very sensitive to oxidation; therefore food products containing these susceptible components are supplemented with antioxidants. Rate of the oxidation

reaction in foods can be affected by numerous factors such as oxygen availability, type and degree of lipids unsaturation, antioxidants, prooxidants, packaging, light and temperature. However, the degree of unsaturation is considered to be the determinant factor for the oxidation rate (deMan, 1999).

Steps in the oxidation reaction in lipids and fats consist of three steps; initiation, propagation and termination. In the initiation step, free radical (R$^{\cdot}$) forms and hydrogen is removed from a lipid molecule (RH);

RH $\rightarrow$ R$^{\cdot}$ + H$^{\cdot}$
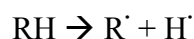
Heat, metal catalysts, singlet oxygen, photochemical reactions, UV or visible light, reaction of molecular oxygen with reductant metabolites or enzymatic processes may influence the formation of free radicals in this first reaction step. These free radicals then react with oxygen and form a peroxyl radical (ROO$^{\cdot}$). The free radicals are self-propagating; therefore a chain reaction occurs. The reactions are as follows:

R$^{\cdot}$ + O$_2$ $\rightarrow$ ROO$^{\cdot}$

ROO$^{\cdot}$ + RH $\rightarrow$ ROOH + R$^{\cdot}$
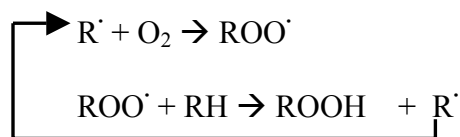
The next step is called propagation. This stage in the reaction chain is where newly formed free radicals abstract hydrogen from another unsaturated lipid molecule. The peroxyl radicals react with lipid and this reaction leads to the formation of hydroperoxides. This reaction is a chain reaction and can repeat up to several thousands times (deMan, 1999).

Final step of autoxidation is the termination step in which the chain reaction is stopped by several mechanisms as shown below. The end products of the termination step are non-reactive.

$$R^{\cdot} + R^{\cdot} \rightarrow R\text{-}R$$
$$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$$
$$nROO^{\cdot} \rightarrow (ROO)_n$$
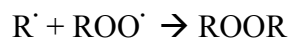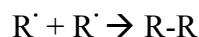
Hydroperoxides formed during the propagation step are primary and major lipid oxidation products. Primary products are not important in flavor deterioration. These products are unstable and they decompose into secondary oxidation products. Secondary oxidation products include carbonyls, aldehydes, alcohols, ketones and acids. It is the secondary products that influence the organoleptic properties of foods. Aldehydes are further oxidized to form fatty acids, and ketones hydrolyze to form short chain acids and aldehydes. Free fatty acids of shorter chain length than the unoxidized original are tertiary oxidation products.

Oxidative deterioration of foods could be prevented by naturally occurring food antioxidants. Antioxidants can slow down the reaction rate very effectively. Antioxidants such as α-tocopherol, β-carotene, ascorbic acid, and thiols can inhibit various oxidation reactions in foods. Tocopherols are the most important one for vegetable oils. Antioxidants can terminate the oxidation reaction at different steps. They may react with free radicals and make the radicals less active for further reactions with other compounds. Trace metals like copper and iron can catalyze the oxidation reaction primarily in the induction step of the reaction to form free radicals. Metal chelating agents like EDTA and citric acid decrease the effect of metals on oxidation. Phenolic compounds have also antioxidant properties. However, at high concentrations they may act as prooxidants. Propyl gallate, BHA, BHT and TBHQ are synthetic antioxidants that can be used in foods. Antioxidants are usually used in combination with each other or in the presence of naturally occurring compounds that are synergistic in their effects.

In addition to lipid oxidation, oxidation of polyphenolic type pigments has also been reported during food processing or storage. Oxidation of pigments may also form off-flavor in addition to discoloration and changes in mouthfeel. Loss of health-functional properties of polyphenols may occur as a result of cross-linking into bigger molecules that are no longer bioavailable (Ory et al., 1985).

REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are also called free radicals. They are molecules with unpaired electrons, and can cause damage on various components of living organisms such as DNA, proteins, lipids and carbohydrates. Some of the common free radicals are shown in the table below (Table 3).

Table 3. Common reactive oxygen species or precursors and their sources (Allard, 2001).

ROS	Half-life (s)	Sources
$O_2^{\cdot-}$ superoxide anion radical	Enzymatic	Oxygen metabolism potentiated by hyperoxia, inflammation, radiation
$OH^{\cdot}$ hydroxyl radical	10^{-9}	Oxygen metabolism potentiated by hyperoxia, inflammation, radiation
H_2O_2 hydrogen peroxide	Enzymatic	Oxygen metabolism potentiated by hyperoxia, inflammation, radiation
$RO^{\cdot}$ alkoxyl radical	10^{-6}	By-products of free radical propagation, lipid peroxidation, prostanoid metabolism
$ROO^{\cdot}$ peroxyl radical	7	By-products of free radical propagation, lipid peroxidation, prostanoid metabolism
$NO^{\cdot}$ nitric oxide radical	1-10	Phagocytes, respiratory burst
$ONOO^{\cdot}$ peroxynitrite	0.05-1	Phagocytes, respiratory burst
$HOCl$ hypochlorite radical	-	Inflammation, phagocytosis
$Q^{\cdot-}$ semiquinones	Days	Mitochondrial electron transport
$^1O_2$ singlet oxygen	10^{-5}	Photosensitization
Fe^{2+}, Cu^{2+} divalent metals	-	Heme (iron) and other metal containing proteins

Peroxyl and Alkoxyl Radicals

Peroxyl radicals can form from a reaction between a free radical and a hydroperoxide. The reaction involved in peroxyl radical formation is as follows:

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R· +O^2 → ROO· or R^+ + ·O^2

Once the hydroperoxides are formed, they undergo the following reactions: (1). Reduction of hydroperoxides with metal ions or a reductant generates alkoxyl radicals and/or hydroxyl radicals (2). At high temperatures, hydroperoxides can give alkoxyl and hydroxyl radicals upon the breakage of the O-O bond (3). Peroxyl radicals can abstract hydrogen from lipids.

1) ROOH + X· → ROO· + XH

2) ROOH $\xrightarrow{[H]}$ RO· +OH- (or RO- + ·OH)

3) ROOH → RO· + ·OH
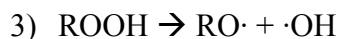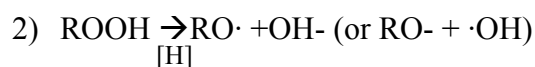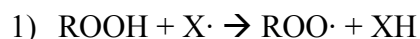
Alkoxyl radicals generated from peroxides or hydroperoxides are more reactive than peroxyl radicals. Their lifetime is however shorter than peroxyl radicals. These radicals can either abstract or add H to double bonds.

Superoxide Ion

Superoxide ions are formed when oxygen reacts with a strong reductant such as a metal, an enzymatic species or another strong reducing agent (Neta and Simic, 1985). Superoxide radical formation in vegetables and fruits may also contribute to their spoilage (Neta and Simic, 1985).

O_2 → O_2-

Hydroxyl Radicals

They are generated from H_2O_2 by a reductant.

$H_2O_2 \xrightarrow{e^-}$ HO· + HO-

Fe^{3+} + O_2- → Fe^{2+} + O_2

H_2O_2 +Fe^{2+} → HO· + OH- + Fe^{3+}
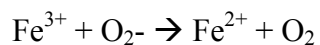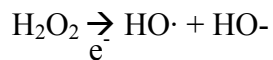
Hydroxyl radicals are an extremely reactive chemical species. Irradiation of water or photolysis of hydrogen peroxide can generate OH radicals (Neta and Simic, 1985). Hydroxyl radicals have a very short life span; therefore the oxidative damage they induce occurs at the site of their generation. Allard (2001) noted that the antioxidative power of phenolic compounds is the result of their ability to scavenge hydroxyl radicals. Hydroxyl radical formation is not associated with any specific enzyme (Allard, 2001).

Singlet Oxygen

Triplet oxygen is the ground state of molecular oxygen. In the singlet form, on the other hand, oxygen is an excited state and its lifetime is longer than that of the triplet form. Singlet oxygen is generated from the ground state by photochemical reactions. It is highly reactive and can react with numerous organic substances.

$$^2O_2 \xrightarrow{\text{H}^+} \, ^3O_2 + H_2O_2$$

$$O_2\text{-} \xrightarrow{[O]} \, ^1O_2$$
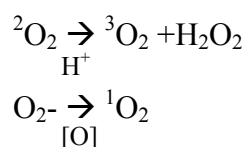
Other Radicals

Biochemical processes in live plant cells and processes used to convert plant materials into foods can cause the formation of numerous free radicals. Radicals can be formed from the oxidation of tyrosine and tryptophan, which in turn can oxidize tocopherols and ascorbic acid in foods. Sulfur-containing aminoacids and peptides can also generate free radicals by the addition of an electron to cystine or by proton abstraction from cysteine. Oxidation of sulfite or bisulfite can form $^\cdot SO_3\text{-}$ radicals, which can oxidize ascorbate and tocopherol (Huie and Neta, 1984).

ANTIOXIDANTS

Antioxidants are substances that can either directly or indirectly scavenge free radical species. They can be natural or synthetic. Antioxidants can be divided into two

categories, primary and secondary antioxidants, depending on their mechanism of action. Primary antioxidants are also called as chain-breaking antioxidants since they can directly react with lipid and peroxyl radicals and convert them into stable non-radical compounds. Their mode of action is through the donation of protons to lipid radicals. Antioxidant radical complex formed as a result of this reaction is more stable and less reactive compared with the lipid or peroxyl radical. Synthetic antioxidants such as BHT, BHA, PG, and TBHQ, and natural antioxidants like tocopherols and carotenoids are examples of the primary antioxidants. Ascorbic acid, α-tocopherol and β-carotene are examples of chain-breaking antioxidants; they scavenge free radicals. Enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase are also known as preventive antioxidants. β-Carotene can also be a preventive antioxidant due to its singlet oxygen quenching capability (Allard, 2001).

$$ROO\cdot + AH \rightarrow ROOH + A\cdot$$

$$RO\cdot + AH \rightarrow ROH + A\cdot$$

$$R\cdot + AH \rightarrow RH + A\cdot$$
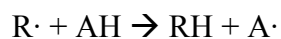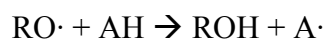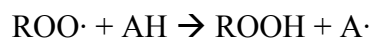
Secondary antioxidants slow down the oxidation rate. However, they are not capable of converting free radicals into nonreactive species. They can chelate metals, provide proton to primary antioxidants, break down hydroperoxides into non-radical products, absorb UV light, deactivate singlet oxygen or scavenge oxygen. Ascorbic acid, ascorbyl palmitate, erythorbic acid, sulfites, lecithin, malic acid, citric acid, tartaric acid, and carotenoids such as β-carotene, lycopene, and lutein are some examples of secondary antioxidants.

Decker (1998) reported that "any compound that has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is capable of donating a hydrogen to that free radical unless the reaction is kinetically unfeasible." The sources of antioxidant with phenolic nature in foods include vegetables, fruits, tea, wine, herbs and spices. Numerous factors can influence the antioxidant activity of plant phenolics. Decker (1998) mentioned some of the factors including "position and degree of hydroxylation, polarity, solubility, reducing potential, stability of the phenolic during food processing operations, stability of the phenolic radical", and acid or ring groups in the phenolic structure. Moreover, glycosylation can also be a factor for the antioxidant activity of plant phenolics (Soleas et al., 2001b).

Metabolic activities in living organisms generate reactive free radical species. Free radicals can also be generated in the human body during microbial infection, and lipid peroxidation may be induced by free radicals like hydrogen peroxide, superoxide anion, singlet oxygen or hydroxyl radical. (Allard, 2001). However, the same reactive oxygen species can also have detrimental effects on cellular components of living organisms. The human body therefore, develops protection against those free radicals including enzymatic and nonenzymatic defense mechanisms (Table 4) (Allard, 2001). In living bodies, superoxide dismutase, catalase, and glutathione peroxidase have protective activities against reactive species such as superoxide ion, H_2O_2, and hydroperoxides, respectively.

During viral infections such as influenza, hepatitis, and HIV, the human body increases the amount of free radicals generated, which in turn, causes an increase in oxidative stress in the body. These conditions weaken the antioxidant defense

mechanism. Although free radicals may have an important role in the elimination of viral infection, they may also enhance the replication of viruses through the activation of some viral transcription factors, e.g. NF-κB (Allard, 2001). Although the mechanism of elimination of viral infection from the body could be very complex, use of antioxidants during infection may be beneficial (Allard, 2001).

Table 4. Enzymatic and nonenzymatic defense mechanisms against reactive oxygen species (Allard, 2001).

System	Role
Nonenzymatic	
Lipid-Soluble	
α-tocopherol	Radical chain breaker/scavenger
β-carotene	Singlet-oxygen quencher
Lycopene/carotenoids	Singlet-oxygen quencher
Ubiquinol-10	Radical scavenger
Water Soluble	
Ascorbate	Radical chain breaker/scavenger
Glutathione	Diverse antioxidant functions
Urate	Radical scavenger
Biliruin	Radical scavenger
Albumin	Radical scavenger
Transferrin, ceruplasmin	Sequesteration of metal by chelation (iron, copper)
Lactoferrin, haptoglobin	Sequesteration of metal by chelation (iron, copper)
Enzymatic	
Superoxide dismutase	Dismutase superoxide
Glutathione peroxidase	Decomposition of H_2O_2 and LOOH
Catalase	Decomposition of H_2O_2
Glutathione-S-transferase	Decomposition of LOOH
Glutathione reductase	Maintaining GSH levels
NADPH-quinone oxidoreductase	Two-electron reduction
NADPH-supply transport systems	GSSG export/thioredoxin reductase
Repair systems	DNA repair enzymes

Antioxidants when taken in combination are more effective than individual antioxidants (Knudsen et al. 1996). Arts et al. (2001) reported that antioxidant activities of quercetin, rutin, catechin and 7-monohydroxyethylrutinoside are not additive because

of the interactions between the antioxidant and other substances present in the environment.

FLAVONOIDS

The flavonoids are defined as "a class of plant secondary metabolites derived from the condensation of a cinnamic acid with three malonyl-CoA groups" (Bloor, 2001). They are generally categorized as phenolics or polyphenols because of their chemical structure (Figure 2). Over 4,000 flavonoids have been identified. Although flavonoids are responsible for the color of fruits and vegetables, there are also colorless flavonoids in nature. Structural differences in their chemistry could be small, their UV spectra could be quite different from each other. This property of flavonoids makes it easy to differentiate one from the other and quantify their concentration in a sample.

Figure 2. Basic structure of flavonoids
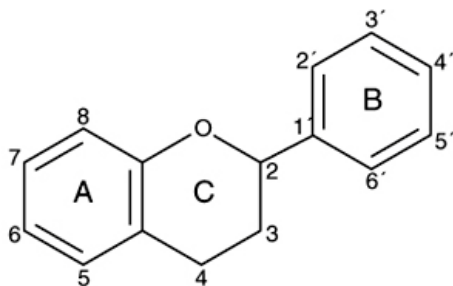
Flavonoids can be divided into 14 classes based on the level of oxidation in the structure of ring C (Seigler, 1995) (Figure 2). However, the major dietary flavonoids are often classified under six groups (Peterson and Dwyer, 1998) shown in Figure 3;

- Anthocyanidins (e.g. delphinidin, cyanidin, petunidin, peonidin, and malvidin). These are colored flavonoids and are responsible for the red, blue and violet coloration of fruits and vegetables such as berries, grapes, cherries, and eggplant, and wine.

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- Flavonols (e.g. quercetin, kaempferol, and quercetagetin). Onions, berries, cherries, broccoli, apples, grapefruit, tea and red wine are rich sources of flavonols.
- Flavanols (also called proanthocyanidins, flavan-3-ols or catechins, e.g. catechin, epicatechin, epicatechin gallate, and epigallocatechin-3-gallate). Black grapes, red wine, and tea are good sources of flavanols.
- Isoflavonoids (isoflavones, e.g. genistein, diadzein, formononetin, and biochanin A, and coumestans, e.g. coumestrol),
- Flavones (e.g. rutin, apigenin, luteolein, and chrysin). Onion, apple skin, berries, tea, lemon, olive, celery, and red pepper are good sources of this group.
- Flavanones (e.g. myricetin, hesperidin, naringin, and naringenin). Citrus fruits, especially orange juice, are rich in flavanones.

Figure 3. Molecular structures of major dietary flavonoids

Anthocyanidins

Flavonols

Flavanols

Isoflavonoids

Flavones

Flavonones
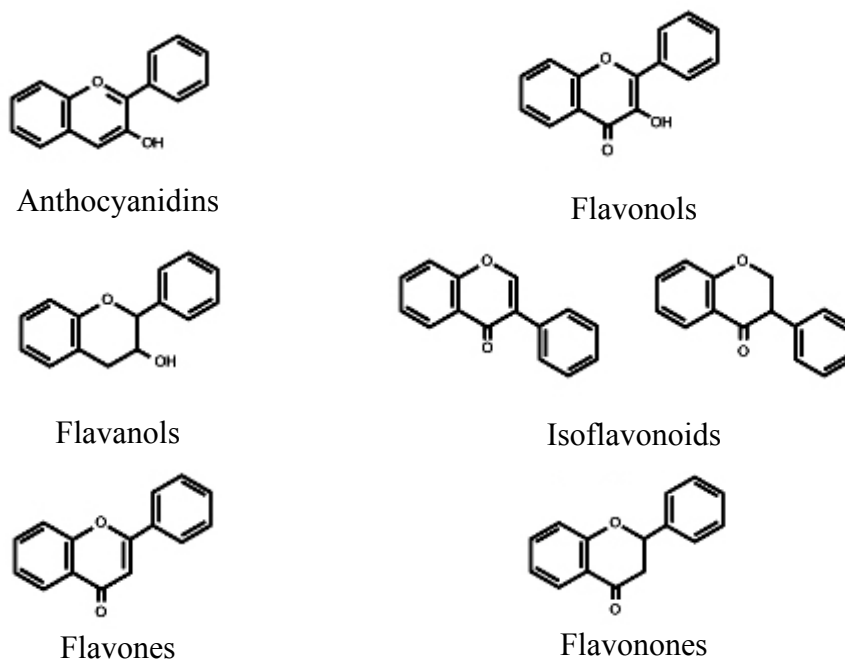
These flavonoids are present in nature as glycosides, and sugar moiety attached to a flavonoid molecule is an important factor in determining its absorption from the

intestinal tract and their bioavailability. Hollman and Katan (1999) reported that glycosides of the flavonoid quercetin from onions are absorbed more than aglycons. Glycosylation lessens the reactivity of flavonoids against free radicals and increases their solubility in water (Rice-Evans et al., 1997). Glucose is the main sugar moiety in glycosylated flavonoids, but galactose, rhamnose, xylose and arabinose can also occur.

Flavonoids may have diverse functions in nature such as protecting plants from UV radiation, insects and mammalian herbivory (Haslam, 1989), antimicrobial activities, medicinal properties (Harborne and Williams, 2000), antioxidant activities and internal signaling properties for plant growth (Woo et al., 2002). Plant polyphenols were thought to be undesirable constituents of plants as a food source due to the astringency in the mouth, which could be as a result of their complexation with glycoproteins (Haslam, 1989). Plant polyphenols may have physiological functions in plants as well as pharmacological functions in living organisms. While the former includes plants chemical defense mechanism, the latter has numerous manifestations. The astringent nature of plant polyphenols makes the plant unattractive to potential predators. Plant polyphenols participate in the perception of food flavors in the mouth. They give an astringent taste to various foods and beverages. The loss of astringency during the ripening of fruits has been attributed to the polymerization of plant tannins. This polymerization can be influenced by several factors such as maturity, variety, and climate.

Some of the polyphenolics are known to have potential antioxidant activities. Many herbal medicines used to treat vascular, viral, gastrointestinal and microbial diseases and inflammatory diseases may contain plant polyphenols (Haslam, 1989). Their

medicinal properties make them a potential group of compounds beneficial to human health. The average daily intake of flavonoids in the US diet may be only a few hundred milligrams (Hollman and Katan, 1999). Dietary intake of flavonoids is usually underestimated because of the difficulty in measuring the amount of all flavonoids present in the consumed foods. Peterson and Dwyer (1998) reported that daily intake of flavonoids could range from 23mg/day to 1000mg/day. Hollman and Katan (1999) reported that in the Netherlands, approximately half of the daily flavonol and flavone intake comes from tea.

The biological effects of flavonoids in reducing the risk of cardiovascular diseases are possibly associated with their antioxidant properties. These effects also include protection of tissues against free radical attack and lipid peroxidation. The effects of flavonoids on atherosclerosis, cancer, inflammation, and plasma cholesterol level have been investigated by many researchers. However, results of epidemiological studies are somewhat conflicting regarding proof of an inverse relation between incidence of cancer and dietary flavonoid intake (Hollman and Katan, 1999).

Teissedre and Landrault (2000) reported that flavonoids scavenge aqueous free radicals easily, which could be due to their amphipatic characteristics (Riou et al., 2002). Polyvalent phenols in flavonoid structures give some of flavonoids a metal chelating ability (Reische et al., 2000). Phenolic acids are precursors of flavonoids. Phenolic acids such as hydroxycinamic (caffeic, coumaric, ferulic, and sinapic acids), hydroxycoumarin (scopoletin), and hydroxybenzoic acids (ellagic, gallic, and vanillic acids) can form metal complexes (Reische et al., 2000). Flavonoids can also scavenge superoxide anions.

Quercetin, myricetin and rutin can effectively inhibit superoxide anions generated by either enzymatically or chemically (Robak and Gryglewski, 1988).

The most common flavanols found in plants are catechin, epicatechin, gallocatechin and epigallocatechin. Besides these, catechin monomers, dimeric, trimeric, oligomeric and polymeric catechins are also present in a number of fruits, vegetables, tea and wine. Oxidation of flavanols by enzymes during the fermentation is responsible for the formation of black pigments, which include mainly theaflavin and its gallate esters and thearubigins. Theacitrin A, which is a yellow unstable thearubigin compound present in black tea leaf was also identified from black tea (Davis et al., 1997).

Catechin

Catechin (C) is a flavanol found in vegetables, fruits, tea and wine. It is also a monomer, which forms dimeric, trimeric and oligomeric proanthocyanidins of grape berries including their skins and seeds. Catechin degrades during fermentation process of black tea and this degradation is dependent on the fermentation temperature (Obanda et al., 2001). Chocolate is also rich in polyphenols such as catechins and procyanidins (Wollgast and Anklam, 2000a and 2000b).

Catechin is reported to have hydroxyl (Moini et al., 2002), peroxyl (Scott et al., 1993), superoxide (Bors and Michel, 1999) and DPPH (Fukumoto and Mazza, 2000) radical scavenging activities. Moreover, it can chelate iron (Morel et al, 1993). Nakao et al. (1998) found that epicatechingallate (ECG), epicatechin and catechin have a peroxyl radical scavenging activity ten times higher than L-ascorbate and β-carotene when tested on bacteria. Nanjo et al. (1996) reported that DPPH radical scavenging activity of catechin and epicatechin is less than epigallocatechin (EGC), ECG, and epigallocatechin gallate (EGCG).

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Cell suspension cultures of *Vitis vinifera* were reported to produce piceid (a resveratrol glucoside), epicatechin and catechin (Teguo et a., 1996).

Epicatechin

Epicatechin (EC) is a monomeric flavanol, which is found in vegetables, fruits, wine and tea. Black tea contains mainly gallates of epicatechin. Obanda et al. (2001) found that ECG and EGCG are 'the main residual catechins in black tea'. In black tea, green tea and oolong tea, EGC, EC, EGCG and ECG are present, but not catechin (Khokhar et al., 1997). Catechin and epicatechin are also present in apples and their concentration is dependent on fruit development and ripening (Awad et al., 2001).

Epicatechin is able to scavenge hydroxyl radicals (Moini et al., 2002), peroxyl radicals (Liu et al., 2000), superoxide radicals (Bors and Michel, 1999), and DPPH radicals (Fukumoto and Mazza, 2000).). Peroxyl radical scavenging activity of EC was found to be ten times higher than L-ascorbate and β-carotene (Nakao et al., 1998).

Gallic Acid

Gallic acid is a phenolic acid found in a variety of plants. Red wine contains gallic acid, trans-resveratrol, quercetin and rutin (Lopez et al., 2001). Gallic acid, catechin, and epicatechin are present in cranberry juice (Chen et al., 2001). Grape juice was reported to have gallic acid at a concentration of 12.5μg/g and ellagic acid at a concentration of 2.5μg/g (Amakura et al., 2000).

Gallic acid is a peroxyl radical (Liu et al., 2000) and DPPH radical (Sanchez-Moreno et al., 1999; Fukumoto and Mazza, 2000) scavenger. Gallic acid was reported to have antioxidant activity at stomach pH (Gunckel et al., 1998). Gallic acid has also antifungal activity (Shukla et al., 1999). This phenolic acid was interestingly detected in an Egyptian mummy (Mejanelle et al., 1997). Tannin from a plant origin used for

mummification in ancient Egypt was reported to be the source of gallic acid and inositol in the mummy.

Gallic acid intake up to 5g/kg body wt was shown to be non-toxic in mice (Rajalakshmi et al., 2001). A toxicological study indicated that NOAEL (no-observed-adverse-effect level) for gallic acid intake was 119 and 128 mg/kg/day for male and female rats, respectively (Niho et al., 2001).

Ellagic Acid

Oak contains gallic acid and ellagic acid esters (Mammela et al., 2000). Phenolic acid ellagic acid is present in green grape, black grape, cherry juices (Shahrzad and Bitsch, 1996), acacia, and chestnut (Bianco and Savolainen, 1997).

BIOAVAILABILITY OF FLAVONOIDS

Bioavailability of flavonoids has been demonstrated by many researchers. Maiani et al. in 1997 successfully determined the levels of tea polyphenols in human plasma such as catechin, epicatechin, caffeic acid, and epigallocatechin using an HPCL with photodiode array and electrochemical coularray detectors. Ruidavets et al. (2000) screened catechin concentrations of plasma obtained from 180 human subjects and found that plasma catechin levels were the highest when diet included vegetable, fruit and wine like in a European Mediterranean diet.

Martinez-Ortega et al. (2001) found that stability of phenols in wine samples was higher than in hydroalcoholic phenolic solution under gastrointestinal conditions. They also reported resistance of resveratrol and related compounds to gastrointestinal treatment (Martinez-Ortega et al., 2001).

Simonetti et al. (2001) found that dietary hydroxycinnamates (caffeic acid) are bioavailable. Soleas et al. (2001a) showed for the first time that some absorption of *trans-*
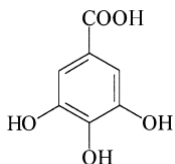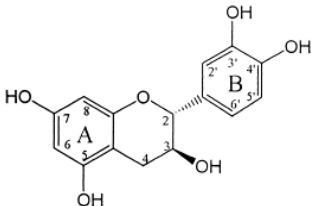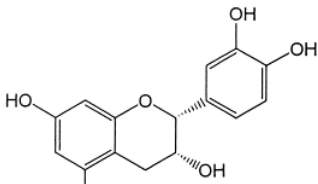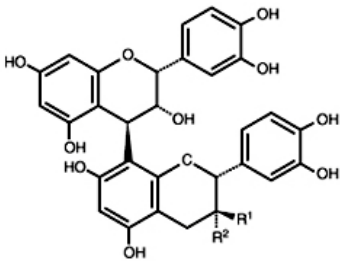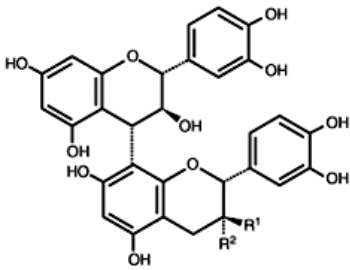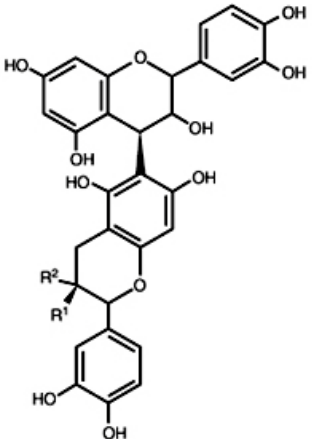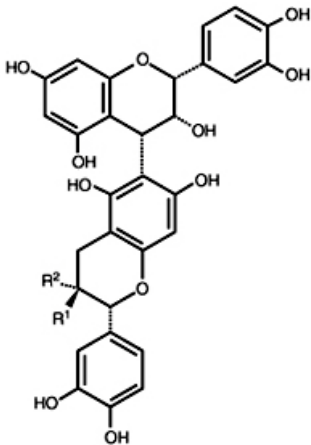
resveratrol is possible in humans. Soleas et al. (2001b) speculated that glycosides of resveratrol could be bioavailable, and would exhibit biological activities similar to that of resveratrol.

Proanthocyanidins are also major polyphenolic components of red wine. Yamakoshi et al. (1999) detected proanthocyanidins in the plasma of cholesterol-fed rabbits but not in the lipoproteins. They also noted that increasing the amount of proanthocyanidins did not increase their level in plasma further. Therefore, the authors concluded that there was a limit in the amount of proanthocyanidins absorbable in blood.

FLAVONOIDS OF GRAPE SKINS, SEEDS AND WINE

Anthocyanins, phenolic acids, and flavanols (catechins) are major flavonoids of grape berries and wine. Red color of wine comes from the anthocyanins of grape berries. Grape skins and seeds have gallic acid, monomeric catechins as well as dimeric catechins (Figure 4). These dimeric catechins include Procyanidins B_1 (epicatechin- (4β-8)-catechin), B_2 (epicatechin- (4β-8)-epicatechin), B_3 catechin- (4α-8)-catechin, and B_4 (catechin- (4α-8)-epicatechin). Besides those monomeric and dimeric catechins, grape skins and seeds have trimeric, oligomeric and polymeric proanthocyanidins. Gallic acid, catechin and epicatechin were identified as the main phenolic compounds in grape seeds (Palma and Taylor, 1999). Resveratrol, a minor grape skin constituent with phenolic structure, received much attention because of its antimutagenic, anticarcinogenic, and antioxidant activities. The stilbene resveratrol is present in nature mostly in its trans form, and fungal infection of grape berries induces its production since it has antifungal activity.

Figure 4. Structures of phenolic acids, monomeric and dimeric proanthocyanidins present in grape seeds and skins

Constituent	Chemical Structure
Phenolic acid	Gallic acid
Monomers	(+)-Catechin (-)-Epicatechin
Procyanidin dimers	B$_1$ R^1=OH, R^2=H B$_3$ R^1=OH, R^2=H B$_2$ R^1=H, R^2=OH B$_4$ R^1=H, R^2=OH B$_5$ R^1=H, R^2=OH B$_6$ R^1=OH, R^2=H B$_7$ R^1=OH, R^2=H B$_8$ R^1=H, R^2=OH

Kennedy et al. (2000) investigated the effect of the maturity of grape berries and vine water status at time of harvest on flavanol monomers and procyanidins contents. The reduction in the polyphenol contents of the berries was 90% for flavanol monomers and 60% for procyanidins during fruit ripening. The most dramatic reduction in monomers was on epicathechin gallate (ECG), then C, and finally EC. Lower flavanol monomer content of seeds was found in grape seed grown in minimally irrigated lands.

Romero-Perez et al. (2001) reported the concentrations of *trans*-resveratrol as 26.25μg/g dry skin in Chardonnay and 38.26μg/g dry skin in Merlot grape berries (Table 5). The total concentration of both isomers of resveratrol and *cis*-piceid in Merlot skins were approximately 13 times higher than in Chardonnay skins.

Table 5. Concentrations of resveratrol and its glycosides in dry Chardonnay and Merlot grape skins (Romero-Perez et al., 2001).

Grape variety	°Brix	μg/g of dry skin			
		trans-resveratrol	*trans*-piceid	*cis*-piceid	Total amount
Chardonnay	18	26.25	12.13	39.68	78.22
Merlot	16	38.26	342.66	645.47	1026.39

Romero-Perez et al. (2001) also reported increased amounts of *trans*-resveratrol (~12fold) and *cis*-piceid (~8fold) in highly infected grape skins. They noted that infection of grapes with *Botrytis cinerea* increased the production of resveratrol in grapes, but the concentration of resveratrol was lower because the resveratrol was degraded by a laccase-like stilbene oxidase. The authors pointed out that this problem could be alleviated by infecting the grape with *Uncinula necator*, which is incapable of degrading resveratrol. The presence of *trans*-piceid was also reported in *Polygonum cuspidatum*. (Romero-Perez et al., (2001).

Palomino et al. (2000) found none or trace levels of polyphenols, both isomers of resveratrol, quercetin, quercitrine and rutine in the pulps of Spanish grape berries. Protection of grapes with a preservative paper after collection was responsible for the poorer polyphenol content compared with the unprotected grapes. Palomino et al. (2000) also found that protected berries had lower *trans*-resveratrol than unprotected ones. Peels from unprotected grape berries had 15μg/100g *trans*-resveratrol, which was twice more than protected berry peels had. In conclusion, skins of grape berries had more rutine, *trans*-resveratrol, and quercetin concentrations than either whole berries or pulp.

Teissedre and Landrault (2000) quantified the levels of various polyphenolic substances in numerous French wines, and found that total phenolic contents of white wines were much less (~0.1 times) than those of red wines (Table 6).

Table 6. Important phenolic substances present in red Merlot and white Chardonnay wines (Teissedre and Landrault, 2000).

Phenolics mg/L	Merlot Wine	Chardonnay Wine
Gallic acid	19.0±5.3	2.0±0.30
Catechin	76.0±36.5	4.0±2.30
Epicathechin	87.0±60.9	1.0±0.90
Dimer B_1	50.0±26.5	0.5±0.10
Dimer B_2	47.0±27.6	0.3±0.67
Dimer B_3	25.0±12.2	0.2±0.14
Dimer B_4	45.0±13.5	0.5±0.45
Malvinidine3-glucoside	91.0±22.3	-
Total phenolic content (mg GAE/L)	2000.0±514.0	245.0±63.00

Resveratrol contents of wines are influenced by various factors like physiological status of the grape berries, and processing conditions during winemaking. Vrhorsek et al. (1997) studied the effect of two yeast strains (one with high β-glucosidase activity), malolactic fermentation or fining reagents on the levels of resveratrol (both free and

bound forms) in Pinot noir (1994) wines from Austria. Yeasts with high β-glucosidase activity increased *cis*- and *trans*-resveratrol concentrations, and decreased *trans*-resveratrol glucoside. Malolactic fermentation, however, had a less significant effect on resveratrol levels than yeasts. Gelatin, on the other hand, did not affect any of the resveratrol forms in wine. Aglycon form of resveratrol binds to polyvinyl-polypyrrolidone (PVPP), a white powdered fining agent, up to 90%. Glucosides, however, show low binding (< 10%) to PVPP (Vrhorsek et al., 1997). PVPP is used to remove polyphenols that cause haze beer or pink color formation in white wine.

Revilla and Ryan (2000) reported that fewer peaks appeared on white wine chromatograms compared to those for red wine. Catechin levels in both grape pulp and white grape skin extracts, and the levels of catechins and oligomeric procyanidins in red grape skins were low. Peaks in chromatogram at 280nm (10-60 min retention time) for grape seed extract were reported to correspond to catechins and oligomeric procyanidins. Revilla and Ryan (2000) detected catechin, epicatechin, procyanidin B_1 and B_2 in grape seed extracts. The authors did not detect *trans*-resveratrol in wines, and they found the concentration of that compound to be very low in grape skin extracts. They reported that low *trans*-resveratrol levels may be a result of reduced incidence of mold infection or physiological stress. Perhaps laccase, an enzyme that degrades stilbenes, reduced the concentration of stilbene *trans*-resveratrol in wine as Romero-Perez et al. (2001) reported. The presence of this enzyme could explain low levels of resveratrol in wines made from grapes highly infected with mold. On the other hand, the reason for the low level of resveratrol in grape skin extract is still unknown.

Riou et al. (2002) studied the aggregation of grape seed tannins into colloidal particles in wines. They reported that hydrophobic (aromatic rings) and hydrophilic (hydroxyl groups) constituents of polyphenols give them amphipathic characteristics. This property of polyphenols provides both hydrophobic effects and the formation of hydrogen bonds. Riou et al. (2002) noted that procyanidin polymers of grape seed tannins are mainly composed of C, EC and ECG units; therefore these polymers could aggregate and form colloidal particles. The size of the particles depends on several factors such as the mean degree of polymerization, concentrations of these molecules and presence of other compounds. The authors also reported that aggregation of seed tannins could be prevented by mannoproteins, whereas rhamnogalacturan increases the particle growth. The authors suggested that the results could be useful to the understanding of aggregation of tannin polymers during aging, clarification and stabilization of wines.

FLAVONOIDS AND HEALTH

Cells of living organisms have two major defense mechanisms against damage induced by free radicals. These are: a) enzymatic, e.g. superoxide dismutase, glutathion peroxidase, catalase and b) nonenyzmatic, e.g., dietary antioxidants such as vitamin E and glutathion. Foods and foodstuffs are the major agents in the non-enzymatic defense system. Fruits, vegetables, tea and wine contain a variety of antioxidants. Flavonoids have dietary importance for humans due to their widespread presence in fruits, vegetables, tea and wine and their antioxidative characteristics. High flavonoid content of grapes makes grapes and grape products important in the diet. Grape seed contains some compounds that are able to scavenge superoxide radicals (Bouhamidi et al., 1998). Grape seed contains high amounts of polyphenol proanthocyanidins, which are the oligomers of

flavan-3-ol units, especially catechin and epicatechin. Dimeric proanthocyanidins are the simplest ones, and they have $4\rightarrow8$ linked monomers. B_1, B_2, B_3 and B_4 are the most common dimers. These are followed by less common $4\rightarrow6$ linked isomers such as B_5, B_6, B_7 and B_8 (Figure 4). Trimers of procyanadins have C_1 isomers. Grape, apple, hawthorn, elderberry, chokeberry, sour cherry and blackcurrant have proanthocyanidin content between 0.3-0.9 g/kg (Wilska-Jeszka, 1996).

Grape polyphenols play an important role in color, taste (especially astringency) and stability of wine. Anthocyanins and proanthocyanidins are main polyphenolic compounds in red wines. Proanthocyanidins are also known as condensed tannins, which can be found in grape skins as procyanidins and prodelphinidins. Grape seeds contain proanthocyanidins. During fermentation of grapes, these tannins are extracted into wines. The winemaking process has a large influence on the type and level of phenolic compounds in wines.

Besides grape seeds which contains the procyanidin B_2, quince is another good source where the dominant procyanidin at a level of 2g/kg is B_2 (Wilska-Jeszka, 1996). Takahashi et al. (1999) evaluated the safety of procyanidin B_2 obtained from apple juice, using bacteria, cell cultures, etc (Table 7).

Table 7. Safety test results for procyanidin B2 (Takahashi et al., 1999).

Effect	Medium	Result
Mutagenicity	*S. typhimurium* and *E. coli*	Negative
Acute subcutaneous injection	Rats	Negative
Dermal irritation	Male rabbits	Negative
Skin sensitization	Guinea pigs	Negative
Eye irritation	Rabbits	Slight irritation of conjuctivae, none in cornea or iris

Procyanidin B_2 was found to be non-mutagenic in bacterial cultures, or mice tested using the micronucleus tests, and the lethal dose was found to be greater than 2g/kg, in animal models (Takahashi et al., 1999). No serious irritation was found for doses up to 2g/kg on skin or eye of animals (Takahashi et al., 1999). Procyanidin B_2 from apples was also found to induce *in vivo* hair growth (Takahashi, 2001).

Using dogs, monkeys and humans, Folts (2002) found that consumption of red wine (5mL/kg) and purple grape juice (5-10mL/kg) resulted in high blood antiplatelet activity. Moreover, consumption of purple grape juice caused further protection in the patients against the oxidation of LDL cholesterol. He suggested that the flavonoids present in purple grape juice and red wine 'may inhibit the initiation of atherosclerosis'.

Regulation of the gene expression by flavonoids were also reported but the level of flavonoids in plasma or tissues is still a concern since "steady state plasma concentrations of flavonoids are usually not much higher than $1\mu M$ even in populations that consume large amounts of plant material" (Kuo, 2002). "This concentration is relatively low compared to the concentrations of flavonoids that were commonly used in cell culture systems to demonstrate their effectiveness. Nevertheless, evidence exists that some flavonoids may accumulate in the cell" (Kuo, 2002).

Flavonoids have been reported to play an important role on the inhibition of carcinogenesis, mutagenesis, and ulcer. These activities of flavonoids are perhaps related to their *in vivo* and *in vitro* antioxidative activities. Hair growth can also be induced by flavonoids.

Antioxidant Activities of Flavonoids

Grape berry components (skin, pulp and seed) contain a variety of flavonoid monomers, dimers, trimers, oligomers and polymers. Because of the wide variety of

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flavonoids in the plant kingdom, the discussion in this dissertation will be limited to the phenolic constituents of grape berries such as gallic acid, (+)-catechin, (-)-epicatechin, procyanidin dimers, trimers, oligomers, and polymers, resveratrol and ellagic acid. However, available information on foods with dietary significance (e.g. tea) will also be presented.

Antioxidant activities of flavonoids can be determined by numerous methods such as trolox equivalent antioxidant capacity (TEAC) (Rice-Evans and Miller, 1995), ferric-reducing ability of plasma assay (FRAP) (Benzie and Strain, 1996), total peroxyl radical trapping parameter (TRAP) (Wayner et al., 1985), oxygen radical absorbing capacity (ORAC) (Cao and Prior, 1999), color reduction of 2,2-Diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid reactive substances (TBARS) test and electron paramagnetic resonance (EPR) spectroscopy (Bors et al., 2001). The last one not only determines the antioxidant activity of flavonoids but also gives information on the structures of radical intermediates. In some of these methods, the free radical is produced with various chemical or enzymatic reagents. 2,2'-Azobis(2-amidinopropane) dihyrochloride (AAPH) is used as a peroxyl radical generator and this reagent produces radicals linearly with time at 37°C. AAPH is used in the ORAC assay. TEAC uses 2,2-azobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS$^{\bullet+}$) to produce the free radical. A mixture of FeCl$_3$-EDTA, hydrogen peroxide (H$_2$O$_2$) and ascorbic acid can also be used to generate unique hydroxyl radicals for an antioxidant assay using DPPH• . The method is called the DPPH method. Horseradish peroxidase can be used in EPR spectroscopy to determine potential antioxidant activity of polyphenolic compounds.

In Vitro Antioxidant Activity

A number of studies involve *in vitro* antioxidant activities of flavonoids found in vegetables, fruits (especially berries) and tea. Structure of flavonoids could be important in determining their antioxidant properties (Morel et al., 1993). Flavonoids are able to scavenge the radicals of hydroxyl ($^\bullet$OH), peroxyl (ROO$^\bullet$), superoxide ($O_2^{\bullet-}$), nitric oxide (NO$^\bullet$) and DPPH. Besides the free radical scavenging activities of the flavonoids, metal chelating properties of phenolic flavonoids are also studied extensively.

Ricardo da Silva et al. (1991) found that monomeric and polymeric grape seed proanthocyanidins including catechin, epicatechin, epicatechin-3-*O*-gallate, procyanidin B_2, B_5, B_2-3-*O*-gallate, B_2-3'-*O*-gallate, C_1, and two trimers scavenge superoxide and hydroxyl radicals but catechin monomers scavenge especially hydroxyl radicals. They noted that esterification position in the oligomeric proanthocyanidins had an effect on the ability of the molecule to scavenge free radicals. Comparing the superoxide and hydroxyl radical scavenging abilities of these compounds with trolox, Ricardo da Silva et al. (1991) concluded that procyanidin B_2-3'-*O*-gallate scavenge the oxygen free radicals the most effectively. Another *in vitro* study indicated that epicatechin, gallic acid, and other green tea polyphenols had peroxyl radical scavenging activity and reduced low density lipoprotein peroxidation induced by benzophenone or AAPH (Liu et al., 2000).

trans-Piceid is a glucoside of resveratrol. Using the DPPH method, Cuendet et al. (2000) found that *trans*-piceid can scavenge free radicals as effectively as BHT. *trans*-Piceid was able to scavenge peroxyl radicals generated by AAPH, but this property was less than that of trolox or chlorogenic acid.

Flavonoids including catechin, quercetin and kaempferol inhibited linoleic acid oxidation more efficiently than methyl linolenate oxidation (Torel et al., 1986). The ability of flavonoids to donate protons terminates the oxidation chain reaction of lipids (Torel et al., 1986). Tedesco et al. (2000) determined the antioxidant activity of resveratrol, quercetin and oak barrel aged red wine extract using human erythrocytes containing free radicals induced by hydrogen peroxide, and found that nonalcoholic constituents of red wine showed antioxidant activity, but oak barrel aged wine had a higher antioxidant activity than resveratrol or quercetin.

Catechin was shown to possess antioxidant activity in human plasma by delaying the degradation of endogenous α-tocopherol and β-carotene and by inhibiting the oxidation of plasma lipids (Lotito and Fraga, 1997).

Ohshima et al. (1998) reported that epigallocatechin gallate, myricetin and quercetagetin induced breaks in DNA single strands in the presence of nitric oxide (NO); but catechin, epicatechin and gallic acid produced fewer breaks on DNA strands than the former compounds. Catechin, epicatechin and gallic acid at a concentration of 0.5mM inhibited DNA strand breaks more than 90% in the presence of 0.5mM peroxynitrite. These three compounds were also reported to reduce the number of DNA strand breaks in the presence of nitroxyl anion (NO$^-$). Ohshima et al. (1998) successfully showed that some flavonoids such as catechin, epicatechin and gallic acid have an *in vitro* antioxidative activity, and these compounds can scavenge nitric oxide, peroxynitrite and nitroxyl anion radicals.

Constituents of green tea, epigallocatechin, epigallocatechin gallate and pyrogallol showed antioxidant activity by scavenging DPPH radicals (Zhu et al., 2001).

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Sanchez-Moreno et al. (1999) found that gallic acid, resveratrol and tannic acid equally inhibited lipid peroxidation while this inhibition was higher than BHA or D-L-α-tocopherol. Using DPPH, they found that gallic acid had the highest free radical scavenging activity while D-L-α-tocopherol and resveratrol had the lowest. The authors also reported higher antioxidant activities in wines, especially red wine than in grape juices.

Polyphenols are able to chelate metal ions. Polyphenols present in tea have iron-chelating activity (Grinberg et al., 1997). Catechol units are reported to have the ability to chelate iron (Hider et al., 2001). Morel et al. (1993) investigated antioxidant and iron-chelating activities of catechin, quercetin and diosmetin (methylated quercetin) using cell cultures. Antioxidant activities were in the order catechin > quercetin > diosmetin on their inhibition of lipid peroxidation. They showed that these three flavonoids could chelate iron *in vitro*, which can explain the reduction in lipid peroxidation.

In Vivo Antioxidant Activity

Antioxidant activities of flavonoids were extensively investigated *in vitro* by many researchers, but not many of the more valuable *in vivo* studies are available in the literature. The majority of researches indicated that flavonoids in foods with plant origin have *in vitro* antioxidative activity against free radicals. TEAC, ORAC, FRAP, or DPPH methods are mostly used by researchers to determine and compare antioxidant activities of plant materials. Some procedures like the ORAC can be also used to determine antioxidant capacity of blood and cellular materials in living organisms (Cao and Prior, 1999). *In vitro* studies also include assays that use cell cultures to test the reaction of cells to potential antioxidant substances. *In vivo* studies, on the other hand, use animal or

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human models to determine the antioxidant or prooxidant potential of food materials under live physiological conditions. These studies are considered more valuable in terms of antioxidative potential of phytochemicals in the body.

In vivo studies involve mostly free radical scavenging properties of flavonoids under live physiological conditions. Free radicals could be formed as a result of metabolic activities. The human body has its own defense mechanism for inhibiting tissue damage from free radicals. The enzymatic defense system includes superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase (Das and Maulik, 1994). A majority of the studies indicated that antioxidants from plant origins for example some flavonoids are part of the non-enzymatic defense system although Zhang et al., (1997) found inhibition of gluthathione reductase by phenolic compounds of plants at various concentrations. Polyphenols in grape seed extracts may reduce the levels of plasma cholesterol in humans with elevated plasma cholesterol levels. Reduction of LDL levels has been reported in humans. An inverse relationship was found between the level of flavonoids in human diet and coronary disease mortality (Palomino et al., 2000).

An ex vivo study showed that grape seed procyanidins (GSPC) might reduce the oxidation of polyunsaturated fatty acids in mouse liver microsomes (Bouhamidi et al., 1998). Addition of 2mg/L GSPC inhibited the oxidation of arachidonic and docosahexaenoic acids significantly upon oxidation induced with UV-C irradiation ($200\mu W/cm^2$ for 24h). Monomers of GSPC, epigallocatechin and epigallocatechin-gallate were shown to be ineffective in protecting microsomal polyunsaturated fatty acids from oxidation compared to natural GSPC which also contains significant amounts of dimers and oligomers of flavanols besides monomers (Bouhamidi et al., 1998).

An *in vivo* study with dog, monkey and humans showed that consumption of purple grape juice caused further protection in the patients against the oxidation of LDL cholesterol; therefore, flavonoids present in purple grape juice and red wine may play a role in the inhibition of initiation of atherosclerosis (Folts, 2002).

Sato et al. (2001) showed that GSPE acted as an *in vivo* antioxidant. Bagchi et al. (1998) found that water-ethanol extracts of red grape seeds reduced the production of free radicals including superoxide anions in mouse macrophages. The reducing effect was more than that of vitamin C, β-carotene or vitamin E succinate at the same concentrations. The same study also indicated that grape seed extracts could reduce lipid peroxidation in liver and brain. The incorporation of grape seed procyanidin extract into mouse diet showed protection against DNA fragmentation and this protection was dose dependent.

Resveratrol is a stilbene, which is produced in response to fungal infections like *Botrytis cinerea* and environmental stress. In rats, inhibition of platelet aggregation and LDL oxidation and protection of liver from lipid peroxidation by resveratrol were reported as well as its anticarcinogenic activities (Palomino et al., 2000).

Tebib et al. (1997) fed rats with a high cholesterol-vitamin E-deficient diet to study the effect of grape seed tannins on antioxidant enzyme activity, total glutathione and the extent of lipid peroxidation in several tissue samples. A vitamin E deficient diet reduced the levels of enzymatic antioxidants such as catalase, glutathione peroxidase and superoxide dismutase in different tissues such as aortic, hepatic, cardiac, intestinal, muscular and renal tissues. Tebib et al. (1997) reported that monomeric tannins were ineffective in restoration of the levels of these enzymatic antioxidants. However, in rats

fed with polymeric tannins, these enzymes were effectively restored. Vitamin E

deficiency reduced the total glutathione level in rat tissues and blood. Polymeric tannin

supplementation of rat diet increased the glutathione level back to its original level.

Polymeric tannins reduced lipid peroxidation in plasma and tissues as effectively as

vitamin E. More interestingly, in rats fed with a diet high in cholesterol and deficient in

vitamin E, incorporation of polymeric grape seed tannins in the diet increased total

glutathione level in blood approximately 4 times compared with rats fed the same diet

with monomeric grape seed tannins. The authors concluded that polymeric grape seed

tannins have *in vivo* antioxidant activity and could be as important as vitamin E in

preventing oxidative damage in tissues.

Cancer

Cancer is defined as "a group of diseases characterized by uncontrolled growth

and spread of cells" by the American Cancer Society (ACS, 2001). Cancer can be

induced by many factors such as lifestyle, environmental and genetic factors. According

to the ACS, lifestyle factors such as diet and regular exercise contribute to the causes of

about one-third of total cancer deaths in 2001. A change in lifestyle could prevent these

deaths. The ACS expects the number of skin cancers to be more than a million in 2001.

Most of these cancers could be prevented by the use of appropriate sun protection

methods. Treatment methods of cancer include surgery, chemotherapy, radiation,

hormones and immunotherapy.

Genes control cell growth and division. Malfunction of genes may induce cancer.

The ACS expects the number of new cancer cases in 2001 to be close to 1.3 million.

Diagnosed cancer cases have reached to 15 million since 1990. After heart disease,

cancer is the second major cause of death in the US where a quarter of all deaths is from

cancer. The National Institutes of Health estimated the cost of cancer to the US economy to be $180.2 billion in the year 2000.

Temple (2000) summarized the relationship between dietary antioxidant/nutrient status and cancer. Antioxidant intake in diet shows an inverse relation with cancer cases according to epidemiological studies. In their review, Kuroda and Hara (1999) indicated that "in Japan, an epidemiological study showed an inverse relationship between habitual green tea drinking and the standardized mortality rates for cancer". Ascorbic acid, β-carotene, lycopene, α-tocopherol and selenium are considered as important dietary micronutrients that could reduce cancer cases or heart disease rates.

Cancer preventing activities of flavonoids have been extensively studied. Components of tea, grape seeds and skins were reported to have anticarcinogenic activities as well as cancer inducing activities. Phenolic compounds present in beans (*Phaseolus vulgaris*) were also shown to be antimutagenic (Mejia et al., 1999). Antitumor, antiplatelet, antiallergic, antiischemic, and antiflammatory activities of flavonoids are mostly associated with the antioxidative properties of plant flavonoids (Shi et al., 2001). Polyphenolic constituents of cacao liquor were found to have an inhibitory effect against oxidative DNA damage, which may indicate antimutagenic and anticarcinogenic activities of cacao liquor extracts (Yamagishi et al., 2001).

Zhao et al. (2001) reported that phenols in the diet such as C, EC, EGC, caffeic acid and quercetin can protect the DNA against damage by nitrite (HNO_2) and peroxynitrite ($ONNO^-$). Nie et al. (2001) reported that green tea polyphenols EC, EGCG, EGC, and ECG showed a protective effect on oxidative DNA damage induced by hydroxyl radicals *in vitro*. Weyant et al. (2000) reported that C showed antitumor activity

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both *in vivo* and *in vitro,* preventing tumor formation. Chen et al. (1998) reported that EGCG, a major polyphenolic constituent of green tea extracts, showed tumor inhibiting activity on colorectal and breast cancer cells lines, Coco-2 and Hs578T, respectively.

Black tea and green tea have antimutagenic activity (Krul et el., 2001). Food eaten with the antioxidant was shown to affect antimutagenic activity of tea antioxidants in the body (Krul et al., 2001). Santana-Rios et al. (2001) made a synthetic tea using nine major substances present in natural green tea, and they found that the natural tea had superior antimutagenic activity in the Salmonella assay compared to the synthetic one. Antioxidant activity of tea catechins were found to be positively related to their antimutagenic activity (Krul et al., 2001).

Tea extracts from green, oolong, and black teas, and tea constituents gallic acid and EGCG showed antimutagenic activity under the Ames test against various chemical mutagens (Hour et al., 1999). Hirose et al. (1997) found green tea catechins to be ineffective in inhibiting rat mammary cancer progression; however, a diet containing EGCG weakly inhibited early promotion of cancer in rats. A similar research indicated that EGCG, ECG, EGC, EC, and green tea water extract showed anticarcinogenic activity during the initiation stage of chemical carcinogenesis (Han, 1997). Anticarcinogenic effect of green tea extract was found to be higher than any of these polyphenolic substances at that stage of carcinogenesis. However, the anticarcinogenic effect of phenolic substances on cancer cells during promotion stage of cancer was higher than tea water extract (Han, 1997).

A number of studies investigated the effect of flavonoids on colon cancer. Flavonoids (+)-catechin and hesperidin were found to have chemoprotective effect in rats

against colon cancer induced with heterocyclic amines (Franke et al., 2002). Using rats, Hirose et al. (2001) indicated that green tea catechins at a level of 40mg/kg body wt is ineffective to inhibit carcinogenesis after colon, lung, or thyroid cancer is initiated. However, catechins increased colon carcinogenesis when rats were fed a diet containing 40mg/kg green tea catechins. On the other hand, Uesato et al. (2001) showed that EC, EGC, and EGCG showed a stronger inhibition activity against colon cancer cells than hepatic epithelial cells *in vitro*. Moreover, anticancer activity of EC on both types of cancer cells was found weaker than the other two epicatechin derivatives.

Apoptosis can be defined as programmed cell death. Apoptosis is necessary for the elimination of damaged or cancerous cells from the body during cancer treatments. Treatment of human Chang liver cells with 25μg/ml grape seed procyanidin extract (GSPE) reduced apoptosis by reducing the expression of p53 and increasing the expression of cellular Bcl-2 (Joshi et al., 2000). Bcl-2 protein is related to apoptosis expression on cells. It is highly expressed in tumor cells, which are resistant to apoptosis. Bcl-2 gene is an antogonist of apoptosis whereas p53 is a proapoptosis gene. Gallic acid was reported to play a role in the induction of apoptosis, programmed cell death, in the body (Sakaguchi et al., 1998). Green tea constituents, EC, EGC, EGCG and ECG showed chemoprotective activity against human prostate cancer cells by suppressing their growth and inducing apoptosis (Chung et al., 2001).

Ohe et al. (2001) showed that tea catechins (C, EC, EGC, EGCG and ECG) should not be responsible for the antigenotoxic activity of teas because of the insignificant correlations between catechin contents of teas and antigenotoxicity of teas against nitroarenes. Interestingly, fermented teas, which have low catechin contents,

showed a high genotoxicity by suppressing activity of nitroarenes. Apostolides et al. (1997) previously reported that gallic acid, ungallated tea catechins (C, EC and EGC), and methyl gallate did not inhibit *in vitro* mutagenesis from direct mutagens; however, theaflavin, gallated catechins, and tannic acid showed antimutagenic activity. Chen and Chung (2000) also found that tannic acid and its hydrolyzed products including phenolic acids, ellagic acid and gallic acid showed no antimutagenic activity against direct mutagens such as 2-nitrofluorene, 1-nitropyrene, and 2-nitro-*p*-phenylenediamine. Moreover, these compounds were found to be non-mutagenic. Muto et al. (2001) reported that EC, EGC, EGCG and ECG inhibited 'metabolic activation of procarcinogens by human cytochrome P450' partially through the inhibition of NADPH-CYP reductase.

Dhawan et al. (2002) evaluated the antigenotoxic properties of grape seed procyanidins B_1, B_2, B_3, B_4 and B'2G and of black tea theaflavins and theafluvins *in vitro*. They reported that monomeric and dimeric flavanols of grape seeds did not cause or prevent damage to lymphocyte DNA induced by Trp-P-2 at a concentration up to $100\mu M$ (micromolar). However, black tea theaflavins and theafluvins at a concentration up to 0.5mg/mL prevented DNA damage in a dose dependent manner. Dhawan et al. (2002) concluded that anticarcinogenic potential of black teas could be associated with their theaflavins and theafulvins content.

Cancer preventing activity of chemical drugs may come from their ability to inhibit cyclooxygenase, which can catalyze the conversion reaction of arachidonic acid into tumor cell growth stimulating substances. Jang et al. (1997) reported that resveratrol showed anticarcinogenic activities in the initiation, promotion and progression steps of

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cancer development in several ways, including inhibition of cyclooxygenase and hydroperoxidase activities.

Bomser et al. (2000) noted that ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis could have "an essential role in diverse biological processes including cell proliferation and differentiation". High levels of ODC are associated with increased risk for cancer. GSE containing mainly oligomeric and polymeric proanthocyanidins was shown to inhibit epidermal ODC activity in mice when it is used before TPA, which is a tumor promoter (Bomser et al., 2000).

Breast Cancer

ACS estimated new breast cancer cases among US women to be about 192,000 in 2001. 1,500 new cases are expected in men. Approximately 40,000 deaths were expected from breast cancer and majority of these deaths was in women. Fat intake was reported to be correlated with breast cancer rates worldwide. 31% of total new cancer incidents in the US among females were expected to be breast cancer cases, while 15% of the expected deaths among the same group were from breast cancer (ACS, 2001).

Nakagawa et al. (2001) studied the effect of resveratrol on the inhibition of the growth of cancerous cells obtained from breast cancer patients (Table 7). Suppression in the growth of these cells by resveratrol was reported to come from apoptosis, programmed cell death.

The p53 gene suppresses tumor formation and induces apoptosis. Modulation of p53 expression by red wine and its content of polyphenolic substances was studied in three human breast cancer cell lines and one colon cancer line by Soleas et al. (2001). Wine polyphenols quercetin, catechin, *trans*-resveratrol and caffeic acid did not affect

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Table 8. The effect of resveratrol concentration on the inhibition of breast cancer cells. Cell lines: Estrogen Receptor (ER)-positive= KPL-1 and MCF-7; Estrogen Receptor (ER)-negative=MKL-F (adapted from Nakagawa et al., 2001).

Resveratrol concentration μM	The effect on breast cancer cells
≤4	- Proliferation in MCF-7
≤22	- Proliferation in KPL-1
≥44	- Suppression in KPL-1, MCF-7 and MKL-F
52-74	- Reduction in the effect of a breast cancer stimulator, linoleic acid
	- Suppression in KPL-1, MCF-7 and MKL-F

p53 gene expression in two of the breast cancer lines; however, resveratrol decreased the expression of this gene in breast cancer cells of a wild type (MCF-7). Although catechin and caffeic acid increased the expression of p53 in wild type breast cancer cells, the increase was independent of concentration. Caffeic acid and resveratrol reduced the expression of p53 in colon cancer cell lines, but this reduction again was not dose-responsive. Soleas et al. (2001) concluded that anticarcinogenic properties of wine should not be attributed to the modulation of p53 gene expression by these wine polyphenolic constituents.

Skin Cancer

New skin cancer cases were expected to be more than a million in 2001 by ACS. Melanoma is the most serious form of skin cancer. Whites were expected to be 10 times more susceptible to skin cancer than blacks (ACS, 2001). Approximately 10,000 deaths were expected to occur from skin cancer in the US and protection could be achieved by limiting outdoor activities between 10am and 4pm, wearing a hat or a long-sleeved shirt and using a sunscreen lotion (ACS, 2001).

Bomser et al. (1999) found that grape seed extract containing mainly oligomeric and polymeric proanthocyanidins showed anti-tumor activity in mouse skin epidermis.

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Cardiovascular Diseases

Coronary heart disease (CHD) is usually associated with high cholesterol levels in blood. CHD is a serious health problem affecting the American population. High levels of low-density lipoprotein (LDL) cholesterol in plasma may play a role on the initiation of atherosclerotic plaque. Physicochemical and biological properties of LDL can be modified through enzymatic modification such as lipases and oxygenases or non-enzymatic modifications such as glycosylation, proteoglycans and immune complexes (Aviram, 1993). In atherosclerosis, lipid peroxidation plays an important role during the initiation and propagation steps of this disease. Serum cholesterol level may not necessarily be a cause of the problem as manifested in the French paradox, but oxidation of cholesterol, especially LDL (Aviram, 1993) could be the problem. Piotrowski et al. (1990) found high levels of cholesterols and lipid peroxidation in tissues and phospholipids in aortic tissues obtained from people with coronary heart diseases. Atherosclerotic plaque could form as a result of lipid oxidation, especially of LDLs in plasma.

LDL peroxidation can be affected by several factors such as presence of copper ions, antioxidant content of cells (both enzymatic and non-enzymatic), and 'the composition and location of polyunsaturated fatty acids' of LDL (Aviram, 1993). Extrinsic factors like antioxidant concentration in blood and other tissues may play an important role on the reduction of coronary heart disease risks. Epidemiological studies show that risk could be reduced by high dietary intake of fruits and vegetables. Phenolic substances found in grapes, wine and other foods are able to block the oxidation of LDL by acting as antioxidants (Aviram, 1993) and may be responsible for their cardioprotective effect (Manthey et al., 2002).

An *in vitro* study indicated that catechin and quercetin have a property to inhibit LDL oxidation (Roland, 1997). Moreover, tannins (tannin acid), flavonols (catechin, quercetin, rutin), cinnamic acids (caffeic and ferulic acid), stilbenes (resveratrol), benzoic acids (gallic acid), anthocyanidins (malvidin) also inhibit the *in vitro* oxidation of LDL in a dose-dependent manner, and this inhibition was better than that of common antioxidants like vitamin E and vitamin C (Sanchez-Moreno et al., 2000).

Dietary phenols were found to be better in inhibiting LDL oxidation *in vitro* than common antioxidants like ascorbic acid and tocopherols (Sanchez-Moreno et al., 2000). Red apples and apple juice containing a variety of polyphenolic compounds was found to protect LDL against oxidation induced by *in vitro* copper (Pearson et al., 1999). Cranberry extracts containing about 1.5g GAE/L reduced the *in vitro* oxidation of LDL induced by cupric sulfate (Wilson et al., 1998). Caldu et al. (1997) showed that red and white wine reduced the oxidation of LDL both *in vivo* and *in vitro*. Moreover, higher inhibition of LDL oxidation by red wine compared to white wine was reported.

Locher et al. (2002) found that constituents of green tea extracts inhibited proliferation of vascular smooth muscle cells caused by increased levels of native LDL in humans. That is, antioxidative activity of green tea protected against growth of smooth muscle cells related to elevated levels of native LDL.

Meyer et al. (1997) showed that inhibition of copper induced LDL oxidation in humans by grape extracts vary in degree, depending on the phenolic content of the extracts. Crushing the seeds and extracting for a longer time increased the amount of flavanols and hydroxybenzoates in the extract. The authors found a positive significant

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correlation between the relative inhibition rate of LDL oxidation and the levels of flavanols, total phenols and hydroxybenzoates.

Studies were also done to determine whether alcohol in wine is responsible for the reduced levels of LDL in blood of moderate wine drinkers. Frankel et al. (1993) diluted red wine in order to get 10μmol/L total phenolics (1000 times dilution) to study the effect of wine phenolics on the *in vitro* inhibition of human LDL oxidation. Diluted wine and 10μmol/L quercetin equally inhibited the human LDL oxidation induced by copper, and this inhibition was from non-alcoholic constituents of red wine. They also indicated that the inhibition was independent of the copper concentration; thus, antioxidant activity was not from the metal chelating activity of wine phenolics. The inhibition was significantly higher than that induced by α-tocopherol.

Platelets are small cells that are able to adhere to damaged arteries and capillaries. Platelets circulate in the blood. By sticking to the damaged area they can prevent bleeding and promote healing. Under normal conditions, platelets do not stick to healthy endothelium because of the release of NO, a platelet inhibitor, by endothelial cells (Rabbani and Loscalzo, 1994). When endothelial cells are damaged, platelets are able to stick and aggregate on the wall of arteries (Folts, 2002). This could lead to CHD.

De-alcoholized red wines showed antiplatelet activity of wine phenolics (Pace-Asciak et al., 1995). Although *trans*-resveratrol and quercetin inhibited platelet aggregation induced with both thrombin and ADP in a dose-dependent manner, ethanol inhibited only aggregation induced with thrombin. (Pace-Asciak et al., 1995).

Sato et al. (1999) investigated cardioprotective properties of grape seed proanthocyanidins in ischemic/reperfused rats. Myocardial infection rate was found lower

in animals fed with GSPE than the control group. They showed that GSPE could protect the heart against ischemic/ reperfusion injury. The authors indicated that this protection may come from the ability of GSPE to scavenge peroxyl and hydroxyl radicals generated during ischemia and reperfusion.

In another study, Sato et al. (2001) showed that 100mg grape seed proanthocyanidin extract (GSPE)/kg/day can reduce the number of apoptotic cells in rats with ischmic/reperfused hearts. There was about 50 and 75% reduction in the production of free radicals in rats fed with 50 and 100mg/kg/day GSPE, respectively. The cardioprotective effect of GSPE was shown to come from the reduced expresssion of JNK-1 factor and c-Jun gene, which are proapoptotic factors in the ischmic/reperfused myocardium. GSPE inhibits the expression of proapoptotic transcription factor and gene, JNK-1 and c-Jun (Sato et al., 2001).

Korthuis and Gute (2002) found that a mixture of flavonoids diosmin (90%) and hesperedin (10%) (Daflon 500mg) exerted anti-inflammatory actions after ischmic reperfusion. During ischemia and reperfusion, free radical generation increases, while the level of both enzymatic and non-enzymatic antioxidants in tissues reduces (Das and Maulik, 1994).

Yamakoshi et al. (1999) found no effect of 1% GSPE (w/w) in diet on serum lipid profiles in rabbits. However, it reduced atherosclerosis in the aorta. Grape seed proanthocyanidins can trap free radicals, especially aqueous peroxyl radicals in plasma and 'interstitial fluid of the arterial wall'. GSPE reduces atherosclerotic activity of LDL by inhibiting its oxidation. Yamakoshi et al. (1999) also found that a diet with 1% (w/w) of catechin showed very weak antiatherosclerotic activity in cholesterol-fed rabbits

compared with procyanidin rich extract. They concluded that preventive activity of grape seed extracts against atherosclerosis comes mostly from proanthocyanidins.

Ulcer

Grape seed extracts of *Vitis vinifera* L. grapes (GSE) containing either high or low flavanol contents showed antiulcer activity in rats (Saito et al., 1998). Protection against stomach injury for GSE with high flavanol content was higher than the one with low flavanol content. Saito et al. (1998) also showed that catechin, procyanidin B3 and dimeric and trimeric procyanidins did not have any protective activity against ulcer. On the other hand, tetramers, pentamers and hexamers of grape seed proanthocyanidins were shown to have antiulcer activities in animal models. The authors speculated that this activity of longer oligomeric procyanidins might come from their ability to bind proteins on the surface of the stomach.

Oestrogenic Activity of Flavonoids

Basly et al. (2000) studied the estrogenic/antiestrogenic effects of resveratrol isomers on the *in vitro* human breast cancer cells (Table 9). *Cis* form was less effective than *trans* in both cell lines. DPPH assay and Fe^{+3} reduction assay showed that these isomers can be both antioxidant and prooxidant, depending on their concentrations (Basly et al., 2000).

Table 9. The effect of resveratrol isomers on human breast cancer cells (adapted from Basly et al. (2000).

Concentration (μM)	Effect
10-25	Increased *in vitro* growth of MCF-7 cell lines
0.1-1	No effect
>50	Cytotoxic, reduced cell growth
Concentration (mM)	
25 *trans*	Reduced proliferation induced by estradieol
10 *cis*	No interference with estrogen receptor
10-25 *trans* and 25 *cis*	"Supergonists of estradieol"

Other Biological Activities of Flavonoids

Khanna et al. (2001) showed that grape seed proanthocyanidin extract containing 5mg/g *trans*-resveratrol induces the expression of vascular endothelial growth factor (VEGF) in keratinocytes. Therefore, GSPE containing resveratrol can be used to treat dermal wounds and other dermal disorders.

The effect of plant flavonoids on intestinal microflora has been investigated. Tebib et al. (1996) reported that monomeric proanthocyanidins of grape seeds did not cause any change in the activities of fecal bacterial enzymes on rats. However, polymeric proanthocyanidins showed 'a beneficial cecal metabolic and colonic protective effect' by reducing colonic enzymatic activity of β-glucosidase, β-glucuronidase, mucinase and nitroreductase 'due to a dilution effect'. Feeding rats with polymeric tannins increased the formation of volatile fatty acids, an indicator of bacterial activity, thus reducing the pH in cecum. Polymeric tannin supplementation was shown to stimulate fermentative activities without increasing the activity of harmful enzymes on animal models. Tannins may also inhibit the growth of some bacteria in human intestines. Chung et al. (1998) reported that tannic acid inhibited the growth of intestinal bacteria such as *Clostridium perfringens*, *Entrobacter cloacae*, *E. coli* and *S. typhimurium* while showing no inhibitory effect on *B. infantis* or lactic acid bacteria *L. acidophilus*. A fluorescence assay was developed to determine the bacterial degradation of flavonoids by Schoefer et al. (2001). This assay could differentiate the abilities of colonic bacteria to degrade various flavonoids. However, the bacterial degradation of catechin could not be determined due to the lack of its quenching effect of the fluorescing compound. Future studies may reveal details on the mechanisms of flavonoid degradation by colonic microflora.

Green tea polyphenolic constituents, isomers of C, EC, EGCG and ECG reduce membrane fluidity, which is typically increased by cancer cells (Tsuchiya, 2001).

POTENTIAL USE OF FLAVONOIDS IN THE FOOD INDUSTRY

Tea catechins have been extensively used to study the antioxidant activity of flavonoids in foods. Chen and Chan (1996) indicated that jasmine tea extracts containing EGCG, EGC, EC, and ECG showed significant superior antioxidant activity compared with BHT in canola oil. Moreover, the thermal stability of tea extract catechins was better than that of BHT. Addition of tea catechins at a level of 300mg/kg was shown to inhibit lipid oxidation significantly in red meat and poultry patties (Tang et al., 2001a). On the other hand, concentrations of tea catechins higher than 300mg/kg were needed for the inhibition of lipid oxidation in samples with high levels of highly unsaturated lipids like fish (Tang et al., 2001a).

Flavonoids can also be used as an alternative to vitamin E as an antioxidant agent. For example, feeding chickens with a diet containing 300mg/kg tea catechins was found to be as effective as 200mg α-tocopheryl acetate/kg feed in protecting frozen chicken meat against long-term oxidation up to 9 months (Tang et al., 2001b). Tea catechin addition had a protective effect on added vitamin E in frozen chicken meat stored for a year, and tea catechins showed Fe^{2+} chelating and DPPH free radical scavenging activity (Tang et al., 2001b).

McCarthy et al. (2001a; 2001b) indicated that tea catechins, rosemary and sage when used at a level less than 0.5% were the most potent antioxidants to reduce lipid oxidation in raw and cooked pork patties from frozen pork meat. Tea catechins were the

most effective antioxidant against lipid oxidation after the cooking process compared with ginseng, mustard, rosemary, sage, BHA/BHT or vitamin E.

Tang et al. (2000) found that feeding chickens with a diet containing tea catechin at a concentration higher than 200mg/kg reduced lipid oxidation in chicken meat, liver and heart. Catechin was found to increase the stability of peanut oil most significantly compared to rosemary, tocopherol, phospholipids and ascorbyl palmitate (Chu and Hsu, 1999).

FLAVONOIDS AS FUNCTIONAL FOODS

Functional foods are considered as foods with a physiological purpose in the body. Sanders (1998) defined a functional food as 'a food or food ingredient that provides a health benefit beyond satisfying traditional nutritional requirement'. The definition implies that functional foods possess physiological effects that improve human health. Phytochemicals can be seen as functional foods since the intake of the plant-derived substances improves health by reducing the risk for numerous diseases. Terminology to define functional foods includes a variety of terms (Table 10), which can sometimes be confusing, since the distinction among the terms is not well established among the different scientific communities.

Consumer interests towards functional foods have recently increased sharply because of the following reasons (Goldberg, 1999; Sanders, 1998);

- Increased consumer awareness on the relationship between health and nutrition
- More media coverage on diet-disease interactions
- Aging population
- High medical cost
- Increased consumer desire to prevent disease rather than cure

- Scientific research
- Others (Nutritional labeling, brand differentiation, and environmental factors)

Table 10. Some of the terms used to define functional foods (adapted from Sanders, 1998).

Term	Definition
Functional food	A modified food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirements
Nutraceutical	A food or part of a food that offers medical and/or health benefits including prevention or treatment of disease
Medical food	A special classification of food dictated in United States food law which: • Must be used under medical supervision • Must be for a disease with well defined, specific nutrient characteristics • Based on recognized scientific principles • Must provide medical evaluation (an example is a formula for dietary management of phenylketonuria)

Dietary supplements are regulated as a class of foods by FDA under the Dietary Supplement Health and Education Act (1994). This act defines dietary supplements and provides structure-function health claims for dietary ingredients. According to this act, plant extracts (e.g. grape seed or skin extract) could be sold as a dietary supplement in the form of liquid, powder, tablet, capsule, or gel (soft or cap).

Plants have been an important part of animal and human diets due to their functionality in the body. Plant derived foods provide energy for metabolic activities, provide precursors for protein synthesis, supply essential micronutrients for the life like vitamins, essential fatty acids, and minerals. Moreover, phenolic constituents of plants are known to have nutritional functions as well as medicinal. Most of these constituents are secondary metabolites (Walton et al., 1999) and are also called phytochemicals.

Phytochemicals can be described as 'plant-derived substances that are nutritionally physiologically and/or medicinally highly active' (Lin, 1999) secondary metabolites. Components of garlic, ginkgo biloba, soybean, tea, grapes and many other fruits and vegetables are phytochemicals with dietary importance for humans. Tea, rich in catechins, is considered to be a functional food for the improvement of oral health (Wu and Wei, 2002).

The beneficial components of grape wine, grape seeds and skins have been discussed previously. Grape berries (seeds, skins and pulp) with their major flavonoids can be used as a potential functional food due to their antioxidant (both in vitro (Zhu et al., 2001) and in vivo (Bagchi et al., 1998)), antimutagenic (Weyant et al., 2000; Krul et al., 2001), antiplatelet (Pace-Asciak et al., 1995), cardioprotective (Sato et al., 2001), antiatherosclerotic (Yamakoshi et al., 1999), antiulcer (Saito et al., 1998) activities and beneficial effect on gastrointestinal health (Chung et al., 1998; Tebib et al., 1996).

REFERENCES

ACS. 2001. Cancer Facts and Figures. American Cancer Society Atlanta. GA

Albano, E., Tomasi, A., Parola, M., Comoglio, A., Ingelman-Sundberg, M. and Dianzani, M.U. 1993. Mechanisms responsible for free radical formation during ethanol metabolism and their role in causing oxidative injury by alcohol. In *Free Radicals and Antioxidants in Nutrition*, Corongiu, F., Banni, S., Dessi, M.A. and Rice-Evans, C. (eds.). Richelieu Press, London, England. pp. 77-96.

Allard, J.P. 2001. Oxidative stress and infections. In *Micronutrients and HIV Infection*, Friis, H. (ed.). CRC Press LLC, Boca Raton, FL.

Amakura, Y., Okada, M., Tsuji, S., and Tonogai, Y. 2000. Determination of phenolic acids in fruit juices by isocratic column liquid chromatography. J. Chromatogr. A. 891:183-188.

Anonymous, 2001. http://www.geocities.com/NapaValley/1172/rotundif.html.Accessed
July 27, 2001.

Apostolides, Z., Balentine, D.A., Harbowy, M.E., Hara, Y., and Weisburger, J.H. 1997.
Inhibition of PhIP mutagenicity by catechins, and by theaflavins and gallate
esters. Mutat. Res. 389:167-172.

Arts, M.J.T.J., Haenen, G.R.M.M., Voss, H.-P., and Bast, A. 2001. Masking of
antioxidant capacity by the interaction of flavonoids with protein. Food Chem.
Toxicol. 39:787-791.

Aviram, M. 1993. Modified forms of low density lipoprotein and atherosclerosis.
Atherosclerosis. 98:1-9.

Awad, M.A., Jager, A., van der Plas, L.H.W., and van der Krol. 2001. Flavonoid and
chlorogenic acid changes in skin of 'Elstar' and 'Jonagold' apples during
development and ripening. Sci. Hortic-Amsterdam. 90:69-83.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J. and Stohs, S.J.
1998. Protective effects of grape seed proanthocyanidins and selected antioxidants
against TPA-induced hepatic and brain lipid peroxidation and DNA
fragmentation, and peritoneal macrophage activation in mice. Gen. Pharmac.
30(5):771-776.

Basly, J.P., Marre-Fournier, F., Le Bail, J.C., Habrioux, G., and Chulia, A.J. 2000.
Estrogenic/antiestrogenic and scavenging activities of (E)- and (Z)-resveratrol.
Life Sci. 66(9):769-777.

Benzie, I.F.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a
measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239:70-76.

Bianco, M.A. and Savolainen, H. 1997. Phenolic acids as indicators of wood tannins. Sci.
Total Environ. 203:79-82.

Bloor, S.J. 2001. Overview of methods for analysis and identification of flavonoids.
Method. Enzymol. 335:3-14.

Bomser, J., Singletary, K. and Meline, B. 2000. Inhibition of 12-*O*-tetradecanoylphorbol-
13-acetate (TPA)-induced mouse skin ornithine decarboxylase and protein kinase
C by polyphenolics from grapes. Chem-Biol. Interact. 127:45-59.

Bomser, J.A., Singletary, K.W., Wallig, M.A. and Smith, M.A.L. 1999. Inhibition of
TPA-induced tumor promotion in CD-1 mouse epidermis by a polyphenolic
fraction from grape seeds. Cancer Lett. 135:151-157.

Bors, W. and Michel, C. 1999. Antioxidant capacity of flavanols and gallate esters: pulse
radiolysis studies. Free Radical Bio. Med. 27(11/12):1413-1426.

Bors, W., Michel, C., and Stettmaier, K. 2001. Structure-activity relationships governing
antioxidant capacities of plant polyphenols. Method. Enzymol. 335:166-180.

Bouhamidi, R., Prevost, V. and Nouvelot, A. 1998. High protection by grape seed
proanthocyanidins (GSPC) of polyunsaturated fatty acids against UV-C induced
peroxidation. Life Sci. 321:31-38.

Caldu, P., Ramon, J.M., Gonzalo, A., Antoli, R., Minguez, S., and Fiol, C. 1997.
Inhibition of LDL oxidation by white and red wine phenolics. 11th International
Symposium on Atherosclerosis. Abstract. Paris, France.

Cao, G. and Prior, R. 1999. Measurement of oxygen radical absorbance capacity in
biological samples. Method. Enzymol. 299:50-63.

Chen, H., Zuo, Y., and Deng, Y. 2001. Separation and determination of flavonoids and
other phenolic compounds in cranberry juice by high-performance liquid
chromatography. J. Chromatogr A. 913:387-395.

Chen, S.C. and Chung, K.T. 2000. Mutagenicity and antimutagenicity studies of tannic
acid and its related compounds. Food Chem. Toxicol. 38:1-5.

Chen, Z.P., Schell, J.B., Ho, C.T., and Chen, K.Y. 1998. Green tea epigallocatechin
gallate shows a pronounced growth inhibitory effect on cancerous cells but not on
their normal counterparts. Cancer Lett. 129:173-179.

Chen, Z.Y. and Chan, P.T. 1996. Antioxidative activity of green tea catechins in canola
oil. Chem. Phys. Lipids. 82:163-172.

Chu, Y.H. and Hsu, H.F. 1999. Effects of antioxidants on peanut oil stability. Food
Chem. 66:29-34.

Chung, K.T., Lu, Z., and Chou, M.W. 1998. Mechanism of inhibition of tannic acid and
related compounds on the growth of intestinal bacteria. Food Chem. Toxicol.
36:1053-1060.

Chung, L.Y., Cheung, T.C., Kong, S.K., Fung, K.P., Choy, Y.M., Chan, Z.Y., and Kwok, Y.Y. 2001. Induction of apoptosis by green tea catechins in human prostate cancer DU145 cells. Life Sci. 68:1207-1214.

Cuendet, M., Potterat, O., Salvi, A., Testa, B. and Hostettmann, K. 2000. A stilbene and dihydrochalcones with radical scavenging activities from *Loiseleuria procumbens*. Phytochemistry. 54:871-874.

Das, D.K. and Maulik, N. 1994. Antioxidant effectiveness in ischemia-reperfusion tissue injury. Method. Enzymol. 233:601-610.

Davis, A.L., Lewis, J.R., Cai, Y., Powell, C., Davis, A.P., Wilkins, J.P.G., Pudney, P., and Clifford, M.N. 1997. A polyphenolic pigment from black tea. Phytochemistry. 46(8):1397-1402.

Decker, E.A. 1998. Antioxidant mechanisms. In *Food Lipids: Chemistry, Nutrition and Biotechnology*. Akoh, C.C. and Min, D.M. (eds). Marcel Dekker, Inc. New York, NY.

deMan, J.M. 1999. *Principles of Food Chemistry*. Aspen Publishers, Gaithersburg, MD.

Dhawan, A., Anderson, D., Pascual-Teresa, S. Santos-Buelga, C., Clifford, M.N., and Ioannides, C. 2002. Evaluation of the antigenotoxic potential of monomeric and dimeric flavanols, and black tea polyphenols against heterocyclic amine-induced DNA damage in human lymphocytes using the Comet assay. Mutat. Res. 400400:1-18 (in press).

Folts, J.D. 2002. Potential health benefits from the flavonoids in grape products on vascular disease. Adv. Exp. Med. Biol. 505:95-111.

Franke, A.A., Custer, L.J., Cooney, R.V., Tanaka, Y., Xu, M., and Dashwood, R.H. 2002. Inhibition of colonic aberrant crypt formation by the dietary flavonoids (+)-catechin and hesperedin. Adv. Exp. Med. Biol. 505:123-133.

Frankel, E.N., Kanner, J., German, J.B., Parks, E. and Kinsella, J.E. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet. 341:454-457.

Fukumoto, L.R. and Mazza, G. 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J. Agr. Food Chem. 48(8):3597-3604.

Goldberg, I., 1999. *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Aspen Publishers, Inc. Gaithersburg, MD.

Grinberg, L.N., Newmark, H., Kitrossky, N., Rahamim, E., Chevion, M., and Rachmilewitz, E.A. 1997. Protective effects of tea polyphenols against oxidative damage to red blood cells. Biochem. Pharmacol. 54:973-978.

Gunckel, S., Santander, P., Cordano, G., Ferreira, J., Munoz, S., Nunez-Vergara, L.J., and Squella, J.A. 1998. Antioxidant activity of gallates: an electrochemical study in aqueous media. Chem-Biol. Interact. 114:45-59.

Han, C. 1997. Screening of anticarcinogenic ingredients in tea polyphenols. Cancer Lett. 114:153-158.

Harborne, J.B. and Williams, C.A. 2000. Advances in flavonoid research since 1992. Pytochemistry. 55:481-504.

Haslam, E. 1989. *Plant Polyphenols, Vegetable Tannins Revisited*. Cambridge University Press, Cambridge, UK.

Hider, R.C., Liu, Z.D., and Khodr, H.H. 2001. Metal chelation of polyphenols. Method. Enzymol. 335:190-203.

Hirose, M., Hoshiya, T., Mizoguchi, Y., Nakamura, A., Akagi, K., and Shirai, T. 2001. Green tea catechins enhance tumor development in the colon without effects in the lung or thyroid after pretreatment with 1,2-Dimethylhydrazine or 2,2'-dihydroxy-di-n-propylnitrosamine in male F344 rats. Cancer Lett. 168:23-29.

Hirose, M., Mizoguchi, Y., Yaono, M., Tanaka, H., Yamaguchi, T., and Shirai, T. 1997. Effects of green tea catechins on the progression or late promotion stage of mammary gland carcinogenesis in female Sprague-Dawley rats pretreated with 7,12-dimethylbenz(a)anthracene. Cancer Lett. 112:141-147.

Hollman, P.C.H. and Katan, M.B. 1999. Dietary flavonoids: Intake, health effects and bioavailability. Food Chem. Toxicol. 37(9-10):937-942.

Holman, P.C.H. 2001. Determination of flavonoids in body fluids. Method. Enzymol. 335:97-103.

Hour, T.C., Liang, Y.C., Chu, I.S., and Lin, J.K. 1999. Inhibition of eleven mutagens by various tea extracts, (-)-epigallocatechin-3-gallate, gallic acid and caffeine. Food Chem. Toxicol. 37:569-579.

Huie, R.E. and Neta, P. 1984. Chemical behavior of SO_3- and SO_5- radicals in aqueous-solutions. J. Phys. Chem-US. 88(3):5665-5669.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W.W., Fong, H.H.S., Farnworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C. and Pezzuto, J.M. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 275:218-220.

Joshi, S.S., Kuszynski, C.A., Bagchi, M. and Bagchi, D. 2000. Chemopreventive effects of grape seed proanthocyanidin extract on Chang liver cells. Toxicology. 155:83-90.

Kennedy, J.A. Matthews, M.A., and Waterhouse, A.L. 2000. Changes in grape seed polyphenols during fruit ripening. Phytochemisty. 55:77-85.

Khanna, S., Roy, S., Bagchi, D., Bagchi, M. and Ken, C.K. 2001. Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. Free Radical Bio. Med. 31(1):38-42.

Khokhar, S., Venema, D., Hollman, P.C., Dekker, M., and Jonge, W. 1997. A RP-HPLC method for the determination of tea catechins. Cancer Letter. 114:171-172.

Knudsen, C.A., Tappel, A.L., and North, J.A. 1996. Multiple antioxidants protect against heme protein and lipid oxidation in kidney tissue. Free Radical Bio. Med. 20(2):165-173.

Kolpan, S., Smith, B.H., and Weiss, M.A. 2001. *Exploring Wine: The Culinary Institute of America's Complete Guide to Wines of the World*. John Wiley & Sons, Inc., New York, NY.

Korthuis, R.J. and Gute, D.C. 2002. Anti-inflammatory actions of a micronized, purified flavonoid fraction in ischemia/reperfusion. Adv. Exp. Med. Biol. 505:181-190.

Krul, C., Luiten-Schuite, A., Tenfelde, A., van Ommen, B., Verhagen, H., and Havenaar, R. 2001. Antimutagenic activity of gree tea and black tea extracts studied in a dynamic *in vitro* gastrointestinal model. Mutat. Res. 474:71-85.

Kuo, S.M. 2002. Flavonoids and gene expression in mammalian cells. Adv. Exp. Med. Biol. 505:191-200.

Kuroda, Y. and Hara, Y. 1999. Antimutagenic and anticarcinogenic activity of tea polyphenols. Mutat. Res. 436:69-97.

Lin, R.I-S. 1999. Phytochemicals and antioxidants. In *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Goldberg, I. (ed.) Aspen Publishers, Inc. Gaithersburg, MD.

Liu, Z.Q., Ma, L.P., Zhou, B., Yang, L., Liu, Z.L. 2000. Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. Chem. Phys. Lipids. 106:53-63.

Locher, R., Emmanuele, L., Suter, P.M., Vetter, W., and Barton, M. 2002. Green tea polyphenols inhibit human vascular smooth muscle cell proliferation stimulated by native low-density lipoprotein. Eur. J. Pharmacol. 434:1-7.

Lopez, M., Martinez, F., Del Valle, C., Orte, C., and Miro, M. 2001. Analysis of phenolic constituents of biological interest in red wines by high-performance liquid chromatography. J. Chromatogr. A. 922:359-363.

Lotito, S.B. and Fraga, C.G. 1997. (+)-Catechin prevents human plasma oxidation. Free Radical Bio. Med. 24(3):435-441.

Maiani, G., Serafini, M., Salucci, M., Azzini, E., and Ferro-Luzzi, A. 1997. Application of a new high-performance liquid chromatographic method for measuring selected polyphenols in human plasma. J. Chromatogr. B. 692:311-317.

Mammela, P., Savolainen, H., Lindroos, L., Kangas, J., and Vartianinen, T. 2000. Analysis of oak tannins by liquid chromatography-electrospray ionisation mass spectrometry. J. Chromatogr. A. 891:75-83.

Manthey, J.A., Buslig, B.S., and Baker, M.E. 2002. Flavonoids in cell function. Adv. Exp. Med. Biol. 505:1-7.

Martinez-Ortega, M.V., Garcia-Parrilla, M.C., and Troncoso, A.M. 2001. Changes in phenolic composition of wines submitted to *in vitro* dissolution tests. Food Chem. 73:11-16.

McCarthy, T.L., Kerry, J.P., Kerry, J.F., Lynch, P.B., and Buckley, D.J. 2001a. Assessment of the antioxidant potential of natural food and plant extracts in fresh and previously frozen pork patties. Meat Sci. 57:177-184.

McCarthy, T.L., Kerry, J.P., Kerry, J.F., Lynch, P.B., and Buckley, D.J. 2001b. Evaluation of the antioxidant potential of natural food/plant extracts as compared

with synthetic antioxidants and vitamin E in raw and cooked pork patties. Meat Sci. 57:45-52.

McLellan, M. R. and Acree, T. 1993. Grape Juice. In *Fruit Juice Processing Technology*, Nagy, S., Chen, C.S., Shaw, P.E. (Eds.). Agscience, Inc., Auburndale, FL.

Mejanelle, P., Bleton, J., Goursaud, S., and Tchapla, A. 1997. Identification of phenolic acids and inositols in balms and tissues from an Egyptian mummy. J. Chromatogr. A. 767:177-186.

Mejia, E.G., Castano-Tostado, E., and Loarca-Pina, G.L. 1999. Antimutagenic effects of natural phenolic compounds in beans. Mutat. Res. 441:1-9.

Meyer, A.S., Yi, O., Pearson, D.A., Waterhouse, A.L. and Frankel, E.N. 1997. Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (*Vitis vinifera*). J. Agric. Food Chem. 45(5):1638-1643.

Moini, H., Guo, Q.O., and Packer, L. 2002. Xanthine oxidase and xanthine dehydrogenase inhibition by the procyanidin-rich French maritime pine bark extract, Pycnogenol®: A protein binding effect. Adv. Exp. Med. Biol. 505:141-149.

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P. and Cillard, J. 1993. Antioxidant and iron chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem. Pharmacol. 45(1):13-19.

Muto, S., Fujita, K., Yamazaki, Y., and Kamataki, T. 2001. Inhibition by green tea catechins of metabolic activation of procarcinogens by human cytochrome P450. Mutat. Res. 479:197-206.

Nakagawa, H., Kiyozuka, Y., Uemura, Y., Senzaki, H., Shikata, N., Hioki, K. and Tsubura, A. 2001. Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic acid, a potent breast cancer cell stimulator. J. Cancer Res. Clin. Oncol. 127:258-264.

Nakao, M., Takio, S., and Ono, K. 1998. Alkyl peroxyl radical-scavenging activity of catechins. Phytochemistry. 49(8):2379-2382.

Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., and Hara, Y. 1996. Scavenging effects of tea catechins and their derivatives on 1,1-diphenyl-2-picrylhydrazyl radical. Free Radical Bio. Med. 21(6):895-902.

NASS. 2002. http://www.usda.gov/nass. Accessed May 26, 2002.

Neta, P. and Simic, M.G. 1985. Free radical chemistry of natural products. In *Chemical Changes in Food during Processing*. Richardson, T. and Finley, J.W. (eds) Van Nostrand Reinhold Co. Inc. New York, NY.

Nie, G., Wei, T., Shen, S., and Zhao, B. 2001. Polyphenol protection of DNA against damage. Method. Enzymol. 335:232-244.

Niho, N., Shibutani, M., Tamura, T., Toyoda, K., Uneyama, C., Takahashi, N., and Hirose, M. 2001. Subchronic toxicity study of gallic acid by oral administration in F344 rats. Food Chem. Toxicol. 39:1063-1070.

Obanda, M., Owuor, P.O., and Mang'oka R. 2001. Changes in the chemical and sensory quality parameters of black tea due to variations of fermentation time and temperature. Food Chem. 75:395-404.

Ohe, T., Marutani, K., and Nakase, S. 2001. Catechins are not major components responsible for anti-genotoxic effects of tea extracts against nitroarenes. Mutat. Res-Gen. Tox. En. 496(1-2):75-81.

Ohshima, H., Yoshie, Y., Auriol, S. and Gilibert, I. 1998. Antioxidant and pro-oxidant actions of flavonoids: effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. Free Radical Bio. Med. 25(9):1057-1065.

Ory, R.L., Angelo, A.J.St., Gwo, Y.Y., Flick, G.J.Jr., and Mod, R.R. 1985. Oxidation-induced changes in foods. In *Chemical Changes in Food during Processing*. Richardson, T. and Finley, J.W. (eds) Van Nostrand Reinhold Co. Inc. New York, NY.

Pace-Asciak, C.R., Hahn, S., Diamandis, E.P., Soleas, G., and Goldberg, D.M. 1995. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implication for protection against coronary heart disease. Clin. Chim. Acta. 235:207-219.

Palma, M. and Taylor, L.R. 1999. Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. J. Chromatogr. A. 849:117-124.

Palma, M., Pineiro, Z., and Barroso, C.G. 2001. Stability of phenolic compounds during extraction with superheated solvent. J. Chromatogr. A. 921:169-174.

Palomino, O., Gomez-Serranillos, M.P., Slowing, E., Carretero, E., and Villar, A. 2000. Study of polyphenols in grape berries by reversed-phase high-performance liquid chromatography. J. Chromatogr. A. 870:449-451.

Pearson, D.A., Tan, C.H., German, J.B., Davis, P.A., and Gershwin, M.E. 1999. Apple juice inhibits human low density lipoprotein oxidation. Life Sci. 64(21):1913-1920.

Peterson, J. and Dwyer, J. 1998. Flavonoids: Dietary occurrence and biochemical activity. Nutr. Res. 18(12):1995-2018.

Piotrowski, J.J., Hunter, G.C., Eskelson, C.D., Dubick, M.A. and Bernhard, V.M. 1990. Evidence for lipid peroxidation in atherosclerosis. Life Sci. 46(10):715-721.

Rabbani, L.E. and Loscalzo, J. 1994. Recent observations on the role of hemostatic determinants in the development of the atherothrombotic plaque. Atherosclerosis. 105(1):1-7.

Rajalakshmi, K., Devaraj, H., and Devaraj, S.N. 2001. Assessment of the no-observed-adverse-effect level (NOAEL) of gallic acid in mice. Food Chem. Toxicol. 39:919-922.

Reische, D.W., Lillard, D.A. and Eitenmiller, R.R. 1998. Antioxidants. In *Food Lipids: Chemistry, Nutrition and Biotechnology*. Akoh, C.C. and Min, D.M. (eds). Marcel Dekker, Inc. New York, NY.

Revilla, E. and Ryan, J.M. 2000. Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. J. Chromatogr. A. 881:461-469.

Ricardo da Silva, J.M., Darmon, N., Fernandez, Y. and Mitjavilla, S. 1991. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. J. Agric. Food Chem. 39(9):1549-1552.

Rice-Evans, C. and Miller, N.J. 1994. Total antioxidant status in plasma and body-fluids. 234:279-293.

Rice-Evans, C.A., Miller, N.J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2(4):152-159.

Riou, V., Vernhet, A., Doco, T., and Moutounet, M. 2002. Aggregation of grape seed tannins in model wine – effect of wine polysaccharides. Food Hydrocolloid. 16:17-23.

Robak, J. and Gryglewski, R.J. 1988. Flavonoids are scavengers of superoxide anions. Biochem. Pharmacol. 37(5):837-841.

Robinson, J. 1986. *Vines, Grapes and Wines*. Alfred A. Knopf, Inc., New York, NY.

Roland, A. 1997. The inhibition of low density lipoprotein oxidation by the flavonoids quercetin and catechin. Abstract for 11th International Symposium on Atherosclerosis, Paris, France.

Romero-Perez, A.I, Lamuela-Raventos, R.M., Andres-Lavueva, C. and Torre-Boronat, M.C. 2001. Methods for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. J. Agric. Food Chem. 49(1):210-215.

Ruidavets, J.B., Teissedre, P.L., Ferrieres, J., Carando, S., Bougard, G., and Cabanis, J.C. 2000. Catechin in the Mediterranean diet: vegetable, fruit or wine? Atherosclerosis. 153:107-117.

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S. and Yamaji, N. 1998. Antiulcer activity of grape seed extract and procyanidins. J. Agric. Food Chem. 46(4):1460-1464.

Sakaguchi, N., Inoue, M., and Ogihara, Y. 1998. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. Biochem. Pharmacol. 55(12):1973-1998.

Sanchez-Moreno, C., Jimenez-Escrig, A., and Saura-Calixto, F. 2000. Study of low-density lipoprotein oxidizability indexes to measure the antioxidant activity of dietary polyphenols. Nutr. Res. 20(7):942-953.

Sanchez-Moreno, C., Larrauri, J.A., Saura-Calixto, F. 1999. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Res. Int. 32:407-412.

Sanders, M.E. 1998. Overview of functional foods: emphasis on probiotic bacteria. Int. Dairy J. 8:341-347.

Santana-Rios, G., Orner, G.A., Amantana, A., Provost, C., Wu, S.Y., Dashwood, R.H. 2001. Potent antimutagenic activity of white tea in comparison with green tea in the Salmonella assay. Mutat. Res. 495:61-74.

Sato, M., Bagchi, D., Tosaki, A. and Das, D.K. 2001. Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. Free Radical Bio. Med. 31(6):729-737.

Sato, M., Maulik, G., Ray, P.S., Bagchi, D. and Das, D.K. 1999. Cardioprotective effects of grape seed proanthocyanidin against ischemic reperfusion injury. J. Mol. Cell Cardiol. 31:1289-1297.

Schoefer, L., Braune, A., and Blaut, M. 2001. A fluorescence quenching test for the detection of flavonoid transformation. FEMS Microbiol. Lett. 204:277-280.

Scott, B.C., Butler, J., Halliwell, B. and Aruoma, O.I. 1993. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Rad. Res. Comms. 19(4):241-253.

Seigler, D.S. 1995. Flavonoids. In *Plant Secondary Metabolism*. Kluwer Academic Publishers, Norwell, MA.

Shahrzad, S., and Bitsch, I. 1996. Determination of some pharmacologically active phenolic acids in juices by high-performance liquid chromatography. J. Chromatogr. A. 741:223-231.

Shi, H., Noguchi, N., and Niki, E. 2001. Galvinoxyl method for standardizing electron and proton donation activity. Method. Enzymol. 335:157-166.

Shrikhande, A.J. 2000. Wine by-products with health benefits. Food Res. Int. 33:469-474.

Shukla, Y.N., Srivastava, A., Kumar, S., and Kumar, S. 1999. Phytotoxic and antimicrobial constituents of *Argyreia speciosa* and *Oenothera biennis*. J. Ethnopharmacol. 67:241-245.

Simonetti, P., Gardana, C., and Pietta, P. 2001. Caffeic acid as biomarker of red wine intake. Method. Enzymol. 335:122-130.

Soleas, G.J., Angelini, M., Grass, L., Diamandis, E.P., and Goldberg, D.M. 2001b. Absorption of *trans*-resveratrol in rats. Method. Enzymol. 335:145-154.

Soleas, G.J., Goldberg, D.M., Grass, L., Levesque, M. and Diamandis, E.P. 2001. Do wine polyphenols modulate p53 gene expression in human cancer lines? Clin. Biochem. 34:415-420.

Soleas, G.J., Yan, J., and Goldberg, D.M. 2001a. Measurement of *trans*-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. Method. Enzymol. 335:130-145.

Souquet, J.M., Cheynier, V., Brossaud, F., and Moutounet, M. 1996. Polymeric proanthocyanidins from grape skins. Phytochemistry. 43(2):509-512.

Takahashi, T. 2001. Biological actions of oligomeric procyanidins: proliferation of epithelial cells and hair follicle growth. Method. Enzymol. 335:361-368.

Takahashi, T., Yokoo, Y., Inoue, T. and Ishii, A. 1999. Toxicological studies on procyanidin B-2 for external application as a hair growing agent. Food Chem. Toxicol. 37:545-552.

Tang, S., Kerry, J.P., Sheehan, D., Buckley, D.J., and Morrissey, P.A. 2001a. Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. Food Res. Int. 34:651-657.

Tang, S.Z. , Kerry, J.P., Sheehan, D., Buckley, D.J., and Morrissey, P.A. 2000. Dietary tea catechins and iron-induced lipid oxidation in chicken meat, liver and heart. Meat Sci. 56:285-290.

Tang, S.Z., Kerry, J.P., Sheehan, D., and Buckley, D.J. 2002. Antioxidant mechanisms of tea catechins in chicken meat systems. Food Chem. 76:45-51.

Tang, S.Z., Kerry, J.P., Sheehan, D., Buckley, D.J., and Morrissey. 2001b. Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. Meat Sci. 57:331-336.

Tebib, K., Besancon, P. and Rouanet, J. 1996. Effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes. Nutr. Res. 16(1):105-110.

Tebib, K., Rouanet, J.M. and Besancon, P. 1997. Antioxidant effects of dietary polymeric grape seed tannins in tissues of rats fed a high cholesterol-vitamin E-deficient diet. Food Chem. 59(1):135-141.

Tedesco, I., Russo, M., Russo, P., Iacomino, G., Russo, G.L., Carraturo, A., Faruolo, C., Moio, L., and Palumbo, R. 2000. Antioxidant effect of red wine polyphenols on red blood cells. J. Nutr. Biochem. 11:114-119.

Teguo, P.W., Decendit, A., Vercauteren, J., Deffieux, G., and Merillon, J.M. 1996. *Trans*-resveratrol-3-*o*-β-glucoside (piceid) in cell suspension cultures of *Vitis vinifera*. Phytochemistry. 42(6):1591-1593.

Teissedre, P. and Landrault, N. 2000. Wine phenolics: contribution to dietary intake and bioavailability. Food Res. Int. 33:461-467.

Temple, N.J. 2000. Antioxidants and disease: more questions than answers. Nutr. Res. 20(3):449-459.

Torel, J., Cillard, J. and Cillard, P. 1986. Antioxidant activity of flavonoids and reactivity with peroxyl radical. Phytochem. 25(2):383-385.

Tsuchiya, H. 2001. Stereospecifity in membrane effects of catechins. Chem-Biol. Interact. 134:41-54.

Uesato, S., Kitagawa, Y., Kamishimoto, M., Kumagai, A., Hori, H., and Nagasawa, H. 2001. Inhibition of green tea catechins against the growth of cancerous human colon and hepatic epithelial cells. Cancer Lett. 170:41-44.

Vine, R.P., Harkness, E.M., Browning, T., and Wagner, C. 1997. *Winemaking: From Grape Growing to Marketplace*. Chapman and Hall, New York.

Vrhorsek, U., Wendelin, S., and Eder, R. 1997. Effects of various vinification techniques on the concentration of *cis*- and *trans*-resveratrol and resveratrol glucoside isomers in wine. Am. J. Enol. Vitic., 48(2):214-219.

Walton, N.J., Rhodes, M.J.C., Michael, A.J., and Parr, A.J. 1999. Characterization and control of plant secondary metabolism. In *Chemical From Plants: Perspective on Plant Secondary Products*. Imperial College Press, River Edge, NJ.

Wayner, D.D.M., Burton, G.W., Ingold, K.U., and Locke, S. 1985. Quantitative measurement of the total, peroxyl radical trapping antioxidant capacity of human blood plasma by controlled peroxidation. FEBS Lett. 187:33-37.

Weyant, M.J., Carothers, A.M., and Bertagnolli, M.M. 2000. (+)-Catechin inhibits tumor formation and modulates integrin-mediated signaling *in vitro* and *in vivo*. Surg. Forum Abstracts. 191(4S):S89.

Wilska-Jeszka, J. 1996. Proanthocyanidins: content in fruits and influence on health. Food Chem. 57(1):57-59.

Wilson, T., Porcari, J.P., and Harbin, D. 1998. Cranberry extract inhibits low density lipoprotein oxidation. Life Sci. 62(24):381-386.

Wollgast, J. and Anklam, E. 2000a. Polyphenols in chocolate: is there a contribution to human health? Food Res. Int. 33:449-459.

Wollgast, J. and Anklam, E. 2000b. Review on polyphenols in *Theobroma cacao*: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Res. Int. 33:423-447.

Woo, H.H., Kuleck, G., Hirsch, A.M., and Hawes, M.C. 2002. Flavonoids: signal molecules in plant development. Adv. Exp. Med. Biol. 505:51-60.

Wu, C.D. and Wei, G.X. 2002. Tea as a functional food for oral health. Nutrition. 18:443-444.

Yamagishi, M., Osakabe, N., Natsume, M., Adachi, T., Takizawa, T., Kumon, H., and Osawa, T. 2001. Anticlastogenic activity of cacao: inhibitory effect of cacao liquor polyphenols against mitomycin C-induced DNA damage. Food Chem. Toxicol. 39:1279-1283.

Yamakoshi, J., Kataoka, S., Koga, T. and Ariga, T. 1999. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. Atherosclerosis. 142:139-149.

Zhang, K., Yang, E.B., Tang, W.Y., Wong, K.P., and Mack, P. 1997. Inhibition of glutathione reductase by plant phenols. Biochem. Pharmacol. 54(9):1047-1053.

Zhao, K., Whiteman, M., Spencer, J.P.E., and Halliwell, B. 2001. DNA damage by nitrite and peroxynitrite: protection by dietary phenols. Method. Enzymol. 335:296-307.

Zhu, N., Wang, M., Wei, G.J., Lin, J.K., Yang, C.S., and Ho, C.T. 2001. Identification of reaction products of (-)-epigallocatechin, (-)-epigallocatechin gallate and pyrogallol with 2,2-diphenyl-1-picrylhydrazyl radical. Food Chem. 73: 345-349.

CHAPTER 3

EFFECT OF SOLVENT TYPE ON EXTRACTION OF GRAPE SEED

POLYPHENOLS AND ANTIOXIDANT ACTIVITIES OF GRAPE WINE INDUSTRY

BYPRODUCTS

ABSTRACT

Grape seed powders, by-products of either wine making or grape processing were prepared from *Vitis vinifera* (Merlot and Chardonnay) and *Vitis rotundifolia* (Muscadine) varieties. Water, ethanol, methanol and acetone were tested as extractants on Muscadine seed powder. Antioxidant capacities of grape seeds and skins were determined using oxygen radical absorbing capacity (ORAC) assay. The results indicated that aqueous solutions containing 60% (190 proof) ethanol, 60 to 70% methanol, and 50 to 75% acetone were better than when a single compound solvent system was used for extraction of total phenols. Significant correlations were found between the total phenol contents and absorbances at 280nm, an indicator of the total tannin in the extracts. Antioxidant capacities of Chardonnay, Merlot and Muscadine grape seeds were 637.8, 344.8 and 310.8 μmol TE/g dry weight in decreasing order ($p<0.05$), respectively. The difference between the ORAC values of Chardonnay and Merlot grape skin powder was not significant ($p>0.05$). Total phenol contents of extracts used to determine the antioxidant capacities of grape seeds and skins had a trend similar to ORAC values. Health functional components of grape skin and seed powders from byproducts of grape/wine industry are comparable to fruits and vegetables; therefore, these byproducts can be utilized to make dietary supplements or used in functional foods.

KEYWORDS: Antioxidant; antioxidant capacity; Chardonnay; grape seed; grape skin; Merlot; Muscadine; ORAC; winery byproduct

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INTRODUCTION

Grapes are a food commodity with an economic value of approximately $3 billion (NASS, 2002). *Vitis vinifera* and *Vitis rotundifolia* are two grape varieties commonly grown in the Unites States (McLellan and Acree, 1993). Half of the grapes grown in the US are processed into wine. Chardonnay and Merlot are respectively, white and red *vinifera* varieties of *Vitis* while Muscadine is the red *rotundifolia* variety. Red skinned Muscadine berries have large seeds. They grow well in the Southeastern US and Mexico. The berries of the *vinifera* varieties of *Vitis* are pressed directly and used mostly used to make wine while the *rotundifolia,* because of their low sugar contents are often processed into juices, jams or preserves. Some processors de-seed the Muscadine before pressing for juice to improve the release of juice from the seed sac. The juice is pressed out of the berries of the white vinifera varieties prior to fermentation into wine. In contrast, the red *vinefera* varieties are crushed, fermented followed by separation of the fermented juice from the skin and seeds to produce red wine. Skins and seeds of grapes are produced in large quantities by the wine making industry. These byproducts have become valuable raw materials for extraction of polyphenols. There were 22 grape seed, 5 grape skin, and 7 red wine powder products commercially available in the US in the year 2000 (Shrikhande, 2000), and the number of commercial products has increased since then. Patents have been awarded to several researchers on processing and preparing commercial forms of grape seeds, skins and related flavonoids because of their commercial potential as health functional agents (Masquelier, 1987; Goodman, 2000 and 2001; Toppo, 2000; Carson et al., 2001; Ray and Bagchi, 2001). Moreover, several other

patents have been awarded for food applications of products from grape seeds and skins (Siderquistm et al., 1990; Diamond, 2000).

The major components of grape skins and seeds include phenolic acids (gallic acid and ellagic acid), flavonoids (catechin, epicatechin, and procyanidins) and stilbenes (resveratrol). These grape seed and skin constituents have been shown to have health-functional activities. Grape seed procyanidin extract (GSPE) has *in vivo* antioxidant activity (Sato et al., 2001) and could be as important as vitamin E in preventing oxidative damage in tissues (Tebib et al., 1997) by reducing the lipid oxidation (Bouhamidi et al., 1998) and/or inhibit the production of free radicals (Bagchi et al., 1998). Animal studies indicated that myocardial infraction rate has been reduced with dietary GSPE supplementation (Sato et al., 1999). Addition of 1% GSPE (w/w) to diet reduced atherosclerosis in the aorta without influencing the serum lipid profiles of rabbits (Yamakoshi et al., 1999). Grape seed extracts of *Vitis vinifera* L. grapes containing either high or low flavanol contents showed antiulcer activity in rats (Saito et al., 1998). GSPE and its phenolic acid gallic acid may play a role in inducing apoptosis or programmed cell death in the body (Sakaguchi et al., 1998; Joshi et al., 2000). Since it induces the expression of vascular endothelial growth factor in keratinocytes, which can stimulate angiogenesis in wounds, GSPE containing 5mg/g *trans*-resveratrol can be used to treat dermal wounds and other dermal disorders (Khanna et al., 2001). Dietary supplementation with polymeric tannins can also stimulate fermentative activities without increasing the activity of harmful enzymes on animal models (Tebib et al., 1996; Chung et al., 1998).

Okuda and Yokotsuka (1996) indicated that white wines from Riesling and Chardonnay grapes contained 2.6 to 4.7% of the total extractable resveratrol in the skins. After eight-day fermentation, red wines of Cabernet Sauvignon and Muscat Bailey A had 13.6% and 35.5% of the resveratrol in the skins, respectively. The authors concluded that resveratrol transfer from grape skins to wines is very low for white wine, while the amount transferred varied in red wines due to the differences in variety and processing conditions. These results give a strong justification for the utilization of winery by-products (grape skins and seeds) as raw materials for the production of dietary supplements high in functional components.

The objectives of this study were to determine the effectiveness of different solvents in extracting phenolics from muscadine grape seed powder, and to evaluate antioxidant capacities as ORACS (Cao and Prior, 1999) of powders of skins and seeds from Chardonnay, Merlot and Muscadine grapes, obtained as byproducts of either wine making or juice processing industry.

EXPERIMENTAL

Production of Grape Skin and Seed Powders

Grape skin and seed samples were obtained from different sources. Merlot and Chardonnay skin and seeds were obtained from Habersham Winery (Helen, GA). The winery fermented crushed Merlot grapes for one week at around 75F, followed by pressing the fermented juice. Chardonnay seed and skin were separated from the juice before fermentation. The seed and skin were collected on the day they were produced and transported to the Department of Food Science and Technology, Athens (GA). Seeds were separated from the skin by rubbing the mixture over a coarse screen. A gas heated

impinger oven (Lincoln Foodservice Products, Inc., Fort Wayne, IN) at 93°C air temperature was used to dry grape skin and seeds for approximately 40min. Chardonnay skin and seeds were separated by hand. Chardonnay skin samples were dried at 93°C for about 90min in an impinger oven while seeds were dried for 60min. The extent of drying was determined by how easy the dried product can be ground into a powder using a hammer mill fitted with a fine (0.127 mm) screen (The Fitzpatrick Co, Elmhurst IL). Muscadine seeds were obtained from a mechanical muscadine-deseeding machine at Paulk Vineyards (Ocilla, GA). The wet seeds with skin and other particles were transported to the University of Georgia Food Science Building and stored frozen until used. After thawing, particles of skin and pulp adhering to the skin were removed by hand, and then seeds were washed with tap water to remove the juice adhering to the seeds. Washed seeds were dried at 93°C in the impinger oven for 40min and ground to a powder in a hammer mill.

Extraction of Polyphenolic Compounds from Grape Skin and Seeds

Solvents consisted of different volumes of deionized water mixed with either 190 proof ethanol, methanol, or acetone.

Known weights of grape skin and seed samples were mixed with solvent at a ratio of 1:10 (w/v). The mixture was sonicated for 15min, and shaken for 30min at room temperature. Then, the mixture was centrifuged at 4°C for 20min at 5000*g*. Supernatants were filtered through a funnel with glass wool, which was washed with 3-4mL of solvent. The volumes of filtered supernatants were recorded to calculate total phenolic contents, brix and absorbances at 280nm.

Total Phenol Contents, Brix and Absorbances

Total phenol contents of supernatants were determined according to Folin-Ciocalteu method (Vernon et al., 1999). Folin and Ciocalteu's phenol reagent was purchased from Sigma® (St. Louis, MO). Gallic acid (Aldrich Chem. Co., Milwaukee, WI) was used as a standard. Spectronic® Genesys™ 2 (Rochester, NY) was used to determine total phenolic contents and absorbances at 280nm of supernatants. Brix of the supernatants was determined with a refractometer (ATAGO PR-201, Tokyo, Japan). Sodium carbonate was purchased from Fisher Scientific (Fair Lawn, New Jersey).

Deoiling of Seeds

Grape seed powder was de-oiled with hexane (1part powder to 10 parts hexane, w/v). After shaking the mixture 10min at room temperature, the liquid was separated from the solid by vacuum filtration through a sintered glass filter (Pyrex® 10-15M). The solid residue was evenly distributed over a tray and kept under the hood in the dark to evaporate the hexane. Skin and seed extracts for analysis were prepared by mixing the powders with 70% methanol-water at a ratio of 1 part powder to 10 parts solvent (w/v). The mixture was sonicated 15min and shaken up to 30min at room temperature followed by centrifugation at 4°C for 20min at 26,000g. Supernatants were decanted and filtered through glass wool. The glass wool was rinsed with 3 to 4 mL of solvent which was mixed with the rest of the filtrate. Total phenolic contents of filtrates were determined accordingly.

Antioxidant Activities of Grape Skin and Seeds

Oxygen radical absorbance capacity (ORAC) assay (Cao et al., 1993; Cao and Prior, 1999) was used to quantify the antioxidant capacity of foods by measuring peroxyl

radical scavenging activity of the compounds found in foods. This assay is based on the chemical damage to β-PE caused by a peroxyl radical producing compound (i.e. AAPH in this assay), reducing the fluorescence emission of β-PE. The presence of antioxidants in the medium can recover the damage and prolong the reduction in the fluorescence emission. Antioxidant capacity of foods can be quantified by using the areas under the relative fluorescence intensity curves.

The assay is done using clear extracts of samples. Extracts for assays were made on the same sample first with water and secondly with acetone (Cao and Prior, 1999). Grape skin or seed powder was mixed with deionized water (1:10 w/v) in a centrifuge bottle, vortexed for 10sec, and sonicated at room temperature for 10min. After centrifugation at 4°C for 30min at 26,000g, water supernatants were collected and volumes were recorded. Acetone (1:10 w/v) was added to the precipitate, and the mixture was shaken at room temperature for 30min. After the bottles were centrifuged at 4°C at 26,000g for 10min, acetone supernatants were collected. Both supernatants were kept under dark at 4°C until analyzed. Water and acetone supernatants were run separately for ORACs.

An optimized version of ORAC assay developed by Cao and Prior (1999) was used to quantify the antioxidant capacity of MRP. β-Phycoerythrine (β-PE) was purchased from Cyanotech Co. (Kailua-Kona, HI) (Lot # 0215100) and prepared according to the recommended reconstitution procedure for purification. Briefly, the contents of the vial received from the supplier (1mg β-PE in 0.2mL buffer mixture) was rinsed with about 3.5mL of phosphate buffer (Stock buffer/deionized water, 1:9, v/v) (Stock buffer; 0.75M K_2HPO_4/0.75M NaH_2PO_4, 61.6:38.9, v/v). Prior to passing the

solution through the column, a Sephadex G-25 column was cleaned with about 20mL-phosphate buffer. The red band eluted out from the column was collected, and it was washed off with buffer. The purity of the β-PE solution was determined according to the recommended procedure. β-PE was diluted further with phosphate buffer. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA), and 0.868g of AAPH was dissolved in 10mL phosphate buffer. It was prepared daily and kept in ice until used.

LS-50B Luminescence Spectrometer (Perkin Elmer, UK) was used for the analyses. 'A four position, motor driven, water thermostatted, stirred cell holder' was installed on the spectrometer, and the temperature of the water bath, which supplied hot water to the cell holder, was set to 37°C. Emission and excitation wavelengths were 565 and 540nm respectively. Stirrer was set to low.

50μL of β-PE in phosphate buffer was incubated at 37°C for 5min in the cuvettes located in the holder, and then an appropriate aliquot sample (50-100μL) was added to the cuvettes. The reaction was started by adding 150μL of AAPH (24mM) to the cuvettes at min 5, and an initial fluorescence reading was taken. Total volume in each cuvette was 2mL. Blanks, which contained phosphate buffer, β-PE and AAPH only, were used for the area corrections. β-PE fluorescence intensities of samples and blanks were recorded every minute. The changes in β-PE fluorescence over time were displayed on the screen. The data were collected until the fluorescence reading declined by 95% of the initial reading. Intensities were converted to relative intensities by dividing the readings with the initial reading. The areas under the curves were calculated using the software supplied by spectrofluorometer manufacturer.

Trolox, a water-soluble analog of vitamin E, was purchased from Aldrich Chem. Co. (Milwaukee, WI). It was dissolved in 10mL ethanol (190 proof), and then diluted to 200mL with phosphate buffer. Trolox solution was prepared weekly and kept at refrigeration temperature. Standard curves were obtained using known concentrations of trolox (0.5 to 3µmol final concentration in cuvette). Corrected areas of relative fluorescence intensities versus trolox concentrations were plotted. The trolox equivalents of the samples were calculated by using the linear portion of the plot after proper dilutions were prepared with phosphate buffer.

Statistical Analyses

Data were analyzed using the Statistical Analysis System software (SAS Institute, Inc., 1990). PROC GLM with Duncan's multiple comparison test was performed to determine significant differences at $\alpha = 0.05$. Correlation coefficients were determined using PROC CORR.

RESULTS AND DISCUSSION

Extraction of Polyphenols with Aqueous Ethanol Solutions

Total phenol content of clarified ethanol extracts from muscadine seed powder extracted with solvent containing 50, 60 and 70%, 190 proof ethanol in water was about 28mg gallic acid equivalent (GAE)/g seed powder. Total phenolic contents in the clarified extract when using 50, 60, and 70%, 190 proof ethanol in water were not statistically significant ($p > 0.05$) (Figure 3.1). 190 proof ethanol alone or deionized water alone was ineffective as a solvent for extraction of phenolic compounds from muscadine seed powder. The trend of absorbances at 280 nm of clarified extracts with ethanol

concentration was similar to that of the total phenols indicating that the absorbance of the extract is directly related to the extractable tannins in solution (Figure 3.1).

Low molecular weight soluble solids which manifest an increase in the Brix reading, were higher in the extracts using solvents containing 50 to 70% (v/v) 190 proof ethanol in water, compared to 0 to 40% and 80 to 100% 190 proof ethanol solvents ($p<0.05$) (Figure 3.2).

High and significant correlation coefficients were found among total phenol contents, absorbances at 280nm and brix values of ethanol supernatants. Correlation coefficient between total phenol content and absorbances at 280nm was 0.949 ($p<0.001$). Brix values were also highly correlated with absorbances and total phenol contents of the clarified ethanol extracts, 0.945 and 0.875 ($p<0.001$) respectively.

Methanol Extracts

Supernatants from a solvent that consisted of a mixture containing 60 or 70% methanol and water yielded about 30mg GAE per g muscadine seed. This was the highest concentration in the extract compared with the other methanol-water mixtures (Figure 3.3). Increasing water in the mixture from 70 to 100% significantly reduced the amount of phenolic compounds extracted ($p<0.05$). Extraction of phenolic compounds from muscadine seed by solvents containing methanol only or deionized water only was extracted less than 1/10 of the amount extracted by the optimum solvent mixture. Clarified extracts using solvent containing 50 or 60% methanol have the highest absorbances at 280nm ($p<0.05$) (Figure 3.3). Brix values of clarified extracts with solvents containing 10 to 60% methanol were significantly higher than those of the other solvents ($p<0.05$) (Figure 3.2). Unlike ethanol-water solvents where the same trend in the

amount extracted vs. ethanol concentration was the same whether evaluated as total phenols, absorbance at 280nm, or Brix values of the extract, methanol-water solvents appear to show differences in solvent concentration optimum when evaluated as total phenolics, absorbance at 290nm or Brix values.

The effect of shaking time of the seed powder and solvent during extraction on amount of phenolic compounds extracted from muscadine seed powder was also studied. Results (not shown) indicated that shaking the mixture of muscadine seed powder in 70% methanol (v/v) at room temperature for 30min or 60min had no effect on the total phenol content in the clarified extracts.

When the muscadine seed powder was de-oiled by shaking for 10min with hexane prior to extraction with 70% methanol-water, the total phenol content of the clarified extracts (28.2±0.33mg gallic acid equivalent (GAE)/g dry weight) was slightly less than when no de-oiling was done. Shaking the mixture of de-oiled muscadine seed powder in 70% methanol (v/v) at room temperature up to 30min had no effect on the total phenol contents in the clarified extracts (p>0.05).

Total phenol contents of clarified ethanol-water extracts were highly correlated with brix (0.950 p<0.001) and absorbances at 280nm (0.670 p<0.001).

Extraction of Polyphenols Using Acetone-Water Solvents

Among acetone-water mixture as solvents, those containing 50 or 75% acetone by volume showed the highest total phenol content (about 40mg GAE/g dry seed) in the clarified extracts compared to the other acetone-water solvents. (Figure 3.4) (p<0.05). Neither acetone nor deionized water alone was as effective as the 50 or 75% acetone solvents. The Brix values of clarified extracts when using 75% acetone solvent was

highest among the other acetone solvents (Figure 3.5) ($p<0.05$). The clarified extracts from solvents containing 50% acetone or more, in general, had higher absorbances at 280nm, meaning that tannin extraction was effective when the extraction solvent contained 50% or more acetone.

Katalinic (1999) determined the catechin, epicatechin and procyanidin dimers of B_1, B_2, B_3 and B_4 using grape skins from 10 different cultivars. Katalinic (1999) found that (+)-catechin was the major catechin monomer in all grape skins with the exception of Cabernet Sauvignon. Most catechins have a maximum absorption at around 280nm. In our extraction of skin and seed phenolics from Merlot and Chardonnay grape seeds and skins we found that absorbance at 280nm is a good indicator of the total phenol contents of the grape seed or skin extracts for all the solvents used.

The correlation between total phenol contents and total tannins (absorbance at 280nm) in grape skin extracts were significant ($r=0.814$, $p<0.01$). If we analyze the data presented by Katalinic (1999), similar high correlations were shown between total phenol contents in the grape skin extracts and the total tannins measured as absorbance at 280nm.

As with muscadine seeds, *vinifera* grape skin and grape seed powders behave similarly in the efficiency of extraction of phenolics by ethanol-water, methanol-water or acetone-water systems. Total phenols extracted from muscadine seeds were 6.8mg GAE for water, 38mg GAE for 75% aqueous acetone, 28mg GAE for 60% ethanol (190 proof), and 31mg GAE for 60% methanol per gram dry seed powder. The effectiveness of 75% acetone in extracting phenolic constituents of seeds was significantly higher than others ($p<0.05$). 60% Ethanol (190 proof) and 60% methanol in water equally extracted

phenolic compounds present in muscadine seeds. Water only extracted the least amount of phenolics from seed powders ($p < 0.05$).

A mono-component solvent system (water, 190 proof ethanol, acetone, methanol) was not as efficient in extracting phenolic constituents of dried muscadine grape seeds as an aqueous solution containing at least 50% water. Jayaprakasha et al. (2001) also found that acetone or methanol alone did not give the optimum level of antioxidants in the extracts from grape seeds.

Grape seeds contain catechin, epicatechin, procyanidin dimers (B_1- B_8), trimers, oligomers and polymers (Ricardo da Silva et al., 1991). Kallithraka et al. (1995) reported that aqueous mixtures of either ethanol or acetone extracted gallic acid, catechin, epicatechin, procyanidins B_1, B_2, and C_1, and epicatechin gallate from *Vitis vinifera* grape seeds better than ethanol or acetone alone. They noted that procyanidin trimer C_1 was extracted best when 70% acetone used as a solvent. The authors also indicated methanol extraction was best for catechin, epicatechin and epigallocatechin.

Our study showed that aqueous mixtures of either ethanol, methanol or acetone was better than a mono-component solvent. Results were in good agreement with Jayaprakasha et al. (2001) and Kallithraka et al. (1995). An aqueous mixture of 50 or 75% acetone was a better solvent than 60% ethanol or 70% methanol to extract phenolic constituents of grape seeds.

Antioxidant Capacities of Grape Skin and Seeds

The extraction of antioxidants from wet fruits and vegetables presents a different system compared to extraction of dried samples as discussed in the preceding sections. Fresh fruits and vegetables generally have very high moisture contents, and typical

processing of these fresh materials into juice or purees extracts primarily the water soluble fractions. However, a significant amount of the antioxidants are left in the residue after the aqueous fraction has been separated, thus a second extraction is need to obtain the total antioxidant content of the sample. Our results in the previous sections indicate that an acetone-water mixture is an effective solvent for the antioxidants. Thus, re-extraction of the wet residue after separation of the aqueous phase will result in an acetone-water solvent system with high affinity for the antioxidants.

Antioxidant capacities of grape skins and seeds are shown in Table 3.1. ORAC assay data revealed that the acetone extracted more compounds with peroxyl radical scavenging activities than water. All samples showed good antioxidant properties in terms of in vitro peroxyl radical scavenging activities.

Grape seeds had 3-10 times higher ORAC values than skin samples on a dry basis ($p<0.05$). ORAC values of Chardonnay seeds were around 650μmole trolox equivalent (TE)/g dry seed powder, which was twice more than other seed powders ($p<0.05$). Merlot skin and seed powders had a higher standard deviation compared with muscadine and Chardonnay samples. Fermentation of Merlot grapes prior to juice extraction might be a reason of high variation in the ORAC values. In addition, small size of Merlot seeds may also increase the variation due to the difficulty in separating seeds from skins and pulp particles.

Our results for ORAC of grape skin and seeds are comparable with ORAC values of fruits. Strawberry, plum, red grape (whole), and orange has ORAC values of 154, 80, 36 and 52μmol TE/g dry matter (Wang et al., 1996). Red raspberries have ORAC values ranged from 91 to 115μmol TE/g dry matter (Wang and Lin, 2000). ORAC values of

blueberries depend on variety but the range was reported to be from 63 to 282 μmol TE/g dry matter (Prior et al., 1998).

Similar trends were also observed for total phenol contents of clarified aqueous phase and acetone extracts used in the ORAC assay (Table 3.2). Total phenol contents of acetone supernatants were higher than water supernatants. Total phenol content of Chardonnay seed was about 53 mg GAE/g dry matter and was the highest among all clarified extracts from samples on a dry basis ($p<0.05$). Interestingly, the difference between the phenol contents of Muscadine seed and Chardonnay skin extracts (total) was not significant but Muscadine seeds showed a higher antioxidant capacity expressed as ORAC than Chardonnay skins. Revilla and Ryan (2000) reported that white grape skins indicated a few peaks on an HPLC chromatograph registered at 280nm, whereas seeds showed numerous peaks corresponding to a variety of catechins and oligomeric procyanidins. In this case, grape seeds have more oligomeric or polymeric procyanidins compared to skins. These polymeric procyanidins may scavenge the free radicals better than monomeric or dimeric procyanidins (Bouhamidi et al., 1998; Tebib et al., 1997; Yamakoshi et al., 1999).

CONCLUSIONS

Our study indicated that byproducts of grape/wine industry have health functional components comparable with those present in fruits and vegetables. Seeds were found to be a better source of antioxidative constituents than skins of grape/wine byproducts. These byproducts could be utilized to produce dietary supplements, which could potentially increase total antioxidant intake of the general population. Prior et al. (1998) reported that the estimated antioxidant intake in terms of ORAC ranges from 1.2 to

1.7mmol TE/day in the US. Consumption of 1-g winery byproduct of grape seeds per day could easily increase this daily intake (ORAC) by more than 30%.

ABBREVIATIONS USED

AAPH, 2,2`-azobis(2-amidinopropane) dihydrochloride; GAE, gallic acid equivalent; ORAC, oxygen radical absorbing capacity; β-PE, β-phycoerythrine; TE, trolox equivalent.

REFERENCES

Bagchi D, Garg A, Krohn RL, Bagchi M, Bagchi DJ, Balmoori J and Stohs SJ, Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. *Gen Pharmac* **30**(5):771-776 (1998).

Bouhamidi R, Prevost V and Nouvelot A, High protection by grape seed proanthocyanidins (GSPC) of polyunsaturated fatty acids against UV-C induced peroxidation. *Life Sci* **321**:31-38 (1998).

Cao G and Prior R, Measurement of oxygen radical absorbance capacity in biological samlples. *Method Enzymol* **299**:50-63 (1999).

Cao G, Alessio HM and Culter RG, Oxygen-radical absorbance capacity assay for antioxidant. *Free Radical Bio Med* **14**:303-311 (1993).

Carson RG, Patel K, Carlomusto M, Bosko CA, Pillai S, Santhanam U, Weinkauf RL, Iwata K and Palanker LR, Cosmetic compositions containing resveratrol. Patent no: US6,270,780B1 (2001).

Chung KT, Lu Z and Chou MW, Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food Chem Toxicol*. **36**:1053-1060 (1998).

Diamond GB, Food spray containing grape seed oil. Patent no: 6,123, 977 (2000).

Goodman DW, Treatment for blood cholesterol with *trans*-resveratrol. Patent no: 6,022,901 (2000).

Goodman DW, Treatment for blood cholesterol with *trans*-resveratrol. Patent no: US6,211,247B1 (2001).

Jayaprakasha GK, Signh RP and Sakariah KK, Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. *Food Chem* **73**:285-290 (2001).

Joshi SS, Kuszynski CA, Bagchi M and Bagchi D, Chemopreventive effects of grape seed proanthocyanidin extract on Chang liver cells. *Toxicology* 155:83-90 (2000)

Kallithraka S, Garcia-Viguera C, Bridle P and Bakker J, Survey of solvents for the extraction of grape seed phenolics, *Phytochem Analysis* **6**:265-267 (1995).

Katalinic V, Grape catechins: natural antioxidants. *J Wine Res* **10**(1):15-23 (1999).

Khanna S, Roy S, Bagchi D, Bagchi M and Ken CK, Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract. *Free Radical Bio Med* **31**(1):38-42 (2001).

Masquelier J, Plant extract with a proanthocyanidins content as therapeutic agent having radical scavenger effect and use thereof. Patent no: 4,698,360 (1987).

McLellan MR and Acree T, Grape juice, in *Fruit Juice Processing Technology*, Ed by Nagy S, Chen CS and Shaw PE. Agscience, Inc., Auburndale, FL (1993).

NASS, http://www.usda.gov/nass. Accessed May 26, 2002 (2002).

Okuda T and Yokotsuka K, *trans*-Resveratrol concentrations in berry skins and wines from grapes grown in Japan. *Am J Enol Vitic* **47**(1):93-98 (1996).

Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner N, Ehlenfeldt M, Kalt W, Krewer G and Mainland CM, Antioxidant capacity as influenced by total phenolics and anthocyanin content, maturity, and variety of *Vaccinium* species. *J Agric Food Chem* **46**(7):2686-2693 (1998).

Ray SD and Bagchi D, Prevention and treatment of acetominophen toxicity with grape seed proanthocyanidin extract. Patent no: US6,245,336B1 (2001).

Revilla E and Ryan JM, Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. *J Chromatogr A* **881**:461-469 (2000).

Ricardo da Silva JM, Darmon N, Fernandez Y and Mitjavilla S, Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. *J Agric Food Chem* **39**(9):1549-1552 (1991).

Saito M, Hosoyama H, Ariga T, Kataoka S and Yamaji N, Antiulcer activity of grape seed extract and procyanidins. *J Agric Food Chem* **46**(4):1460-1464 (1998).

Sakaguchi N, Inoue M and Ogihara Y, Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. *Biochem Pharmacol* **55**(12):1973-1998 (1998).

SAS Institute, SAS User's Guide: Statistics. Version 6.4[th] ed. Cary, NC: SAS Institute (1990).

Sato M, Maulik G, Ray PS, Bagchi D and Das DK, Cardioprotective effects of grape seed proanthocyanidin against ischemic reperfusion injury. *J Mol Cell Cardiol* **31**:1289-1297 (1999).

Sato M, Bagchi D, Tosaki A and Das DK, Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. *Free Radic Biol Med* **31**(6):729-737 (2001).

Shrikhande AJ, Wine by-products with health benefits. *Food Res Inter* **33**:469-474 (2000).

Siderquistm CA, Kelly JA and Mandel FS, Gallic acid as an oxygen scavenger. Patent no: 4,968,438 (1990).

Tebib K, Besancon P and Rouanet J, Effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes. *Nutr Res* **16**(1):105-110 (1996).

Tebib K, Rouanet JM and Besancon P, Antioxidant effects of dietary polymeric grape seed tannins in tissues of rats fed a high cholesterol-vitamin E-deficient diet. *Food Chem* **59**(1):135-141 (1997).

Toppo F, Treatment for blood cholesterol with *trans*-resveratrol. Patent no: 6,048,903 (2000).

Vernon LS, Orthofer R and Lamuela-Raventos RM, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Method Enzymol* **299**:152-178 (1999).

Wang H, Cao G and Prior RL, Total antioxidant capacity of fruits. *J Agric Food Chem* **44**(3):701-705 (1996).

Wang SY and Lin HS, Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and development stage. *J Agric Food Chem* **48**(2):140-146 (2000).

Yamakoshi J, Kataoka S, Koga T and Ariga T, Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **142**:139-149 (1999).

FIGURE CAPTIONS

Figure 3.1. Effect of ethanol on the total phenol contents and absorbances at 280nm of supernatants from dried and ground muscadine seeds

Figure 3.2. Extraction of soluble solids from dried and finely ground muscadine seeds using aqueous ethanol or methanol

Figure 3.3. Effect of methanol on the total phenol contents and absorbances at 280nm of supernatants from dried and ground muscadine seeds

Figure 3.4. Effect of acetone on the total phenol contents and absorbances at 280nm of supernatants from dried and ground muscadine seeds

Figure 3.5. Extraction of soluble solids from dried and finely ground muscadine seeds using acetone

Table 3.1. Antioxidant capacities of various grape skin and seed powders as byproducts of food industry

Type	ORAC[1] ± Standard Deviation (μmol TE/g dry weight)
Seed	
Muscadine	310.8 ± 3.7^b
Merlot	344.8 ± 99.9^b
Chardonnay	637.8 ± 7.4^a
Skin	
Merlot	69.8 ± 33.2^c
Chardonnay	102.8 ± 7.0^c

[1]Averages of at least 3 values. Superscripts with different letters within the column show significant differences at α=0.05, using Duncan's multiple range test.

Table 3.2. Total phenol contents in water and acetone supernatants used to determine antioxidant capacities of grape seed and skins

Type	Total Phenol Content[1] (mg GAE/g dry weight)		
	Supernatant		
	Water	Acetone	Total
Seed			
Muscadine	4.78 ± 0.89	27.35 ± 0.74	32.13 ± 1.63^{bc}
Merlot	2.30 ± 1.17	36.15 ± 8.03	38.45 ± 9.20^b
Chardonnay	5.98 ± 0.49	46.69 ± 1.50	52.67 ± 1.98^a
Skin			
Merlot	2.35 ± 1.26	12.64 ± 4.72	14.99 ± 5.97^d
Chardonnay	5.21 ± 0.39	15.09 ± 2.00	20.30 ± 2.39^{cd}

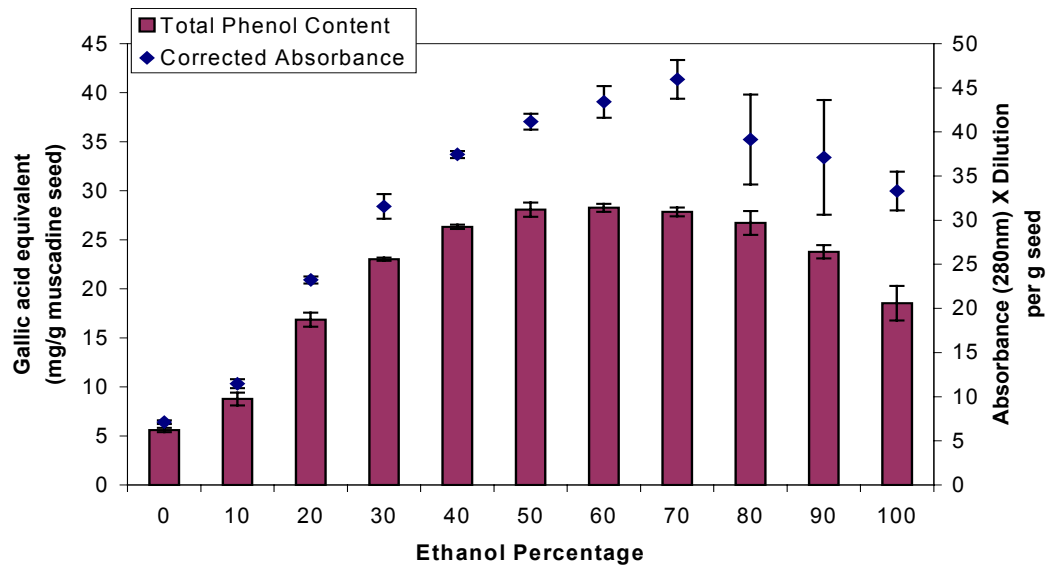[1]Averages of at least 3 values. Superscripts with different letters within the column show significant differences at α=0.05, using Duncan's multiple range test.

Figure 3.1.

Figure 3.2.

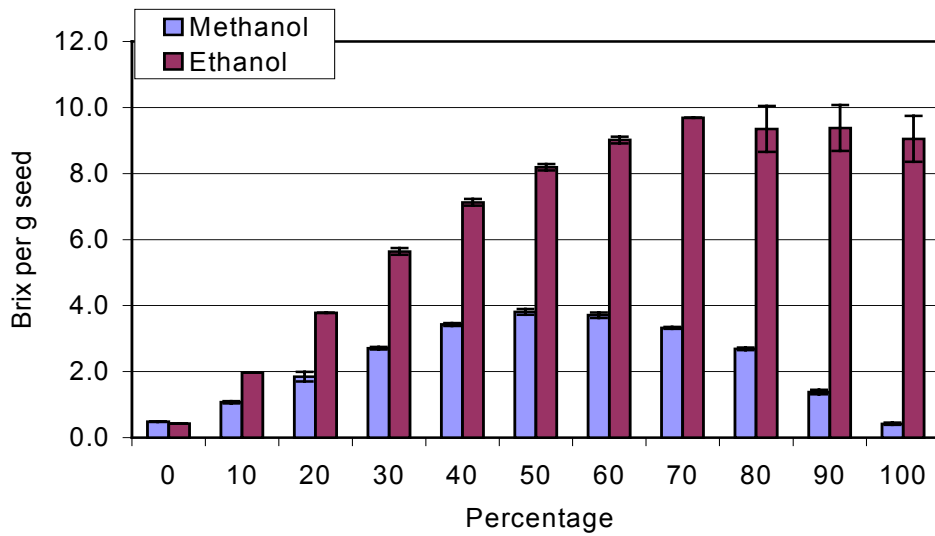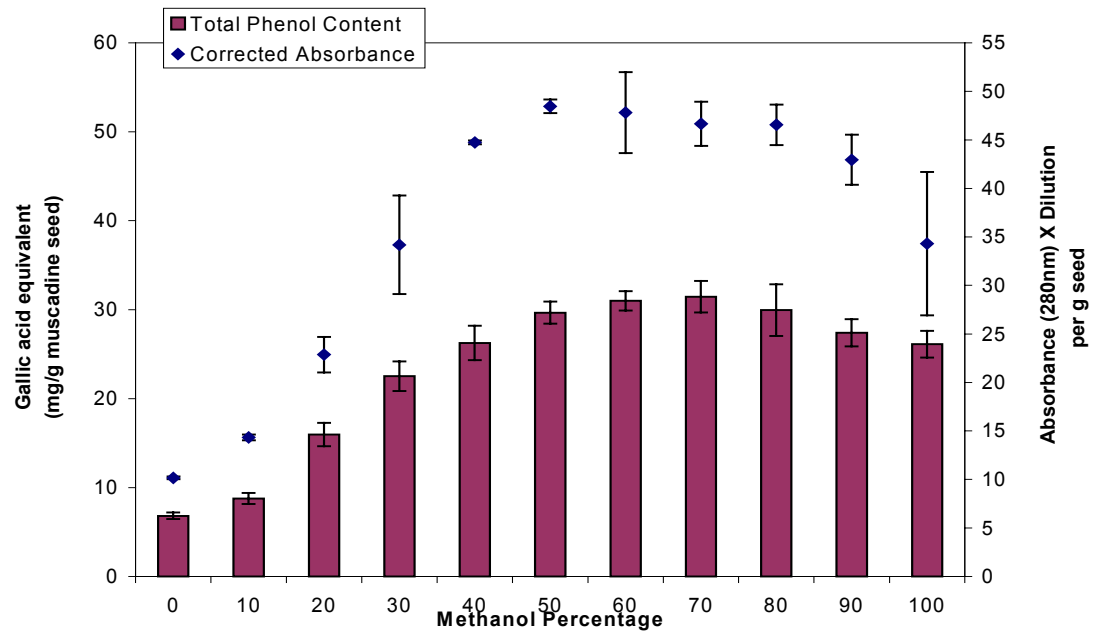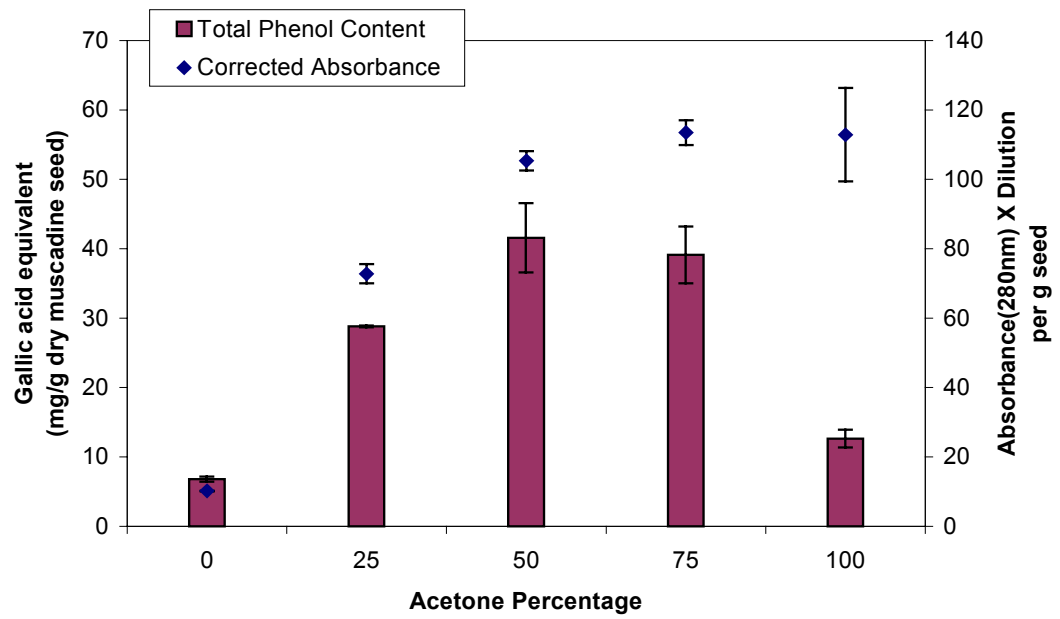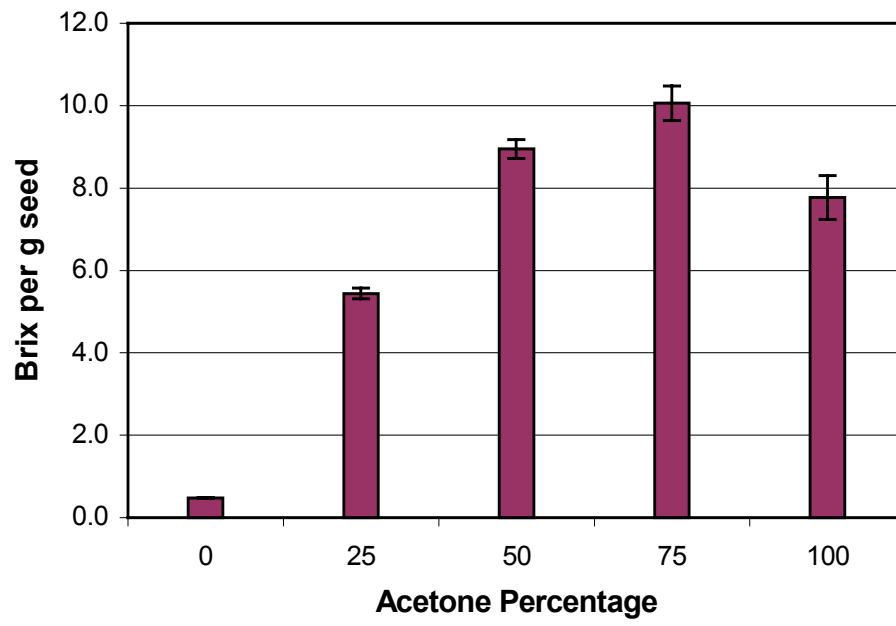Figure 3.3.

Figure 3.4.

Figure 3.5.

CHAPTER 4

MAJOR FLAVONOIDS IN GRAPE SEEDS AND SKINS: ANTIOXIDANT

CAPACITY OF CATECHIN, EPICATECHIN AND GALLIC ACID

ABSTRACT

Grape seeds and skins are good sources of phytochemicals such as gallic acid, catechin and epicatechin and are suitable raw materials for production of antioxidative dietary supplements. Seeds and skins from grapes of *Vitis vinifera* varieties Merlot and Chardonnay and in seeds from grapes of *Vitis rotundifolia* variety Muscadine were analyzed for the major monomeric flavanols and phenolic acids. The contribution of the major monomeric flavanols and phenolic acid to the total antioxidant capacity of grape seeds and skins was also determined. Gallic acid, catechin and epicatechin concentrations were 68, 7, and 69mg/100g d.m. in Muscadine seeds, 10, 211, and 303mg/100g d.m. in Chardonnay seeds, and 7, 74, and 83mg/100g d.m. in Merlot seeds, respectively. Concentrations of these three compounds were lower in winery byproduct grape skins than seeds. These three major phenolic constituents of grape seeds contributed less than 17% to the antioxidant capacity measured as ORAC. Peroxyl radical scavenging activities of phenolics present in grape seeds or skins in decreasing order were resveratrol > catechin > epicatechin = gallocatechin > gallic acid = ellagic acid. The results indicated that dimeric, trimeric, oligomeric or polymeric procyanidins account for most of the superior antioxidant capacity of grape seeds.

KEYWORDS: Antioxidant; antioxidant capacity; catechin; Chardonnay; epicatechin; gallic acid; galvinoxyl; grape seed; grape skin; Merlot; Muscadine; ORAC

INTRODUCTION

Grape seeds and skins are considered good sources of polyphenolic tannins that provide the astringent taste to wine. The phenolic acid, gallic acid, and flavanol monomers, catechin and epicatechin are the main phenolic compounds in grape seeds (Palma and Taylor, 1999). These are also the major flavonoids present in grape skins in addition to various anthocyanins. Terminal units of polymeric procyanidins of grape skins contain 67% (+)-catechin while extension units 60% (-)-epicatechin (Souquet et al., 1996).

(+)-Catechin shows antioxidant activity in human plasma by delaying the degradation of endogenous α-tocopherol and β-carotene and by inhibiting the oxidation of plasma lipids (Lotito and Fraga, 1997). (+)-Catechin has hydroxyl (Moini et al., 2002), peroxyl (Scott et al., 1993), superoxide (Bors and Michel, 1999) and DPPH (Fukumoto and Mazza, 2000) radical scavenging activities. Moreover, it can chelate iron (Morel et al, 1993). (-)-Epicatechin is able to scavenge hydroxyl radicals (Moini et al., 2002), peroxyl radicals (Liu et al., 2000), superoxide radicals (Bors and Michel, 1999), and DPPH radicals (Fukumoto and Mazza, 2000). Nakao et al. (1998) found that (+)-catechin and (-)-epicatechin have a peroxyl radical scavenging activity ten times higher than L-ascorbate and β-carotene when tested on bacteria.

Gallic acid is a phenolic acid which can scavenge peroxyl radicals (Liu et al., 2000) and DPPH radicals (Sanchez-Moreno et al., 1999; Fukumoto and Mazza, 2000). Gallic acid has antioxidant activity at stomach pH (Gunckel et al., 1998). Gallic acid has also antifungal activity (Shukla et al., 1999).

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Grape seed procyanidin extract (GSPE) has *in vivo* antioxidant activity (Sato et al., 2001) and could be as important as vitamin E in preventing oxidative damage in tissues (Tebib et al., 1997) by reducing lipid oxidation (Bouhamidi et al., 1998) and/or blocking the production of free radicals (Bagchi et al., 1998). Animal studies indicated that GSPE supplementation of the diet reduced myocardial infection rate (Sato et al., 1999) and atherosclerosis in the aorta without influencing the serum lipid profiles (Yamakoshi et al., 1999). Grape seed extracts of *Vitis vinifera* L. grapes showed antiulcer activity in rats (Saito et al., 1998). GSPE and the phenolic acid gallic acid may play a role in the induction of apoptosis or programmed cell death, in the body (Sakaguchi et al., 1998; Joshi et al., 2000). Polymeric tannin supplementation can also stimulate fermentative activities without increasing the activity of harmful enzymes on animal models (Tebib et al., 1996; Chung et al., 1998).

Monomeric procyanidins were found to be ineffective in the inhibition of atherosclerosis in rabbits compared with grape seed procyanidin extract (Yamakoshi et al., 1999). Procyanidins are considered superior antioxidants compared to their corresponding monomers (Ursini et al., 2001).

In the present work, we analyzed the major monomeric flavanols present in seeds and skins of *Vitis vinifera* variety Merlot and Chardonnay, and seeds of *Vitis rotundifolia* variety Muscadine. The main objective of this study was to determine the contribution of the major monomeric flavanols and phenolic acid to the total antioxidant capacity of grape seeds and skins measured as ORAC (Cao and Prior, 1999).

MATERIALS AND METHODS

Production of Grape Skin and Seed Powders

Grape skin and seed samples were obtained from different sources. Merlot and

Chardonnay skin and seeds were obtained from Habersham Winery (Helen, GA). The

Merlot samples were residue left after separating the wine following fermentation of the

crushed grapes for a week at around 24°C. The residue which included pulp, skin and

seeds were collected and transported to the University of Georgia, Athens (GA). Seeds

were separated from grape skin by rubbing against a screen. A gas-fired impinger oven,

(Lincoln Impinger, Lincoln Foodservice Products, Inc., Fort Wayne, IN) was used to dry

grape skin and seeds at 93°C for approximately 40min. Chardonnay residue left after

pressing the grapes prior to fermentation were also obtained from Habersham Winery and

transported to the University of Georgia. Since the skins were firm and the seeds were

small, the seeds were manually picked out of the skins. Both Merlot and Chardonnay

samples were stored at 2°C and processed within 48h after receipt at the University of

Georgia. Chardonnay skin and seeds were dried at 93°C in the impinger oven for 90 and

60 min, respectively. The extent of drying was determined by ease with which the dried

samples can be ground to a fine powder. Muscadine seeds were separated on a

mechanical de-seeder while crushing the grapes for juice and pulp at Paulk Vineyards

(Ocilla, GA). Seeds were transported to the University of Georgia and stored at -20°C

until used. Before drying, the muscadine seed was thawed by transferring to a cooler at

2°C and holding for about 4 days. The thawed seeds were spread out on a tray and skin,

bits of pulp, stems and twigs were removed manually. The seeds were washed with tap

water to remove the grape juice adhering to the seeds, drained, and dried 40min at 93°C in the impinger oven.

A hammer mill (FitzMill, The Fitzpatrick Co., Elmhurst, IL) fitted with 0.2 mm screens was used to grind grape seed and skin into a powder.

Preparation of Grape Skin and Seed Extracts

Grape seed powder was de-oiled with hexane (1part powder to 10 parts hexane, w/v). After shaking the mixture 10min at room temperature, the liquid was separated from the solid by vacuum filtration through a sintered glass filter (Pyrex® 10-15M). The solid residue was evenly distributed over a tray and kept under the hood in the dark to evaporate the hexane. Skin and seed extracts for analysis were prepared by mixing the powders with 70% methanol-water at a ratio of 1 part powder to 10 parts solvent (w/v). The mixture was sonicated 15min and shaken 30min at room temperature followed by centrifugation at 4°C for 20min at 26,000g. Supernatants were decanted and filtered through glass wool. The glass wool was rinsed with 3 to 4 mL of solvent which was mixed with the rest of the filtrate. The extract was concentrated in a vacuum rotary evaporator 40°C. Volume of the concentrate was then adjusted to obtain a concentration of 1-g solids/mL by adding a predetermined volume of 25% methanol. In a separate experiment, known quantities of gallic acid, catechin and epicatechin were added to the Chardonnay seed and skin powders before the start of the extraction process to determine how well these compounds could be recovered from the sample using the applied extraction and analytical procedure.

High Pressure Liquid Chromatography

A Shimadzu LC-10AT liquid chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, ML) with a Shimadzu SPD-10AV UV-VIS detector at dual wavelength was used to determine the monomeric phenolic constituents of grape skin and seed extracts. A Waters Spherisorb ODS-2 5u (250mm x 4.6mm) column (Alltech Associates, Inc., Deerfield, IL) with a Waters Spherisorb ODS-2 (C18) 5u guard column was used for the HPLC analyses. A gradient pump (Shimadzu FCV-10AL, Shimadzu Scientific Instruments, Inc., Columbia, ML) was used to create solvent gradient in the column (Table 4.1). Flow rate was set to 0.75mL/min. A sample volume of 20μL was injected to the column using Rheodyne syringe injector (Rheodyne 7725i, Syringe Loading Sample Injector). At least three replicates of each extract obtained from two seed or skin samples were analyzed.

Dual wavelengths were used to detect the eluent as follows; from the start to 25min at 280nm to detect gallic acid, catechin and epicatechin from 25min to the end at 360nm to detect other compounds like ellagic acid. Standards of gallic acid, catechin and epicatechin, and ellagic acid were obtained from Aldrich Chem. Co. (Milwaukee, WI); Sigma®(St. Louis, MO) and Fluka Chemical Co. (Ronkonkoma, NY), respectively. Compounds were quantified from a chromatograph of the standards mixture as shown in Figure 4.1.

Verification of the presence of gallic acid, catechin, epicatechin, ellagic acid and resveratrol was made with a photodiode array detector and fluorescence detector using another HPLC equipment (a Hewlett-Packard (Avondale, PA), model 1090 liquid chromatograph with quaternary pumps).

Total Phenol Contents

Total phenol contents of supernatants were determined by the Folin-Ciocalteu method (Vernon et al., 1999). Folin and Ciocalteu's phenol reagent was purchased from Sigma$^®$ (St. Louis, MO). Gallic acid from Aldrich Chemical Co, Inc. (Milwaukee, WI) was used as a standard. Spectronic$^®$ Genesys™ 2 (Rochester, NY) was used to determine total phenolic contents. Sodium carbonate was purchased from Fisher Scientific (Fair Lawn, NJ).

Antioxidant Activities of Grape Skin and Seed Extracts

An optimized version of ORAC assay developed by Cao and Prior (1999) was used to quantify the antioxidant capacity of grape skin and seed extracts. β-Phycoerythrine (β-PE) was purchased from Cyanotech Co. (Kailua-Kona, HI) (Lot # 0215100) and prepared according to the supplier- recommended reconstitution procedure for purification. Briefly, the vial (1mg β-PE/0.2mL buffer mixture) was rinsed with about 3.5mL of phosphate buffer (Stock buffer/deionized water, 1:9, v/v) (Stock buffer; 0.75M K_2HPO_4/0.75M NaH_2PO_4, 61.6:38.9, v/v). Prior to passing the solution through the column, a Sephadex G-25 column was cleaned with about 20mL-phosphate buffer. The red band eluted out from the column was collected, and it was washed off with buffer. The purity of the β-PE solution was determined according to the recommended procedure. β-PE was diluted further with phosphate buffer. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA), and 0.868g of AAPH was dissolved in 10mL phosphate buffer. It was prepared daily and kept in ice until used.

The LS-50B Luminescence Spectrometer (Perkin Elmer, UK) was used for the analyses. 'A four position, motor driven, water thermostatted, stirred cell holder' was installed on the spectrometer, and the temperature of the water bath, which supplied hot water to the cell holder, was set to 37°C. Emission and excitation wavelengths were 565 and 540nm respectively. Stirrer was set to low.

50μL of β-PE in phosphate buffer was incubated at 37°C for 5min in the cuvettes located in the holder, and then appropriate diluted aliquot sample (50-100μL) was added to the cuvettes. The reaction was started by the addition of 150μL of AAPH (24mM) to the cuvettes at min 5, and initial reading was taken. Total volume in each cuvette was 2mL. Blanks, which contained phosphate buffer, β-PE and AAPH only, were used for the area corrections. β-PE fluorescence intensities of samples and blanks were recorded every minute. The changes in β-PE fluorescence over time were displayed on the screen. The data were collected until the fluorescence reading declined by 95% of the initial reading. Intensities were converted to relative intensities by dividing the readings with the initial reading. The areas under the curves were calculated using the software supplied by spectrofluorometer manufacturer.

Trolox, a water-soluble analog of vitamin E, was purchased from Aldrich Chem. Co. (Milwaukee, WI). It was dissolved in 10mL ethanol (190°), and then diluted to 200mL with phosphate buffer. Trolox solution was prepared weekly and kept at refrigeration temperature. Standard curves were obtained using known concentrations of trolox (0.5 to 3μmol final concentration in cuvette). Corrected areas of relative fluorescence intensities versus trolox concentrations were plotted. The trolox equivalents

of the samples were calculated by using the linear portion of the plot after proper

dilutions were prepared with phosphate buffer.

Galvinoxyl Method

The method developed by Shi et al. (2001) was used to determine the number of

hydrogen molecules available for antioxidant donation in pure compounds. Stock

solution of galvinoxyl (160μM; Aldrich Chem. Co., Milwaukee, WI) was prepared in

HPLC grade ethanol. Total volume of each cuvette was 2mL. Ethanol was used as a

blank. Final concentration of galvinoxyl was 8μM. Reaction was carried out at room

temperature. Absorbances at 428nm were recorded every minute up to 20min using

Spectronic® Genesys™ 2 (Rochester, NY). Different concentrations of gallic acid,

catechin, epicatechin, resveratrol, ascorbic acid, and ellagic acid were used to determine

the number of available hydroxyl groups for proton donation. The following formula was

used to determine the number of hydrogen molecules (N) available for antioxidative

donations;

$$N = \frac{\Delta A}{(\varepsilon I [IH])}$$

where ΔA, absorbance difference; ε, molar extinction coefficient of galvinoxyl

($\lambda_{428nm} = 1.5 \times 10^5$ M^{-1}cm^{-1} in ethanol; I, cell length; [IH], concentration of antioxidant.

Statistical Analysis

Data were analyzed using the Statistical Analysis System software (SAS Institute,

Inc., 1990). PROC GLM with Duncan's multiple comparison test was performed to

determine significant differences at $\alpha = 0.05$.

RESULTS AND DISCUSSION

High Pressure Liquid Chromatography

Fuleki and Ricardo da Silva (1997), Escribano-Bailon et al. (1992), Oszmianski and Sapis (1989), Revilla and Ryan (2000) and Santos-Buelga et al. (1995) reported that concentrations of gallic acid, catechin and epicatechin are significantly high in grape seeds. We also found that these three phenolic compounds are present in seeds from Muscadine, Merlot and Chardonnay grapes at concentrations higher than those of other compounds in the samples (Figure 4.2). Recoveries of gallic acid, catechin and epicatechin were, respectively, 69, 59, and 72% when known quantities were added to seeds, and 79, 70, and 78% when they were added to the skins of Chardonnay grapes. The percentage of the added phenolic compounds that were recovered by the extraction and analytical procedure was lower from the seed powder than from the skin powder. The difference is likely due to losses during the deoiling process of the seed powder prior to preparation of the extracts for analysis. Seeds were de-oiled prior to preparation of the extracts for HPLC analysis to prevent fouling of the column by the oil. Because of the less than 100% recovery, the actual concentrations of these three phenolic compounds in the seed or skin powder would be higher than those shown in Figure 4.2.

Catechin and epicatechin contents of *Vitis vinifera* grapes were higher than in *Vitis rotundifolia* grapes but the latter contained more gallic acid (Table 4.2). In general, grape seeds had much higher monomeric flavanol contents than skins. Catechin and epicatechin concentrations in Chardonnay grape skins were three times higher than in Merlot grape skins (Table 4.2).

Fuleki and Ricardo da Silva (1997) found that catechin and epicatechin concentrations on a wet basis were 64 and 79 mg/100g in Merlot seeds (Ontario, Canada), and 42 and 99 mg/100g in Chardonnay seeds, respectively. Using the byproducts of winery industry, we obtained about 75mg catechin and 83mg epicatechin/100g dry matter in Merlot seeds while Chardonnay seeds obtained had about 211mg catechin and 302.64 mg epicatechin/100g dry matter. Although the authors did not determine the amount of these two flavanols in grape skins, we found that skins from Merlot or Chardonnay grapes also contain considerable amounts of gallic acid, catechin and epicatechin. Using acid hydrolysis technique, Akoh and Pastrana-Bonilla (2002) reported that gallic acid, catechin, and epicatechin contents of Muscadine grape seeds were 6.56, 789 and 1234mg/100g on a wet basis, respectively. On the other hand, we found that our Muscadine seed powder contained less catechin and epicatechin and more gallic acid than the fresh Muscadine seeds.

Ector et al. (1996) reported that seeds of Muscadine grapes contained about 45μg *trans*-resveratrol/g on a wet basis. We attempted to detect resveratrol using a different HPLC unit with a fluorescence detector since resveratrol fluoresces. However, we found that resveratrol was not present in seeds of *Vitis rotundifolia* var. Muscadine. We detected resveratrol only in *Vitis vinifera* grape skins.

Peroxyl Radical Scavenging Activities of Gallic acid, (+)-Catechin, (-)-Epicatechin, trans-Resveratrol, Ellagic acid and (-)-Gallocatechin

The antioxidant capacity of the pure compounds revealed the ability of the stilbene, resveratrol, and the flavanols, catechin and epicatechin, to scavenge the peroxyl

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radicals generated by AAPH. The peroxyl radical scavenging activity of resveratrol (29µmol TE/mg) was the highest among all compounds tested (Table 4.3) ($p<0.05$). In general, flavanols scavenged peroxyl radicals of AAPH much better than phenolic acids.

Peroxyl radical scavenging activities of resveratrol (Cuendet et al., 2000; Tadolini et al., 2000), catechin (Torel et al., 1986; Scott et al., 1993; Nakao et al., 1998), epicatechin (Scott et al., 1993; Nakao et al., 1998; Liu et al., 2000), and gallic acid (Liu et al., 2000) were previously reported. Flavonoids have the ability to donate hydrogen atoms to peroxyl radicals (Torel et al., 1986). Most of studies involving peroxyl radical scavenging activity of these phenolics are rather comparative than quantitative. Using bacterial culture, Nakao et al. (1998) reported that epicatechin showed 25% higher peroxyl radical activity than catechin. Guo et al. (1997) reported that antioxidant activities of catechin and epicatechin were 2.49±0.07 and 2.36±0.13µmol TE/ µmol of compound, respectively, in terms of ORAC values. These values correspond to approximately 8.6±0.24µmol TE for catechin and 8.1±0.45µmol TE for epicatechin per mg compound. Our values for peroxyl radical scavenging activities of the important phenolic compounds in grape seeds and skins were in the order: resveratrol > catechin > epicatechin = gallocatechin > gallic acid = ellagic acid.

Antioxidant Activities of Grape Skin and Seed Extracts

Oxygen radical absorbance capacity (ORAC) assay was developed by Cao and Prior (1999) in order to quantify the antioxidant capacity of foods by measuring peroxyl radical scavenging activity of the compounds found in foods. This assay is based on the chemical damage to β-PE caused by a peroxyl radical producing compound (i.e. AAPH

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in this assay), reducing the fluorescence emission of β-PE. The presence of antioxidants in the medium can recover the damage and prolong the reduction in the fluorescence emission. Antioxidant capacity of foods can be quantified by using the areas under the relative fluorescence intensity curves (Cao and Prior, 1999).

Grape seeds from *Vitis vinifera* varieties Chardonnay and Merlot from *Vitis rotundifolia* variety Muscadine have potential as a source of nutritional supplement because of their high antioxidant capacities measured as ORAC (Table 4.4). Grape seed extracts had higher ORAC values compared with grape skin extracts. Using peroxyl radical scavenging capacities of pure gallic acid, catechin and epicatechin (Table 4.3), we calculated the contribution of these polyphenols to the total antioxidant capacity of the samples. The results indicated that these three compounds contributed less than 17% to total ORAC values of grape seed or skin extracts (Table 4.4). Thus the high antioxidant capacities of grape seeds and skins are most likely from the polymeric procyanidins rather than the monomers.

Hydrogen Donor Activity by Galvinoxyl Method

The Galvinoxyl method developed by Shi et al. (2001) determines the antioxidant activity of compounds that can donate hydrogen. A solution of galvinoxyl in ethanol has a yellow color with a strong absorption at 428nm. As its odd electron is protonoted, decolorization takes place. Hydrogen-donating antioxidants can donate proton(s) to galvinoxyl molecules and the proton donation is measured as a loss of absorbance at 428 nm.

We found that resveratrol is non-reactive towards galvinoxyl therefore its proton donor activity could not be determined with this method. Three hydroxyl groups were

available for antioxidative donation by catechin, epicatechin, gallic acid, and ellagic acid while ascorbic acid and two available (Table 4.5).

Oligomeric or polymeric procyanidins of grape seed or skin extracts have been shown to be beneficial to human health. Saito et al. (1998) reported that oligomeric procyanidins of grape seed extracts showed antiulcer activity in animal models while monomeric, dimeric and trimeric procyanidins do not exhibit this activity. Polymeric tannin supplementation stimulates fermentative activities without increasing the activity of harmful enzymes on animal models (Tebib et al., 1996; Chung et al., 1998). Grape seed extract containing mainly oligomeric and polymeric proanthocyanidins was shown to inhibit epidermal ornithine decarboxylase activity in mice when it is used before 12-*O*-tetradecanoylphorbol-13-acetate, which is a tumor promoter (Bomser et al., 2000). Moreover, a study indicated that 1% GSPE (w/w) in diet reduced atherosclerosis in the rabbit aorta while 1% catechin (w/w) showed very weak antiatherosclerotic activity in cholesterol-fed rabbits (Yamakoshi et al., 1999). Activity of grape seed extracts in preventing atherosclerosis is likely because proanthocyanidins inhibit oxidation (Yamakoshi et al., 1999). An *ex vivo* study showed that grape seed procyanidins might reduce the oxidation of polyunsaturated fatty acids in mouse liver microsomes (Bouhamidi et al., 1998). Monomers of grape seed procyanidins, epigallocatechin and epigallocatechin-gallate were shown to be ineffective to protect microsomal polyunsaturated fatty acids, but natural grape seed procyanidins, which also contains significant amounts of dimers and oligomers of flavanols was effective (Bouhamidi et al., 1998). Our results indicated that monomeric procyanidins could account for less than 17% of the antioxidant capacity of grape seeds. Superior antioxidant capacity of grape

seeds is most likely from their dimeric, trimeric, oligomeric and/or polymeric procyanidins. Our results could be useful in explaining why polymeric procyanidins of grape seeds are more beneficial to health compared with the corresponding monomers.

ABBREVIATIONS USED

AAPH, 2,2`-azobis(2-amidinopropane) dihydrochloride; GAE, gallic acid equivalent; ORAC, oxygen radical absorbing capacity; β-PE, β-phycoerythrine; TE, trolox equivalent.

LITERATURE CITED

Akoh, C.C. and Pastrana-Bonilla, E. 2002. Polyphenolics and antioxidant capacity of muscadine grape cultivars grown in South Georgia. Abstract at Annual Meeting and Food Expo, Anaheim, CA.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J. and Stohs, S.J. 1998. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. Gen. Pharmac. 30(5):771-776.

Bors, W. and Michel, C. 1999. Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies. Free Radical Bio. Med. 27(11/12):1413-1426.

Bouhamidi, R., Prevost, V. and Nouvelot, A. 1998. High protection by grape seed proanthocyanidins (GSPC) of polyunsaturated fatty acids against UV-C induced peroxidation. Life Sci. 321:31-38.

Cao, G. and Prior, R. 1999. Measurement of oxygen radical absorbance capacity in biological samples. Method Enzymol. 299:50-63.

Chung, K.T., Lu, Z., and Chou, M.W. 1998. Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. Food Chem. Toxicol. 36:1053-1060.

Cuendet, M., Potterat, O., Salvi, A., Testa, B. and Hostettmann, K. 2000. A stilbene and dihydrochalcones with radical scavenging activities from *Loiseleuria procumbens*. Phytochemistry. 54:871-874.

Ector, B.J., Magee, J.B., Hegwood, C.P., and Coign, M.J. 1996. Resveratrol concentration in muscadine berries, juice, pomace, purees, seeds and wines. Am. J. Enol. Vitic. 47(1):57-62.

Escribano-Bailon, T., Gutierrez-Fernandez, Y., Rivas-Gonzalo, J.C.R., and Santos-Buelga, C. 1992. Characterization of procyanidins of *Vitis vinifera* variety Tinta del Pais grape seeds. J. Agric. Food Chem. 40(10):1794-1799.

Fukumoto, L.R. and Mazza, G. 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J. Agric. Food Chem. 48(8):3597-3604.

Fuleki, T. and Ricardo da Silva, J.M. 1997. Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. J. Agric. Food Chem. 45(4):1156-1160.

Gunckel, S., Santander, P., Cordano, G., Ferreira, J., Munoz, S., Nunez-Vergara, L.J., and Squella, J.A. 1998. Antioxidant activity of gallates: an electrochemical study in aqueous media. Chem-Biol. Interact. 114:45-59.

Guo, C., Cao, G., Sofic, E. and Prior, R.L. 1997. High-performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: relationship to oxygen radical absorbance capacity. J. Agric. Food Chem. 45(5):1787-1796.

Joshi, S.S., Kuszynski, C.A., Bagchi, M. and Bagchi, D. 2000. Chemopreventive effects of grape seed proanthocyanidin extract on Chang liver cells. Toxicology. 155:83-90.

Liu, Z.Q., Ma, L.P., Zhou, B., Yang, L., Liu, Z.L. 2000. Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. Chem. Phys. Lipids. 106:53-63.

Lotito, S.B. and Fraga, C.G. 1997. (+)-Catechin prevents human plasma oxidation. Free Radical Bio. Med. 24(3):435-441.

Moini, H., Guo, Q., and Packer, L. 2002. Xanthine oxidase and xanthine dehydrogenase inhibition by the procyanidin-rich French maritime pine bark extract, Pycnogenol®: a protein binding effect. Adv. Exp. Med. Biol. 505:141-149.

Morel, I., Lescoat, G., Cogrel, P., Sergent, O., Pasdeloup, N., Brissot, P., Cillard, P. and Cillard, J. 1993. Antioxidant and iron chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem. Pharmacol. 45(1):13-19.

Nakao, M., Takio, S., and Ono, K. 1998. Alkyl peroxyl radical-scavenging activity of catechins. Phytochemistry. 49(8):2379-2382.

Oszmianski, J. and Sapis, J.C. 1989. Fractionation and identification of some low molecular weight grape seed phenolics. J. Agric. Food Chem. 37(5):1293-1297.

Palma, M. and Taylor, L.R. 1999. Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. J. Chromatogr. A. 849:117-124.

Revilla, E. and Ryan, J.M. 2000. Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. J. Chromato. A. 881:461-469.

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S. and Yamaji, N. 1998. Antiulcer activity of grape seed extract and procyanidins. J. Agric. Food Chem. 46(4):1460-1464.

Sakaguchi, N., Inoue, M., and Ogihara, Y. 1998. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. Biochem. Pharmacol. 55(12):1973-1998.

Sanchez-Moreno, C., Larrauri, J.A., Saura-Calixto, F. 1999. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Res. Int. 32:407-412.

Santos-Buelga, C., Francia-Aricha, E.M., and Escribano-Bailon, T.M. 1995. Comparative flavan-3-ol composition of seeds from different grape varieties. Food Chem. 53:197-201.

SAS Institute. 1990. SAS User's Guide: Statistics. Version 6.4th ed. Cary, NC: SAS institute.

Sato, M., Bagchi, D., Tosaki, A. and Das, D.K. 2001. Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. Free Radical Bio. Med. 31(6):729-737.

Sato, M., Maulik, G., Ray, P.S., Bagchi, D. and Das, D.K. 1999. Cardioprotective effects of grape seed proanthocyanidin against ischemic reperfusion injury. J. Mol. Cell Cardiol. 31:1289-1297.

Scott, B.C., Butler, J., Halliwell, B., and Aruoma, O.I. 1993. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Rad. Res. Comm. 19(4):241-253.

Shi, H., Noguchi, N., and Niki, E. 2001. Galvinoxyl method for standardizing electron and proton donation activity. Method. Enzymol. 335:157-166.

Shukla, Y.N., Srivastava, A., Kumar, S., and Kumar, S. 1999. Phytotoxic and antimicrobial constituents of *Argyreia speciosa* and *Oenothera biennis*. J. Ethnopharmacol. 67:241-245.

Souquet, J., Cheynier, V., Brossaud, F. and Moutounet, M. 1996. Polymeric proanthocyanidins from grape skins. Phytochemistry. 43(2):509-512.

Tadolini, B., Juliano, C., Piu, L., Franconi, F., and Cabrini, L. 2000. Resveratrol inhibition of lipid peroxidation. Free Rad. Res. 33:105-114.

Tebib, K., Besancon, P. and Rouanet, J. 1996. Effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes. Nutr. Res. 16(1):105-110.

Tebib, K., Rouanet, J.M. and Besancon, P. 1997. Antioxidant effects of dietary polymeric grape seed tannins in tissues of rats fed a high cholesterol-vitamin E-deficient diet. Food Chem. 59(1):135-141.

Torel, J., Cillard, J. and Cillard, P. 1986. Antioxidant activity of flavonoids and reactivity with peroxyl radical. Phytochem. 25(2):383-385.

Ursini, F., Rapuzzi, I., Toniolo, R., Tubaro, F., and Bontempelli, G. 2001. Charaterization of antioxidant effect of procyanidins. Method. Enzymol. 335:338-350.

Vernon, L.S., Orthofer, R. and Lamuela-Raventos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Method. Enzymol. 299:152-178.

Yamakoshi, J., Kataoka, S., Koga, T. and Ariga, T. 1999. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atheroschlerosis in cholesterol-fed rabbits. Atherosclerosis. 142:139-149.

FIGURE CAPTIONS

Figure 4.1. Chromatograph of standard mixture containing gallic acid, catechin, epicatechin, ellagic acid and resveratrol. Wavelength program: time 0-25, 280nm; time 25-55, 306nm; time 55-70min, 360nm.

Figure 4.2. Chromatographs of seed extracts from Muscadine (A), Merlot (B) and Chardonnay (C) grapes as byproducts of food industry. Wavelength program: time 0-25, 280nm; time 25-75min, 360nm.

Figure 4.3. Chromatographs of Chardonnay and Merlot skin extracts as food industry byproducts. Wavelength program: time 0-25, 280nm; time 25-75min, 360nm.

Table 4.1. Solvent gradient used for the analyses of major phenolic compounds in grape seeds and skins.

Time (min)	A	B
0	100	0
30	82	18
45	72	28
65	60	40
75	40	60
85	0	100

A: 25% aqueous methanol in 1% acetic acid
B: 75% aqueous methanol in 1% acetic acid

Table 4.2. Gallic acid, catechin and epicatechin contents of seeds and skins of grapes from *Vitis vinifera* and *rotundifolia* varieties

Grape Sample	Flavonoid Content mg/100g dry matter		
	Gallic acid	Catechin	Epicatechin
Seed			
Muscadine	68	7	69
Chardonnay	10	211	303
Merlot	7	75	83
Skin			
Chardonnay	4	42	34
Merlot	2	11	10

Table 4.3. Comparison of pure phenolic compounds in terms of their peroxyl radical scavenging activities.

Compound	Peroxyl Radical Scavenging Capacity µmol TE/mg
Stilbene	
Resveratrol	29.06 ± 3.54 [a]
Flavanols	
Catechin	20.53 ± 0.10 [b]
Epicatechin	10.20 ± 0.87 [c]
Gallocatechin	11.58 ± 0.71 [c]
Phenolic acids	
Gallic acid	4.26 ± 1.57 [d]
Ellagic acid	3.88 ± 1.04 [d]

Superscripts with different letters show significant differences at α=0.05, using Duncan's multiple range test.

Table 4.4. Contribution of catechin, epicatechin and gallic acid to total antioxidant capacity of grape seed and skin extracts

Grape Sample	ORAC µmol TE/g dry matter	Percentage of ORAC from catechin, epicatechin and gallic acid
Seed		
Muscadine	245.91 ± 6.77	4.6
Chardonnay	450.51 ± 74.02	16.6
Merlot	272.84 ± 43.27	8.8
Skin [a]		
Chardonnay	102.81 ± 7.02	3.1
Merlot	69.81 ± 33.17	18.1

[a] Sample extraction according to Cao and Prior (1999)

Table 4.5. Number of hydrogen atoms, which each pure compound can donate for antioxidant reaction

Compound	Total Number of Hydroxyl Groups	Number of Protons Available for Antioxidative Donation (N)
Resveratrol	3	NA[*]
Ascorbic acid	4	1.8
Catechin	5	2.9 (3[a])
Epicatechin	5	3.1
Quercetin	5	4.0[a]
α-Tocopherol	1	1.0[a]
Gallic acid	4	3.2
Ellagic acid	4	3.4

[*] NA, not available, non-reactive with galvinoxyl
[a] Shi et al. (2001)

Figure 4.1. Chromatograph of standard mixture containing gallic acid, catechin, epicatechin, ellagic acid and resveratrol. Wavelength program: time 0-25, 280nm; time 25-55, 306nm; time 55-70min, 360nm.
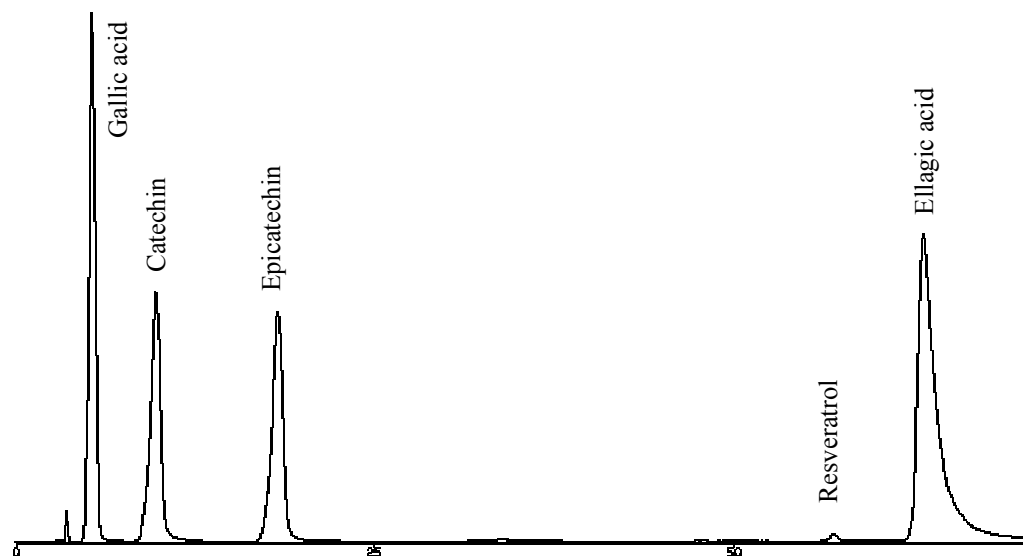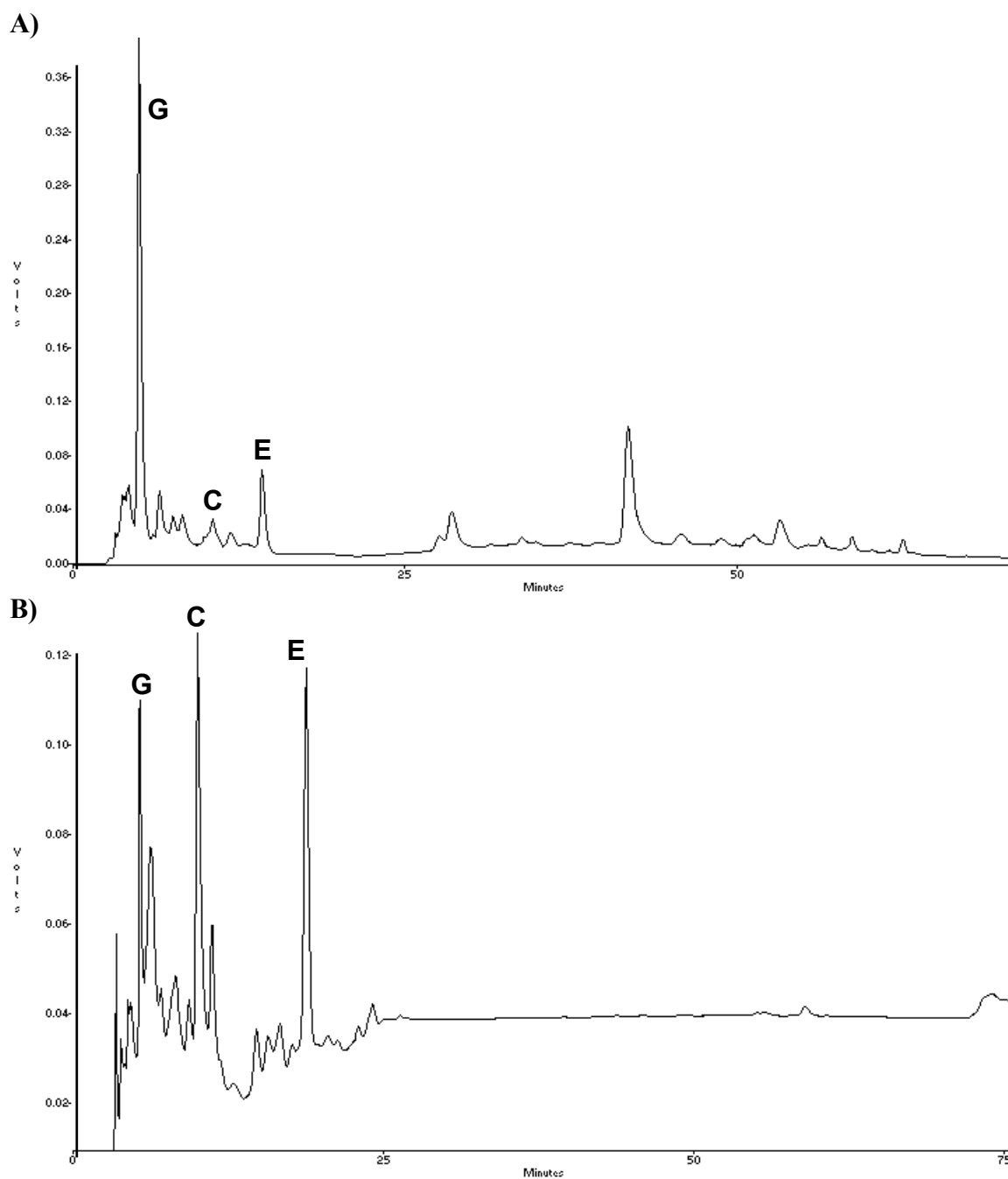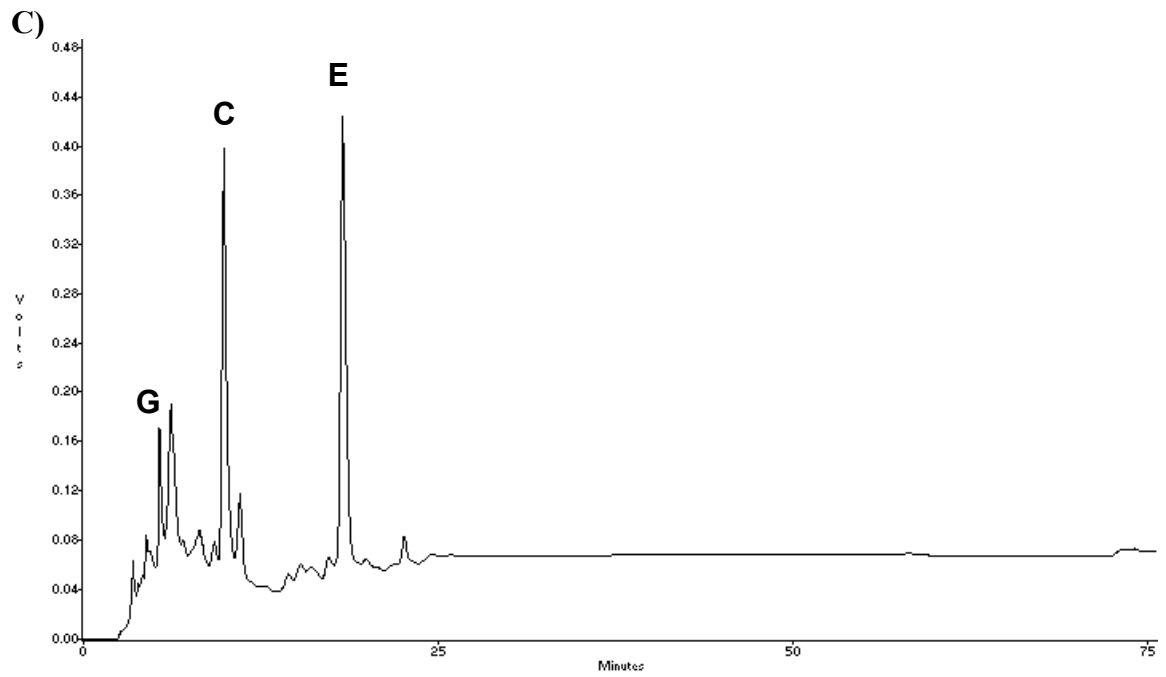
Figure 4.2. Chromatographs of seed extracts from Muscadine (A), Merlot (B) and Chardonnay (C) grapes as byproducts of food industry. Wavelength program: time 0-25, 280nm; time 25-75min, 360nm.

A)

B)

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G, gallic acid; **C**, (+)-catechin; **E**, (-)-epicatechin

Figure 4.3. Chromatographs of Chardonnay (A) and Merlot (B) skin extracts as food industry byproducts. Wavelength program: time 0-25, 280nm; time 25-75min, 360nm

A)

B)
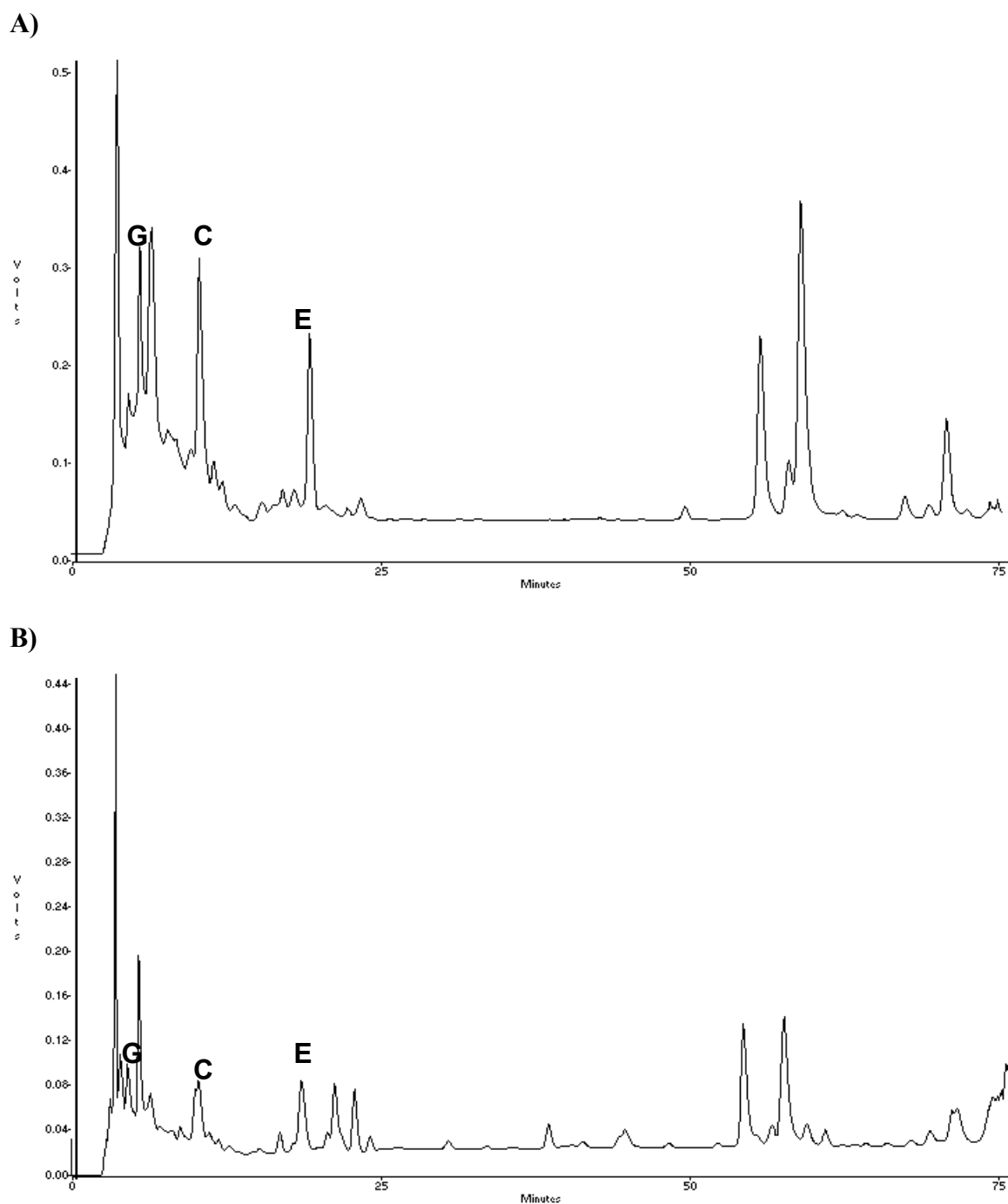
G, gallic acid; **C**, (+)-catechin; **E**, (-)-epicatechin

CHAPTER 5

STORAGE STABILITY OF POLYPHENOLIC ANTIOXIDANTS IN

MUSCADINE SEED EXTRACT USED AS A FUNCTIONAL FOOD INGREDIENT

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ABSTRACT

Plants are an important part of animals and human diets and phenolic constituents of plants are known to have nutritional as well as medicinal roles. The storage stability of phenolic grape seed antioxidants in an actual food product was determined. Aqueous ethanolic extract of Muscadine seed powder (4mg powder/mL) was added to a puffed rice-based snack bar that was stored at either 19° or 37°C up to three months. Phenolic constituents of muscadine seed extract in a rice-based cereal product were more stable at low storage temperatures over a period of three months. Antioxidant capacities of cereal squares measured as oxygen radical absorbing capacity (ORAC) reduced over time. However, the effect of storage temperature on ORAC values was not significant. Therefore, antioxidant capacities of products were stable over storage temperatures we studied. Moreover, the use of muscadine seed extracts provided a natural antioxidant activity by inhibiting lipid peroxidation in cereal product. Supplementation of cereal products with muscadine seed extract as a functional ingredient may increase dietary antioxidant intake for health conscious consumers with the benefit of higher acceptability compared with powder forms of grape skins and seeds.

KEYWORDS: Antioxidant; antioxidant capacity; grape seed; Muscadine; ORAC; TBARS

INTRODUCTION

Plants are an important part of animal and human diets due to their functional role in maintaining the health of body tissues. Plant derived foods provide metabolic energy, precursors for protein synthesis, essential micronutrients involved in enzyme activity and regulation such as vitamins and minerals, and essential fatty acids. Moreover, phenolic constituents of plants are known to have nutritional functions as well as medicinal. Most plant constituents that show activity in health maintenance are secondary plant metabolites (Walton, Rhodes, Michael & Parr, 1999) which are also referred to as phytochemicals. Phytochemicals can be described as 'plant-derived substances that are nutritionally physiologically and/or medicinally highly active' (Lin, 1999). Components of garlic, ginkgo biloba, soybean, tea, grapes and many other fruits and vegetables are phytochemicals with dietary importance for humans.

A functional food can be defined as 'a food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirement' (Sanders, 1998). Recently, consumer interest towards functional foods has increased sharply (Goldberg, 1999; Sanders, 1998). Grape seeds can be a functional food ingredient, and they are currently marketed in the US as a dietary supplement in powder form. The Dietary Supplement Health and Education Act (FDA, 1994) defines dietary supplements and lists allowed structure-function health claims for dietary ingredients. According to this act, plant extracts could be sold as a dietary supplement in the form of liquid, powder, tablet, capsule, or gel (soft or cap). Health conscious customers are primary targets for dietary supplements in the form of grape seed or skin powder capsules. However, for the general public, dietary supplements from grape seeds have not gained widespread acceptability.

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Although the legal implications of adding what is now allowed as a dietary supplement to any food product in the US are still not defined, one potential use of seed extracts (concentrates) is in cereal bars, a popular snack product favored by all age groups. The use of grape seed extracts in foods offer the advantage of an easily consumable source of dietary antioxidant. In addition, commercial manufacturing of value added food products and processing of the grape seeds from winery byproducts will contribute to the economy.

Grape seeds and skins contain various flavonoids. Gallic acid, catechin and epicatechin were identified as the main phenolic compounds in grape seeds (Palma and Taylor, 1999). Seeds, skins or pulps of grape berries with their major flavonoids can be used as a potential functional food due to their *in vitro* (Zhu, Wang, Wei, Lin, Yang & Ho, 2001) and *in vivo* antioxidant (Bagchi et al., 1998) properties, antimutagenic (Weyant, Carothers & Bertagnolli, 2000; Krul, Luiten-Schuite, Tendelfe, van Ommen, Verhagen & Havenaar, 2001), antiplatelet (Pace-Asciak, Hahn, Diamandis, Soleas & Goldberg, 1995), cardioprotective (Sato, Bagchi, Tosaki & Das, 2001), antiatherosclerotic (Yamakoshi, Kataoka, Koga & Ariga, 1999), antiulcer (Saito, Hosoyama, Ariga, Kataoka & Yamaji, 1998) activities and beneficial effect on gastrointestinal health (Chung, Lu & Chou, 1998; Tebib, Besancon & Rouanet, 1996).

Breakfast cereal products are very popular among all ages in the US, and are mostly consumed as a breakfast food. Since the market size of hot and cold cereals dropped from $8.6 billion in 1995 to $8.1 billion in 1999, cereal producers in the US have developed new snacks that could be eaten at any time of a day (Roberts, 2002). Rice-based snack Rice Krispies Treats® by Kellogg's® is one of those new snacks that

has become the most popular snack cereal bar in the US, with more than $140 million in annual sales (Roberts, 2002).

The storage stability of phenolic antioxidants of grape seeds in foods will be different from that in the natural environment in the seeds. Therefore, the primary purpose of this study was to determine if phenolic grape seed constituents will retain their antioxidant capacity during when added as a seed extract to an actual food product. We also tested the inhibition of lipid oxidation by the seed extract.

MATERIALS AND METHODS

Preparation of Muscadine Seed Extract

Muscadine seeds were obtained from a mechanical muscadine-deseeding machine at Paulk Vineyards (Ocilla, GA). Adhering pulp and skin particles were manually removed and adhering juice was removed by rinsing with tap water. Seeds were dried in an impinger oven (Lincoln Impinger, Lincoln Foodservice Products, Inc., Fort Wayne, IN) at 93°C for 45 min. In order to facilitate the extraction of phenolics, dried seeds were ground into a powder with a hammer mill (FitzMill, The Fitzpatrick Co., Elmhurst, IL) fitted with 0.2 mm screens. Muscadine seed powder was stored in the dark at room temperature.

Seed powder was weighed then a 30% water-70% (v/v) ethanol (190 proof) solvent was added at 1:10 (w/v) powder to solvent ratio. The mixture was shaken briefly to disperse the powder and sonicated for 15min at room temperature. Then it was shaken in a shaker for 30min at room temperature. Finally, the mixture was centrifuged at 5,000g for 20min and supernatants were collected.

Solvent was removed from the supernatants by evaporation in a rotary evaporator at 40°C under vacuum. The volume of concentrate was adjusted to obtain 0.25mL extract per gram of powder used in the extraction. The extract was kept frozen at -18°C until use.

Production of Puffed-rice Cereal Squares

Table 5.1 gives the formula for the puffed- rice cereal squares. Margarine was first melted by adding to the bowl of a counter-top mixer immersed in a boiling water bath and holding 1 min. Then marshmallows were added and allowed to melt an additional 2.5min. Water or muscadine seed extract was added to the mixture and held 0.5min. The bowl was then removed from the water bath and quickly attached to the mixer. Puffed rice (Rice Krispies[®], Kellogg's[®], Battle Creek, MI) was added to the mixture slowly. The mixture was mixed 30-45sec at the 1 to3 speed setting on the mixer (Kitchen Aid[®] Stand Mixer Model KGPA with 4½-Qt bowl with handle, St. Joseph, MI). Upon mixing, the mixture was quickly transferred to a sheet of wax paper, and the mix was shaped into a rectangular block approximately 1.3cm thick. The block was allowed to cool at room temperature for 20min, then transferred to a 2°C room to set the shape. Squares, 4.5x4.5cm were then cut and each square was separately placed in aluminum-metalized polyester bags (Silverpaks, Kapak Co., Minneapolis, MN). Bags were flushed with nitrogen and heat-sealed using a hand sealer (Scotchpak[®], Kapak Co., Minneapolis, MN). Half of the bags were stored at 19±1°C and the other half were stored at 37±1°C for up to three months. Triplicates of the experiment were performed. Samples were withdrawn randomly every 15 days, and ORAC, TBARS, total phenolic content, and absorbance of extracts at 280nm were determined.

ORAC Assay

Puffed-rice cereal bars were ground with a coffee grinder (Kitchen Aid® Blade
Coffee Grinder, St. Joseph, MI). After a 2 g sample of powder from each was weighed
into a centrifuge bottle, 70% ethanol (190 proof) (1:10 w/v) was added. Then the bottle
was vortexed for 10sec, sonicated at room temperature for 10 min, centrifuged at 4°C for
20min at 26,000g. The volume of each collected supernatant was recorded.

The ORAC assay developed by Cao and Prior (1999) was modified and used to
quantify the antioxidant capacity of extracts from puffed-rice squares. β-Phycoerythrine
(β-PE) was purchased from Cyanotech Co. (Kailua-Kona, HI) (Lot # 0215100) and
prepared according to the supplier recommended reconstitution and purification
procedure. Briefly, the vial (1mg β-PE/0.2mL buffer mixture) was rinsed with about
3.5mL of phosphate buffer (Stock buffer/deionized water, 1:9, v/v) (Stock buffer; 0.75M
K_2HPO_4/0.75M NaH_2PO_4, 61.6:38.9, v/v). Prior to passing the solution through the
Sephadex G-25 column, it was cleaned with about 20mL-phosphate buffer. The red band
eluted out of the column was collected, and the column was washed with phosphate
buffer. The purity of the β-PE solution was determined according to the supplier
recommended procedure. The purified β-PE was diluted further with phosphate buffer to
the desired concentration. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was
purchased from Wako Chemicals (Richmond, VA). Every day of the test, 0.868g of
AAPH was dissolved in 10mL-phosphate buffer and kept in ice until used.

LS-50B Luminescence Spectrometer (Perkin Elmer, UK) was used for the
analyses. 'A four position, motor driven, water thermostatted, stirred cell holder' was
installed on the spectrometer, and the temperature of the water bath, which supplied hot

water to the cell holder, was set to 37°C. Emission and excitation wavelengths were 565 and 540nm respectively. Stirrer was set to low.

50μL of β-PE in phosphate buffer was incubated at 37°C for 5min in the cuvettes located in the holder, and then the appropriate sample aliquot (50-100μL) was added to the cuvettes. The reaction was started by the addition of 150μL of AAPH (24mM) to the cuvettes at min 5, and the initial florescence reading was taken. Total volume in each cuvette was 2mL. Blanks, which contained phosphate buffer, β-PE and AAPH only, were used for the area corrections. β-PE fluorescence intensities of samples and blanks were recorded every minute. The changes in β-PE fluorescence over time were displayed on the screen. The data were collected until the fluorescence reading declined by 95% of the initial reading. Intensities were converted to relative intensities by dividing the readings with the initial reading. The areas under the curves were calculated using the software supplied by spectrofluorometer manufacturer.

Trolox, a water-soluble analog of vitamin E, was purchased from Aldrich Chem. Co. (Milwaukee, WI). 10mg Trolox was dissolved in 10mL ethanol (190 proof), and then diluted to 200mL with phosphate buffer. Trolox solution was prepared weekly and kept at refrigeration temperature. Standard curves were obtained using known concentrations of trolox (0.5 to 3μmol final concentration in cuvette). Corrected areas of relative fluorescence intensities versus trolox concentrations were plotted. The trolox equivalents of the samples were calculated by using the linear portion of the plot after proper dilutions were prepared with phosphate buffer.

TBARS

A modified procedure developed by Botsoglou, Fletouris, Papageorgious, Vassilopoulos, Mantis & Trakatellis (1994) was used to determine TBA reactive substances (TBARS) in puffed-rice cereal squares. A whole bar (about 20 g) was ground to a powder and 2 g samples were carefully placed into centrifuge bottles. After the addition of 5mL of 0.8% BHT (butylated hydroxyl toluene) (Sigma® Chemical Co., St. Louis, MO) in hexane, the tube was vortexed briefly and shaken thoroughly. Eight mL of 5% TCA (trichloroacetic acid) (Aldrich Chem. Co., Inc, Milwaukee, WI) (pH≅0.92) was added and the tube was vortexed briefly and shaken thoroughly. The tubes were centrifuged at 10,000g for 15min, and then hexane layers were discarded. 2.5mL of aqueous layer was transferred into a test tube and 1.5mL of 0.3% TBA (2-thiobarbituric acid) (Eastman Organic Chemicals, Rochester, NY) was added. Tubes were shaken for a few seconds and incubated at 70°C for 30min. Tubes were cooled down by immersion in a beaker with cold tap water, and absorbances of mixtures were measured at 532nm. Using a calibration curve for malonaldehyde, equivalent TBARS in samples were calculated. TMP (tetramethoxypropane or malonaldehyde bis-(dimethyl acetal)) (Aldrich Chem. Co., Inc, Milwaukee, WI) was dissolved in 40% ethanol (HPLC grade) to prepare standard solutions. Dilutions of TMP were made with 5% TCA solutions.

Total Phenol Contents and Absorbances at 280nm

Total phenol contents of 70% ethanol supernatants were determined according to Folin-Ciocalteu method (Vernon, Orthofer & Lamuela-Raventos, 1999). Folin and Ciocalteu's phenol reagent was purchased from Sigma® (St. Louis, MO). Gallic acid (Aldrich Chem. Co. Inc., Milwaukee, WI) was used as a standard. Spectronic®

Genesys™ 2 (Rochester, NY) was used to determine total phenolic contents and absorbances at 280nm of supernatants. Sodium carbonate was purchased from Fisher Scientific (Fair Lawn, NJ).

Statistical Analysis

Analysis was done on a split-plot design with the whole plot in a completely randomized design with three replicates of treatments (control and extract). Subplots consisted of the combination of temperature by time effects. Data were analyzed using the Statistical Analysis System software (SAS Institute, Inc., 1990). PROC MIXED was performed to determine significant differences at $\alpha= 0.05$. A macro, Pdmix612[©] (Saxton, 1997), was used to generate the letters for significant differences among the means.

RESULTS AND DISCUSSION

Fruits and vegetables are considered as good sources of antioxidants of a phenolic nature. Strawberry, plum, red grape (whole), red raspberry and orange has ORAC values ranging from 36 to 154µmol TE/g dry matter (Wang, Cao & Prior, 1996; Wang and Lin, 2000). ORAC values of blueberries depend on variety but the range was reported to be from 63 to 282 µmol TE/g dry matter (Prior et al., 1998). Our previous study (Yilmaz and Toledo, 2002) indicated that grape byproducts of the juice or wine industry contain polyphenolic antioxidants having oxygen radical absorbing capacity comparable to those present in fruits and vegetables. We have found that the antioxidant capacity of Muscadine seed as ORAC was approximately 310µmol TE /g dry matter. Seeds were found to be a better source of antioxidant compounds than byproduct skins of the grape juice/wine industries. Prior et al. (1998) reported that the estimated dietary ORAC antioxidant intake in the US ranges from 1200 to 1700µmol TE/day. Consumption of

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one gram dried grape seed winery byproduct per day could increase the daily ORAC intake by more than 30%. For example, our Muscadine seed powder extract added to a puffed- rice snack bar at 2.2% would provide an ORAC of about 400μmol TE/100g product.

Changes of Antioxidant Capacity of Rice-Based Cereal Squares with Muscadine Seed Extract

Statistical analyses of data indicated that puffed-rice cereal bars with muscadine seed extract had significantly higher antioxidant capacities than control squares ($p<0.05$) when the extract was used at 2.2% (w/w) (Table 5.2 and Figure 5.1). Addition of muscadine seed extract to the product doubled the ORAC value from the approximately 20μmol/g already present before extract addition. Commercial Rice Krispies[®] already contains synthetic antioxidants like ascorbic acid and BHT.

In general, ORAC values of both supplemented and non-supplemented rice-based cereal bars decreased with storage time ($p<0.01$). Storage time interacted with treatments ($p<0.01$). The reduction was more obvious for those supplemented with muscadine seed extract. However, antioxidant capacities of cereal squares were not affected by temperature over the three- month storage time ($p>0.05$). The effect of storage temperature only on ORACS of cereal squares was not significant ($p>0.05$). Considering the lack of three way interaction (Table 5.2), we can conclude that our Muscadine seed extract was stable at either 19±1°C or 37±1°C when used at 2.2% concentration in the product.

Effect of Muscadine Seed Extract on Lipid Oxidation

The reaction of malonaldehyde with 2-Thiobarbituric acid (TBA) produces colored products, which could be monitored at 532nm wavelength. TBA assay measures secondary oxidation products, mainly aldehydes. Since TBA can react with a various substances such as ketones, acids, sugars, amino acids, oxidized proteins, pyridines etc, these substances are called TBA reactive substances (TBARS) (Guillen-Sans and Guzman-Chozas, 1998).

Addition of muscadine seed extract to puffed-rice cereal bars reduced lipid oxidation monitored with TBARS (Figure 5.2). The product fortified with the extract had significantly lower TBARS value than control samples ($p<0.05$). Storage temperature also affected the oxidation rate. At $37\pm1°C$, TBARS values were significantly higher than at $19\pm1°C$ ($p<0.05$) at the same storage time. Addition of extract significantly inhibited lipid oxidation at high as well as low storage temperature. Although the control samples of Rice Krispies[®] already had antioxidants, addition of seed extract reduced oxidation further.

Changes in Total Phenol Contents of Puffed-Rice Cereal Squares Containing Muscadine Seed Extract

Total phenol contents of the cereal bars were affected by temperature and storage time (Table 5.2). When averaged over storage time, the total phenol contents of the product containing muscadine seed extract was significantly lower by 6.5% in the high temperature compared to low temperature storage. Total phenols in control samples at any time were unchanged (Figure 5.3) with storage temperature.

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Changes in Absorbances at 280nm

Absorbance values at 280nm demonstrated a similar trend to total phenol contents (Table 5.2). Over storage time, absorbance at 280nm of extracts from samples supplemented with muscadine seed extracts decreased more at 37°C than at 19°C storage temperature (Figure 5.4). At the end of the three-month storage period, extracts from products stored at 37°C had significantly lower absorbance compared with initial($p<0.05$). The absorbance of extracts from samples with muscadine seed extract stored at 19°C were higher than those stored at 37°C.

Phenolic constituents of plants have found their way in food applications due to their potential antioxidant activities against lipid oxidation. Jasmine tea extract (Chen and Chan, 1996) was reported to be as effective an antioxidant as BHT in canola oil while tea catechins inhibited lipid oxidation in chicken, fish and pork meats (Tang, Kerry, Sheehan, Buckley & Morrissey, 2001a and 2001b; McCarthy, Kerry, Kerry, Lynch & Buckley, 2001a and 2001b). Having good antioxidant activity, muscadine seed extract could serve a dual function in foods, as an antioxidative agent against lipid oxidation and as a functional ingredient with beneficial health effects as radical scavengers in the human body.

In conclusion, puffed-rice cereal bars supplemented with 2.2% (w/w) muscadine seed extract (0.25mL extract/ g seed powder) exhibited significant lowering of TBARS during storage as well as exhibit stability in the ORAC values with storage. Thus, the extracts can help increase the ORAC dietary intake of health conscious consumers. Incorporation of the grape seed extract in a food product will be much easier for consumers to increase their dietary ORAC compared with a powdered dietary

supplement. Moreover, muscadine seed extracts could also be utilized in numerous foods as a natural lipid oxidation inhibitor. However, at the present time, the regulations on functional foods in the US are not as well defined as those for dietary supplements, therefore industry may not use of this food suplementation practice. However, individual consumers may use the extracts if made available in food supplement stores in making the ORAC fortified product themselves at home.

ABBREVIATIONS USED

AAPH, 2,2`-azobis(2-amidinopropane) dihydrochloride; GAE, gallic acid equivalent; ORAC, oxygen radical absorbing capacity; β-PE, β-phycoerythrine; TE, trolox equivalent.

LITERATURE CITED

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, D.J., Balmoori, J. & Stohs, S.J. (1998). Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. Gen. Pharmac. 30(5), 771-776.

Botsoglou, N.A., Fletouris, D.J., Papageorgious, G.E., Vassilopoulos, V.N., Mantis, A.J. & Trakatellis, A.G. (1994). Rapid, sensitive, and specific thiobarbituric acid method for measuring lipid peroxidation in animal tissue, food and feedstuff samples. *Journal of Agricultural and Food Chemistry*, 42, 1931-1937.

Cao, G. & Prior, R. (1999). Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology*, 299, 50-63.

Chen, Z.Y. & Chan, P.T. (1996). Antioxidative activity of green tea catechins in canola oil. *Chemistry and Physics of Lipids*, 82, 163-172.

Chung, K.T., Lu, Z. & Chou, M.W. (1998). Mechanism of inhibition of tannic acid and related compounds on the growth of intestinal bacteria. *Food and Chemical Toxicology*, 36, 1053-1060.

Goldberg, I. (1999). *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Maryland: Aspen Publishers, Inc.

Guillen-Sans, R. & Guzman-Chozas, M. (1998). The thiobarbituric acid (TBA) reaction in foods: a review. *Critical Reviews in Food Science*, 38(4), 315-330.

Krul, C., Luiten-Schuite, A., Tenfelde, A., van Ommen, B., Verhagen, H., & Havenaar, R. (2001). Antimutagenic activity of gree tea and black tea extracts studied in a dynamic *in vitro* gastrointestinal model. *Mutation Research*, 474, 71-85.

Lin, R.I-S. (1999). Phytochemicals and antioxidants. In I. Goldberg, *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Maryland: Aspen Publishers, Inc.

McCarthy, T.L., Kerry, J.P., Kerry, J.F., Lynch, P.B. & Buckley, D.J. (2001a). Assessment of the antioxidant potential of natural food and plant extracts in fresh and previously frozen pork patties. *Meat Science*, 57, 177-184.

McCarthy, T.L., Kerry, J.P., Kerry, J.F., Lynch, P.B. & Buckley, D.J. (2001b). Evaluation of the antioxidant potential of natural food/plant extracts as compared with synthetic antioxidants and vitamin E in raw and cooked pork patties. *Meat Science*, 57, 45-52.

Pace-Asciak, C.R., Hahn, S., Diamandis, E.P., Soleas, G. & Goldberg, D.M. (1995). The red wine phenolics *trans*-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implication for protection against coronary heart disease. *Clinica Chimica Acta*, 235, 207-219.

Palma, M. & Taylor, L.R. (1999). Extraction of polyphenolic compounds from grape seeds with near critical carbon dioxide. *Journal of Chromatography A*, 849, 117-124.

Prior, R.L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G. & Mainland, C.M. (1998). Antioxidant capacity as influenced by total phenolics and anthocyanin content, maturity, and variety of *Vaccinium* species. *Journal of Agricultural and Food Chemistry*, 46(7), 2686-2693.

Roberts, W.A. (2002). Breakfast cereals: a vulnerable position. *Prepared Foods*, 171(2), 13-14,16.

Saito, M., Hosoyama, H., Ariga, T., Kataoka, S. & Yamaji, N. (1998). Antiulcer activity of grape seed extract and procyanidins. *Journal of Agricultural and Food Chemistry*. 46(4), 1460-1464.

Sanders, M.E. (1998). Overview of functional foods: emphasis on probiotic bacteria. *International Dairy Journal*, 8, 341-347.

SAS Institute. (1990). SAS User's Guide: Statistics. Version 6.4[th] ed. Cary, North Carolina: SAS Institute.

Sato, M., Bagchi, D., Tosaki, A. & Das, D.K. (2001). Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by inhibiting ischemia/reperfusion-induced activation of JNK-1 and C-JUN. *Free Radical Biology and Medicine*, 31(6), 729-737.

Saxton, A. (1997). Pdmix612[®] macro, version 1.1. Tennessee: The University of Tennessee, Knoxville.

Tang, S., Kerry, J.P., Sheehan, D., Buckley, D.J. & Morrissey, P.A. (2001a). Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. *Food Research International*, 34, 651-657.

Tang, S.Z., Kerry, J.P., Sheehan, D., Buckley, D.J. & Morrissey. (2001b). Antioxidative effect of dietary tea catechins on lipid oxidation of long-term frozen stored chicken meat. *Meat Science*, 57, 331-336.

Tebib, K., Besancon, P. & Rouanet, J. (1996). Effects of dietary grape seed tannins on rat cecal fermentation and colonic bacterial enzymes. *Nutrition Research*, 16(1), 105-110.

Vernon, L.S., Orthofer, R. & Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, 299, 152-178.

Walton, N.J., Rhodes, M.J.C., Michael, A.J. & Parr, A.J. (1999). Characterization and control of plant secondary metabolism. In *Chemical From Plants: Perspective on Plant Secondary Products*. New Jersey: Imperial College Press.

Wang, H., Cao, G. & Prior, R.L. (1996). Total antioxidant capacity of fruits. *Journal of Agricultural and Food Chemistry*, 44(3), 701-705.

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Wang, S.Y. & Lin, H.S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and development stage. *Journal of Agricultural and Food Chemistry*, 48(2), 140-146.

Weyant, M.J., Carothers, A.M. & Bertagnolli, M.M. (2000). (+)-Catechin inhibits tumor formation and modulates integrin-mediated signaling *in vitro* and *in vivo*. *Surgical Forum Abstracts*, 191(4S), S89.

Yamakoshi, J., Kataoka, S., Koga, T. & Ariga, T. (1999). Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis*, 142, 139-149.

Yilmaz, Y. & Toledo, R. (2002). Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin and gallic acid. Ph.D. dissertation, Georgia: The University of Georgia, Athens.

Zhu, N., Wang, M., Wei, G.J., Lin, J.K., Yang, C.S. & Ho, C.T. (2001). Identification of reaction products of (-)-epigallocatechin, (-)-epigallocatechin gallate and pyrogallol with 2,2-diphenyl-1-picrylhydrazyl radical. Food Chemistry, 73, 345-349.

FIGURE CAPTIONS

Figure 5.1. Antioxidant capacity (as ORACS) of puffed-rice cereal bars fortified with muscadine seed extract during storage (Error bars on data points indicate standard deviations).

Figure 5.2. Lipid oxidation during storage of puffed-rice cereal bars fortified with muscadine seed extract (Error bars on data points indicate standard deviations).

Figure 5.3. Total phenol contents at various storage times for puffed-rice cereal bars fortified with muscadine seed extract (* indicates significance ($p<0.05$) compared to initial values; error bars indicate standard deviation).

Figure 5.4. Absorbances at 280nm of extracts from puffed-rice cereal bars over three months storage at 19° and 37°C (* indicates significance ($p<0.05$) compared to initial values; error bars indicate standard deviation).

Table 5.1. Formulations used to make puffed-rice cereal bars

INGREDIENTS	PERCENTAGE (by weight)	
	Control	Treatment
Marshmallow	43.3	43.3
Rice Krispies® (Kellogg's®)	43.3	43.3
Margarine	11.1	11.1
Muscadine seed extract (4g seed/mL)	-	2.2
Water	2.2	-

Table 5.2. ANOVA of data from storage stability test of puffed-rice cereal bars fortified with muscadine seed extract.

Source	Variables			
	ORAC	TBARS	TPC[a]	ABS[b]
Treatment	**	**	**	**
Rep (Treatment)	ns	**	**	**
Temperature	ns[c]	**	**	**
Treatment x Temperature	ns	**	**	**
Time	**	**	**	**
Treatment x Time	**	**	ns	**
Temperature x Time	ns	**	**	**
Treatment x Temperature x Time	ns	ns	*	*

* and ** means significantly differ at $p<0.05$ and $p<0.01$, respectively;
[a] total phenol content; [b] absorbances at 280nm; [c] not significant.

Figure 5.1.
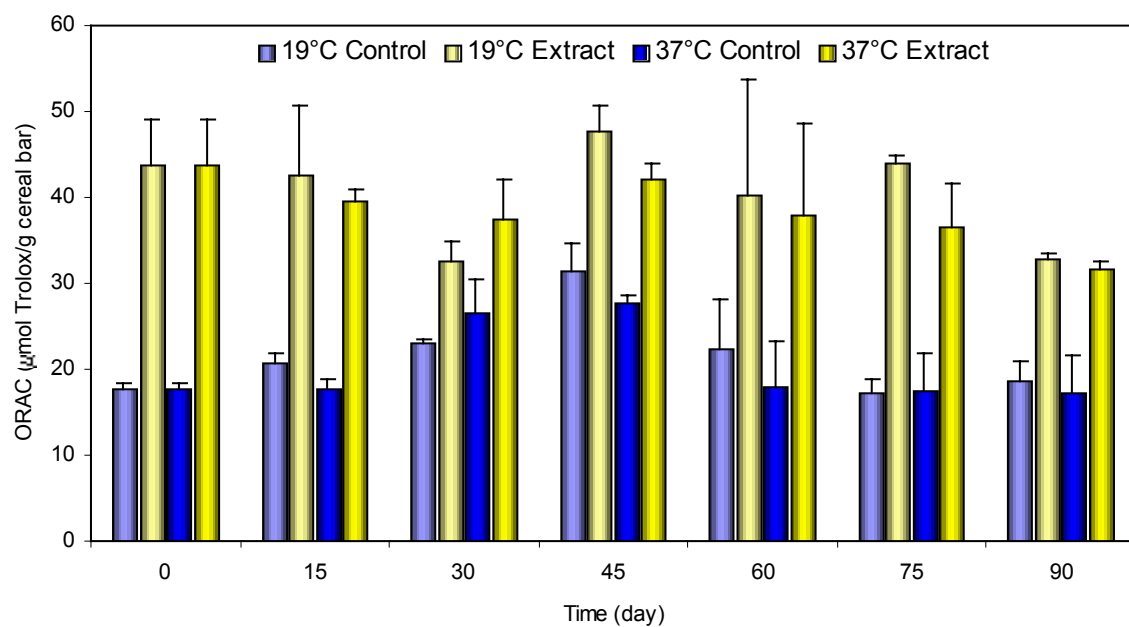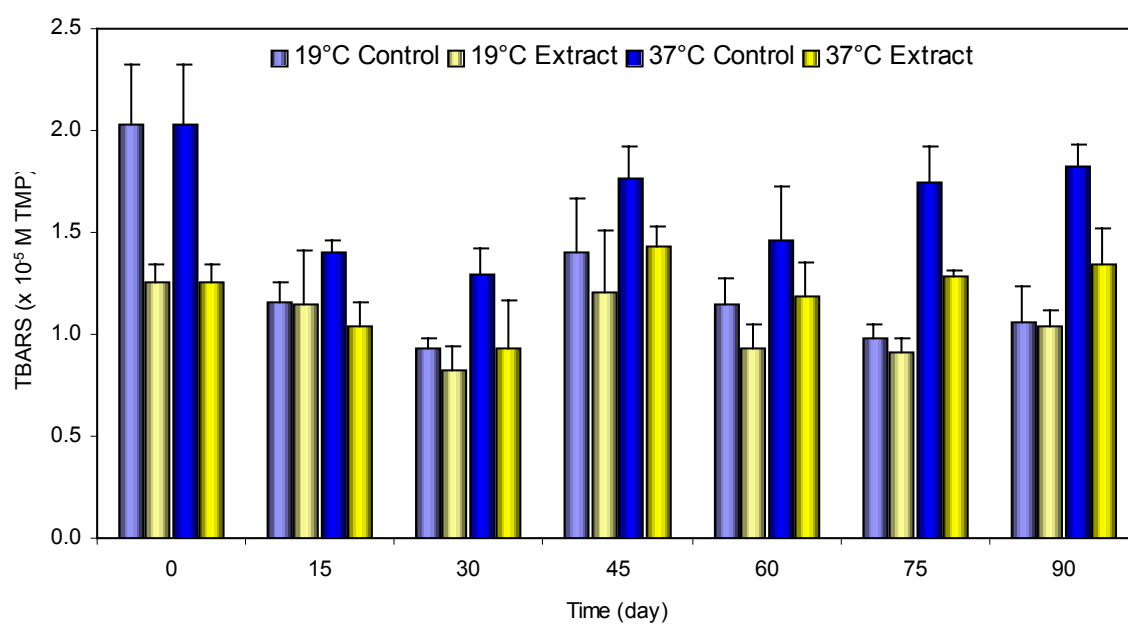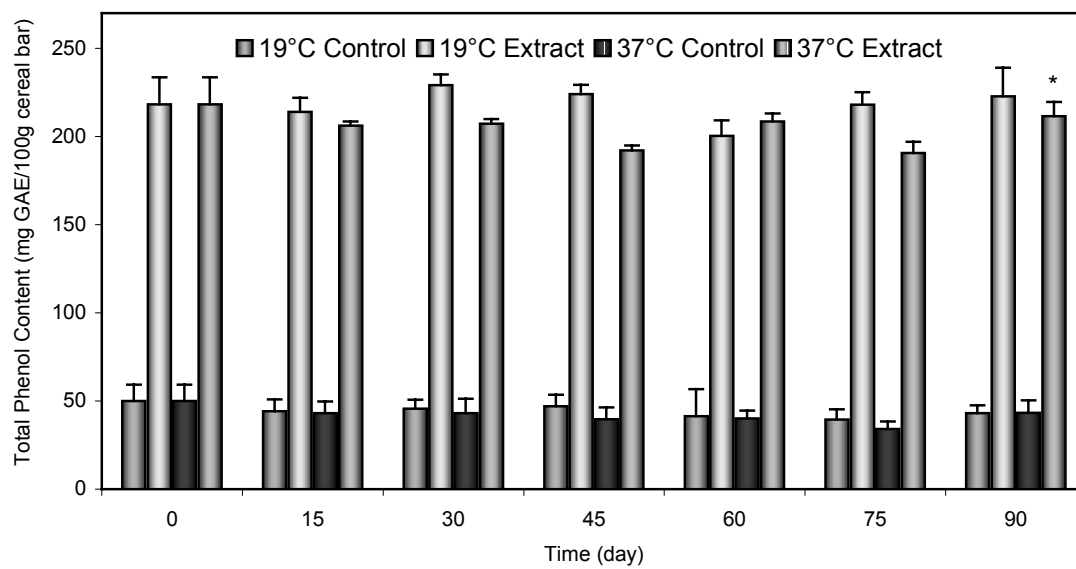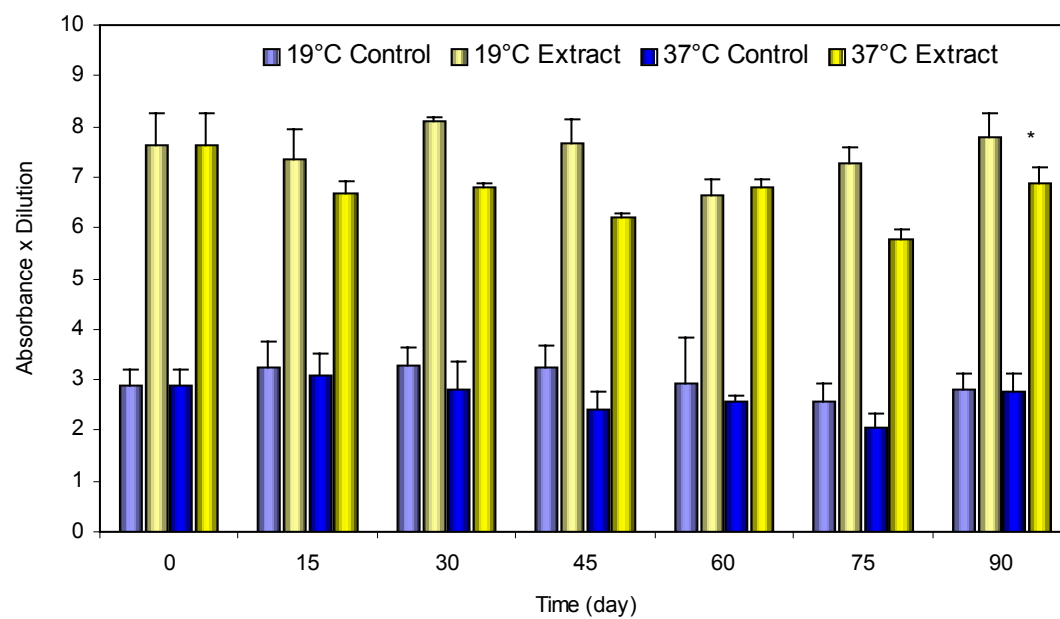
Figure 5.2

Figure 5.3

Figure 5.4.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Aqueous solutions containing 60% ethanol (190 proof), 60 to 70% methanol, and 50 to 75% acetone were better than any single compound solvent system in terms of extracting phenolics from muscadine grape seed powder. Significant correlations were found between the total phenol contents and absorbances at 280nm, an indicator of the total tannin in the extracts. Antioxidant capacities of Chardonnay, Merlot and Muscadine grape seed powders were 637.8, 344.8 and 310.8μmol TE/g dry weight ($p<0.05$), respectively. The difference between the ORAC values of Chardonnay and Merlot grape skin powder was not significant ($p>0.05$). Total phenol contents of extracts used to determine the antioxidant capacities of grape seeds and skins had a trend similar to ORAC values.

Gallic acid, catechin and epicatechin concentrations were 68, 7, and 69mg/100g d.m. in Muscadine seeds, 10, 211, and 303mg/100g d.m. in Chardonnay seeds, and 7, 74, and 83mg/100g d.m. in Merlot seeds, respectively. Concentrations of these three compounds were lower in winery byproduct grape skins than seeds. These three major phenolic constituents of grape seeds contributed less than 17% to the antioxidant capacity measured as ORAC. Peroxyl radical scavenging activities of phenolics present in grape seeds or skins in decreasing order were resveratrol > catechin > epicatechin = gallocatechin > gallic acid = ellagic acid.

Phenolic constituents of muscadine seed extract in a puffed rice cereal bar were more stable at low storage temperatures over a period of three months. Antioxidant capacities of the food product supplemented with the muscadine seed extract measured as oxygen radical absorbing capacity (ORAC) was reduced over time with no difference attributed to storage temperature ($p>0.05$). The antioxidant capacities of products as ORAC were stable over the storage temperatures we studied. Moreover, the use of muscadine seed extracts provided a natural antioxidant activity by inhibiting lipid peroxidation in the cereal product.

Health functional components of grape skin and seed powders from the byproducts of grape/wine industry are comparable to fruits and vegetables; therefore, these byproducts can be utilized to make dietary supplements or used in functional foods. The results indicated that dimeric, trimeric, oligomeric or polymeric procyanidins account for most of the superior antioxidant capacity of grape seeds. Supplementation of cereal products with muscadine seed extract as a functional ingredient may increase dietary antioxidant intake for health conscious consumers with the benefit of higher acceptability compared to food supplement in the forms of grape skin or grape seed powder.