Inhibition of Human Low-Density Lipoprotein Oxidation in Relation to Composition of Phenolic Antioxidants in Grapes (*Vitis vinifera*)

Anne S. Meyer,*[†] Ock-Sook Yi,[†] Debra A. Pearson,[†] Andrew L. Waterhouse,[‡] and Edwin N. Frankel[†]

Department of Food Science and Technology and Department of Viticulture and Enology, University of California, Davis, California 95616

Antioxidant activities of phenolic extracts from fourteen different types of fresh grapes were investigated by measuring the inhibition of human low-density lipoprotein (LDL) oxidation *in vitro*. The inhibition of LDL oxidation ranged from 22% to 60% at 10 μM gallic acid equivalents (GAE) of total phenols and from 62% to 91% at 20 μM GAE. The antioxidant activities were thus comparable to those previously found for wines. The relative LDL antioxidant activity correlated with the concentration of total phenols ($r = 0.89$, $p < 0.01$), with the level of anthocyanins ($r = 0.56$, $p < 0.05$) and flavonols ($r = 0.54$, $p < 0.05$) in the grape extracts as analyzed by high-performance liquid chromatography (HPLC). When seed crushing and longer extraction times were employed, high amounts of flavan-3-ols and hydroxybenzoates were extracted. With these extracts relative LDL antioxidant activity correlated highly with the levels of flavan-3-ols ($r = 0.86$, $p < 0.01$), total phenols ($r = 0.79$, $p < 0.05$), and hydroxybenzoates ($r = 0.77$, $p < 0.05$).

Keywords: *LDL oxidation; flavonoids; hydroxycinnamates; anthocyanins; flavan-3-ols; flavonols; HPLC*

INTRODUCTION

Phenolic compounds in wine, notably red wine, have been shown to inhibit *in vitro* oxidation of human low-density lipoprotein (LDL) (Frankel et al., 1993, 1995; Kanner et al., 1994; Teissedre et al., 1996). Oxidative modification of LDL is considered a primary event in the pathogenesis of atherosclerosis (Steinberg et al., 1989; Steinberg, 1992). Hence, the ability of wine phenolics to inhibit LDL oxidation have been suggested to be a possible mechanism explaining the "French Paradox" (Frankel et al., 1993; Kinsella et al., 1993). The French Paradox refers to the epidemiological finding that in certain parts of France coronary heart disease mortality is low despite a high intake of saturated fats and relatively high plasma cholesterol levels in the population (Renaud and de Lorgeril, 1992).

The phenolic substances present in wine mainly originate from grapes and include non-flavonoid compounds such as hydroxycinnamates, hydroxybenzoates (benzoic acids), and stilbenes in addition to flavonoids such as flavan-3-ols (catechins), anthocyanins, flavonols, and polyphenolic tannins (Singleton, 1982). However, the phenolic profile of wine is not the same as that of fresh grapes because significant changes in phenolic composition occur during the wine making process, both very early at the grape crushing step and during wine fermentation and ageing (Singleton, 1987).

Although much is known about the impact of these mainly oxidative and hydrolytic changes of grape phenolics on the quality and sensory attributes of wine, there is very little knowledge about how the antioxidative properties of the phenolic compounds are affected when fresh grapes are processed for wine making. Thus, to determine if consumption of fresh grapes may also reduce the risk of coronary heart disease, it is important first to compare the relative antioxidant properties of grapes and wines. Secondly, it is relevant to consider if the phenolic composition and antioxidant activities of table grapes differ from wine grapes.

Epidemiological studies have shown that a high intake of fresh fruits and vegetables is associated with a lowered risk of coronary heart disease mortality (Criqui and Ringel, 1994). Recently, the intake of flavonoids was shown to be inversely related to coronary heart disease mortality (Hertog et al., 1993, 1995; Knekt et al., 1996). These results suggest that increased dietary intake of flavonoids may have an important impact in reducing mortality from coronary heart diseases.

Fruit juices, including grape juice, have been demonstrated to exert significant antioxidant activity in an artificial peroxyl radical model system (Wang et al., 1996). However, the evaluation of natural antioxidants by such artificial radical model systems provide no information on what lipid or protein is protected.

Little is known about the relationship between the composition of phenolic constituents in grapes and other fruits and their antioxidant potential against LDL oxidation.

The objective of this study was to evaluate the antioxidant activity toward LDL oxidation *in vitro* of phenolic compounds extracted from twelve different varieties of grapes (*Vitis vinifera*), including seven wine grape varieties and five table grape varieties. Further, the antioxidant activity of different grape extracts was related to their phenolic composition as determined by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Grapes. In previous studies antioxidants and reducing agents were used during extraction of phenolic compounds to minimize their oxidation (Lee and Jaworski, 1990; Santos-

* Corresponding author. Permanent address: Department of Biotechnology, Building 221, Technical University of Denmark, 2800 Lyngby, Denmark [fax (+45) 45 88 49 22; e-mail AM@ibt.dtu.dk].

[†] Department of Food Science and Technology.

[‡] Department of Viticulture and Enology.

S0021-8561(96)00721-2 CCC: $14.00 © 1997 American Chemical Society

Table 1. Grapes (*V. vinifera*) Evaluated for Antioxidant Activities

grape variety	abbreviation	type of grape	°Brix at harvest
Calzin early	Cae	red wine	17.8
Calzin late	Cal	red wine	23.3
Petite Sirah early	PSe	red wine	19.0
Petite Sirah late	PSl	red wine	23.4
Cabernet Sauvignon	CS	red wine	23.2
Cabernet Franc	CF	red wine	23.0
Flame seedless	Fs	red table	18.0
Merlot	Me	red wine	22.0
Sauvignon blanc	Sb	white wine	22.6
Emperor	Em	blush table	17.0
Thompson seedless	Ts	white table	18.0
Chardonnay	Ch	white wine	23.4
Red Globe	RG	blush table	17.5
Red Malaga	RM	blush table	17.0

Buelga et al., 1995; Singleton et al., 1985; Somers and Ziemelis, 1985). In the present study, the use of antioxidants would be confounding and was therefore avoided. To minimize oxidation the grape samples were extracted anaerobically as rapidly as possible. The seeds were also not crushed during extraction to obtain samples representing the actual phenolic compounds consumed in fresh grapes. Grapes were harvested from the experimental vineyards of the Department of Viticulture and Enology at University of California, Davis in the fall 1995. Twelve different varieties of *V. vinifera* grapes were selected, including seven wine grape varieties, Calzin, Petite Sirah, Cabernet Sauvignon, Cabernet Franc, Merlot, Sauvignon Blanc, and Chardonnay, and five table grape varieties, Flame seedless, Emperor, Thompson seedless, Red Globe, and Red Malaga (Table 1). The grapes were picked at their optimum commercial maturity, i.e., table grapes within sugar contents of 16.5−18 °Brix and wine grapes within 22−24 °Brix. Two wine grape varieties, Calzin and Petite Sirah, were also tested at two different stages of maturity (Table 1).

Chemicals. Catechin, gallic acid, caffeic acid, rutin (quercetin rutinoside), hexanal, and Folin−Ciocalteu phenol reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Malvin (malvidin 3,5-diglucoside) was purchased from Pfaltz and Bauer (Waterbury, CT). Copper sulfate was from Fisher Scientific (Fairlawn, NJ).

Sample Preparation and Extraction of Phenols. Whole grapes were thoroughly washed with distilled water, dried, cut in half, the seeds removed, and homogenized for 1 min at maximum speed in a Waring blender. After homogenization, the seeds were added back into the homogenate, and grape samples with intact seeds were divided in smaller aliquots and stored at −30 °C until use.

Phenolic compounds were extracted by shaking grape samples (2 g) with 60% aqueous methanol (10 mL) for 1 min. After filtration (Whatman No. 1), methanol was removed by rotary evaporation under vacuum at 45 °C. The extracts were then diluted with doubly distilled water to a final volume of 5.0 mL and filtered through a 0.45 μm PTFE (polytetrafluoroethylene) filter prior to oxidation and HPLC analysis.

In timed extraction studies with seed crushing, whole grapes were homogenized with seeds for 1 min at maximum speed in a Waring blender. Phenolic constituents were subsequently extracted with 60% aqueous methanol with contact times of 1 min, 1 h, 4 h, 24 h, and 165 h, but otherwise processed as described above.

Analyses of Phenolics. The concentration of total phenols in grape extracts was determined by the Folin−Ciocalteu procedure (Singleton and Rossi, 1965). Total phenols were expressed as mg/L gallic acid equivalents (GAE). The phenolic composition of grape extracts was analyzed by HPLC as described by Lamuela-Raventos and Waterhouse (1994). On the basis of spectral identification, the phenolic compounds were divided into four classes and quantified by calibrating with the following standard authentic compounds: hydroxycinnamates as caffeic acid equivalents (CFAE), peak area 316 nm; anthocyanins as malvin equivalents (ME), peak area 520 nm; flavan-3-ols as catechin equivalents (CCE), peak area 280 nm; flavonols as rutin equivalents (RUE), peak area 365 nm. In the extraction studies, hydroxybenzoates (benzoic acids) were quantified as gallic acid equivalents (GAE), peak area 280 nm.

Isolation of Human LDL. LDL was prepared from the blood of three normolipidemic males as described previously (Frankel et al., 1992).

Inhibition of Human LDL Oxidation. The antioxidant activity of grape extracts to inhibit copper-catalyzed oxidation of human LDL was assayed by monitoring production of hexanal by static headspace gas chromatography as described earlier (Frankel et al., 1992). The grape extracts were diluted with doubly distilled water to standard concentrations of 10 and 20 μM total phenols as GAE. The results obtained after replicate analyses were expressed as percent relative inhibition:

$$(\%\ \mathrm{In}) = [(C - S)/C] \times 100$$

where C was the amount hexanal formed in the control and S was the amount of hexanal formed in the sample.

Statistical Analysis. Differences in antioxidant activities were tested by one-way analysis of variance (Minitab Statistical Software, Addison-Wesley, Reading, MA). Relative percent inhibition of LDL oxidation was calculated by multiplying the inhibition at 10 μM GAE by the dilution factor used for the extract in the LDL oxidation assay and by setting the highest inhibition value as 100% (Frankel et al., 1995). Correlation coefficients of relative percent inhibition of LDL oxidation versus concentration of total phenols and concentration of phenolics in different classes were determined by linear regression analysis. The statistical significance of the correlations were tested by the dose−response F-test (Berry and Lindgren, 1996). For the inhibition data (at 10 μM GAE) there was equality of variances at a 5% level of significance as evaluated by Bartlett's test (Montgomery, 1991).

RESULTS

Antioxidant Activities. The antioxidant activities of all fourteen different grape extracts were compared at the same molar phenol concentrations. The inhibition of copper-induced LDL oxidation varied from 22% to 60% at 10 μM GAE and from 62% to 91% at 20 μM GAE (Table 2). At 10 μM GAE the majority of extracts exerted between 39% and 49% inhibition, and at 20 μM between 70% and 79% inhibition (Table 2). Pure catechin used as a reference was consistently more active than the grape extracts. The Calzin late was the most active of all grape extracts and inhibited LDL oxidation by 60% at 10 μM and by 91% at 20 μM GAE. The inhibition at 20 μM GAE was, however, not significantly different from the antioxidant activity of the Calzin early, which exerted 90% inhibition (Table 2).

The antioxidant activities of extracts of the white and blush table grape varieties Thompson seedless and Red Globe, were significantly lower with 30% inhibition at 10 μM and 62% and 70% inhibition at 20 μM, respectively (Table 2). At 10 μM also the blush table grape variety Emperor showed only 22% inhibition.

Total Phenols and Phenolic Composition. The level of total phenols varied widely in the different grape extracts, and ranged from 169−272 mg/L GAE in the extracts of white and blush grape varieties to 497−1215 mg/L GAE in the dark red wine varieties (Table 2). These results confirm that red wine grape varieties tend to have higher contents of phenolic substances compared to white and blush table and wine grapes (Singleton, 1982; Kanner et al., 1994).

The degree of maturity as measured from the difference in sugar level in °Brix (Table 1) influenced the total amounts as well as the distribution of phenols in

Table 2. Inhibition (% In) of LDL Oxidation *in Vitro* and Phenolic Profiles of Aqueous Extracts of Whole Grapes[a]

grape variety	Folin GAE[b] (mg/L)	% In at 10 μM GAE[b]	% In at 20 μM GAE[b]	cinnamates CFAE[c] (mg/L)	anthocyanins ME[d] (mg/L)	flavan-3-ols CCE[e] (mg/L)	flavonols RUE[f] (mg/L)
Calzin early	859	44.0 ± 6.4[b]	89.5 ± 0.3[c]	2.3 (0.6%)	386.9 (96.8%)	8.9 (2.2%)	1.4 (0.4%)
Calzin late	1215	60.4 ± 0.6[c]	91.3 ± 0.3[c]	3.3 (0.4%)	888.3 (98.2%)	10.2 (1.1%)	21.9 (2.4%)
P. Sirah early	678	40.5 ± 1.2[b]	77.7 ± 1.1[b]	9.8 (0.9%)	1127.0 (97.9%)	0.0 (0.0%)	14.4 (1.3%)
P. Sirah late	963	39.0 ± 2.5[b]	75.0 ± 2.7[b]	10.0 (0.6%)	1708.2 (97.2%)	5.9 (0.3%)	33.5 (1.9%)
Cab. Sauvignon	575	44.3 ± 3.4[b]	73.3 ± 2.7[b]	1.0 (0.1%)	718.0 (98.9%)	0.0 (0.0%)	7.3 (1.0%)
Cab. Franc	529	47.2 ± 6.3[b]	79.3 ± 9.5[bc]	0.8 (0.1%)	881.0 (97.8%)	0.0 (0.0%)	18.9 (2.1%)
Flame seedless	498	48.5 ± 2.0[b]	75.7 ± 5.1[b]	4.8 (5.3%)	72.5 (80.6%)	0.0 (0.0%)	12.6 (14.0%)
Merlot	497	47.0 ± 1.2[b]	85.6 ± 6.8[bc]	4.8 (1.1%)	414.2 (93.2%)	0.0 (0.0%)	25.6 (5.8%)
Sauv. Blanc	272	44.2 ± 3.2[b]	79.4 ± 2.4[bc]	6.2 (40.0%)	0.0 (0.0%)	0.0 (0.0%)	9.3 (60.0%)
Emperor	245	22.4 ± 0.0[a]	88.3 ± 1.8[bc]	3.5 (21.3%)	10.6 (64.6%)	0.0 (0.0%)	2.3 (14.0%)
Thompson seedless	242	30.2 ± 2.6[a]	62.3 ± 4.1[a]	5.5 (34.6%)	0.0 (0.0%)	0.0 (0.0%)	10.4 (65.4%)
Chardonnay	218	46.0 ± 0.9[b]	74.1 ± 1.4[b]	1.7 (26.2%)	0.0 (0.0%)	0.0 (0.0%)	4.8 (73.8%)
Red Globe	173	29.6 ± 4.4[a]	69.7 ± 0.0[ab]	2.6 (14.8%)	12.6 (71.6%)	0.0 (0.0%)	2.4 (13.6%)
Red Malaga	168	41.7 ± 4.7[b]	nd	4.2 (12.6%)	27.6 (82.9%)	0.0 (0.0%)	1.5 (4.5%)
catechin (control)[g]		97.3 ± 0.2[c]	98.7 ± 0.0[c]				

[a] Antioxidant activities (% In) are shown as mean values ± SD. Values in the same column followed by the same roman superscript letter are not significantly different at $P < 0.05$. [b] GAE, gallic acid equivalents. [c] CFAE, caffeic acid equivalents, average SD = 0.2. [d] ME, Malvin equivalents, average SD = 4.0. [e] CCE, catechin equivalents, average SD = 0.1. [f] RUE, rutin equivalents, average SD = 0.1. [g] Catechin was tested at 10 and 20 μM, i.e., not as GAE. nd, not determined. Values in parentheses are relative to the total phenols determined by HPLC.

extracts of both Calzin and Petite Sirah. As expected, extracts of the more mature grapes had higher contents of total phenols and higher absolute levels of both hydroxycinnamates, anthocyanins, flavan-3-ols, and flavonols (Table 2).

Anthocyanins produce the red and purple colors in red grapes and contributed the majority of phenolic compounds as detected by HPLC in extracts of red grape varieties, ranging from 73 mg/L in Flame seedless to 1708 mg/L in Petite Sirah late (Table 2). Anthocyanins were not present in extracts of white grape vareties and were found in amounts of only 11–27 mg/L in extracts of blush grapes (Table 2). The contents of cinnamates were relatively constant and low in all extracts, ranging from 1 to 10 mg/L mainly as *S*-glutathionylcaftaric acid. This compound was identified earlier as the main oxidation product formed during grape crushing decreasing the level of caftaric acid (Singleton et al., 1985). The amounts of flavonols were higher, ranging from 1 to 34 mg/L (Table 2). On the basis of spectral evaluation, rutin (quercetin rutinoside) appeared to be the major flavonol compound. Surprisingly, flavan-3-ols were virtually absent in our extracts prepared with short extraction time of 1 min (Table 2). Hydroxybenzoates, including gallic acid, were not detected in any of the extracts.

Because of the large span in total phenol contents and types of different phenolic compounds, the relative levels of different compound classes varied considerably among extracts of different grape varieties (Table 2, values in parentheses). Thus, although the amounts of cinnamates and flavonols were high in, e.g., the extract of Petite Sirah late, the relative contents of these compounds were only 0.6% and 1.9% respectively, because of the very high anthocyanin fraction of 97.2% (Table 2). In contrast, the relative levels of cinnamates and flavonols were high in extracts of white grape varieties, cinnamates ranging from 26.2% to 40.0% and flavonols from 60.0% to 73.8% in Sauvignon Blanc and Chardonnay (Table 2).

Linear regression analysis of the relative LDL antioxidant activity at 10 μM GAE with the concentration of total phenols and compounds in each class, respectively, gave the following correlation coefficients, $r = 0.89$ ($p < 0.01$) for total phenols (Figure 1), $r = 0.56$ ($p < 0.05$) for anthocyanins, and $r = 0.54$ ($p < 0.05$) for flavonols, but no correlation for cinnamates. Correlation for flavan-3-ols could not be analyzed because of the limited amounts detected.

Effect of Prolonged Extraction Time and Seed Crushing. Flavan-3-ols, including catechin and epicatechin, were shown previously to be the dominant phenolic class in red wines (Frankel et al., 1995). Gallic acid, a hydroxybenzoate (or benzoic acid), also occurred in relatively high concentrations in red wines. Catechin, epicatechin, and gallic acid exert significant antioxidant effects toward human LDL oxidation *in vitro* (Frankel et al., 1995; Teissedre et al., 1996), and these compounds were shown earlier to be particularly abundant in grape skin and seeds (Bourzeix et al., 1986; Santos-Buelga et al., 1995; Singleton, 1982). Because both flavan-3-ols and hydroxybenzoates were virtually absent in our samples of fresh grapes prepared by a 1 min extraction we determined the effect of crushing of seeds and solvent contact time during extraction on antioxidant activity and phenolic profiles of extracts of whole grapes of Petite Sirah late and Cabernet Sauvignon. These grapes were chosen because the corresponding wines were shown previously to contain large amounts of catechin, epicatechin, and gallic acid (Frankel et al., 1995).

For both varieties, grapes extracted with the seeds crushed produced a significantly higher initial phenol content than grapes extracted with intact seeds (Table 3). Extraction of grapes with intact seeds produced a high increase in total phenols only after 165 h (1 week) solvent contact time (Table 3). Further, extraction of grapes with crushed seeds resulted in more significant increases in the levels of different phenolic classes than prolonged extraction. However, with Petite Sirah late extracts, the contents of anthocyanins and flavonols remained relatively constant with seed crushing or even decreased (Table 3). Except for Cabernet Sauvignon with intact seeds the trend was that cinnamate contents decreased with prolonged extraction time. Such decrease may be the result of degradation during prolonged extraction. Significant amounts of flavan-3-ols and, to a lesser degree, hydroxybenzoates, were observed with grapes with intact seeds only after 24 h of extraction (Table 3). In samples with intact seeds, high amounts (99 mg/L) of flavan-3-ols were released only from Petite Sirah after 165 h of extraction.

trans-Caftaric acid (*trans*-caffeoyl-L-(+)-tartaric acid) was found in relatively high amounts (14%–51%) with

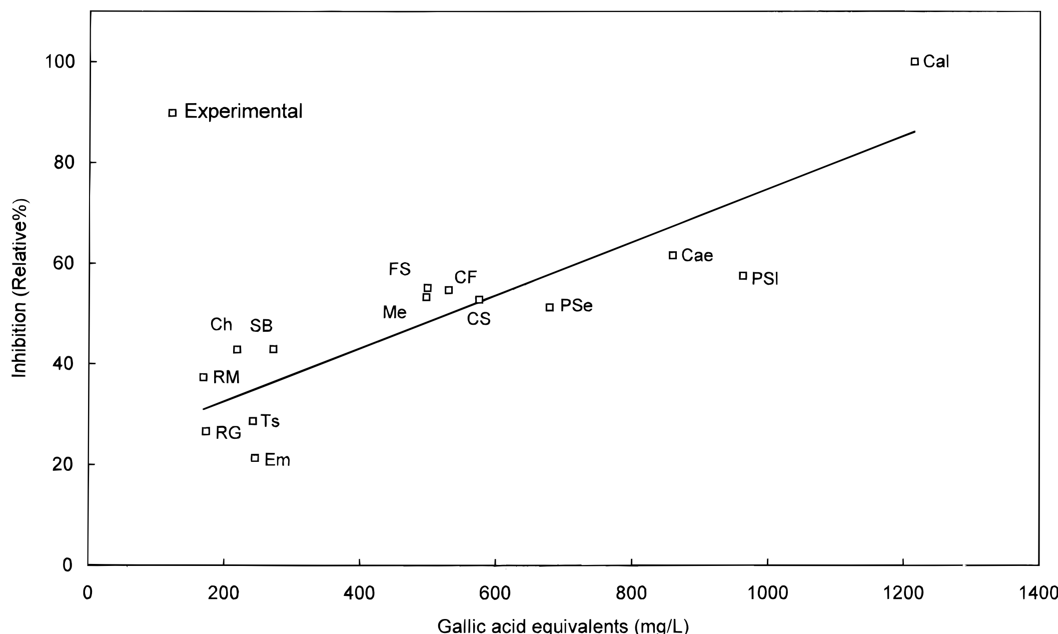

Figure 1. Relative percent inhibition of LDL oxidation by grape extracts versus total phenol content as gallic acid equivalents. For abbreviations, see Table 1. Linear regression equation, calculated as $Y = 0.053X + 22.08$; correlation coefficient, $r = 0.89$.

Table 3. Effects of Different Extraction Times and Crushing of Seeds on Antioxidant Activity and on Phenolic Profiles of Grape Extracts[a]

	total phenols GAE[b] (mg/L)	% In at 10 μM GAE[b]	% In at 20 μM GAE[b]	benzoic acids GAE[c] (mg/L)	cinnamates CFAE[d] (mg/L)	anthocyanins ME[e] (mg/L)	flavan-3-ols CCE[f] (mg/L)	flavonols RUE[g] (mg/L)
Cab. Sauvignon (intact seeds)								
1 min	565	31.3 ± 0.3[a]	nd	0.0	1.0	718.0	0.0	7.3
1 h	686			0.0	1.0	696.0	0.0	6.9
4 h	771			0.0	1.0	793.8	0.0	7.8
24 h	737	49.9 ± 4.2[b]	82.4 ± 0.6[b]	1.0	0.9	705.8	8.9	7.5
165 h	890			2.4	0.9	746.4	0.0	16.9
Cab. Sauvignon (crushed seeds)								
1 min	1780	49.0 ± 2.2[b]	92.6 ± 0.9[c]	4.7	7.6	791.7	133.7	16.7
1 h	1868			3.2	2.3	879.9	122.7	19.7
4 h	1930			2.3	6.1	867.8	140.9	20.1
24 h	2015	61.0 ± 1.2[c]	92.0 ± 0.7[c]	3.2	4.6	856.2	156.0	19.7
165 h	2138			5.0	4.6	775.2	167.4	19.2
P. Sirah late (intact seeds)								
1 min	1115	32.1 ± 1.7[a]	nd	0.0	10.0	1708.2	5.9	33.5
1 h	1136			1.0	9.4	1483.6	0.0	30.7
4 h	1163			1.4	9.0	1558.9	5.5	32.4
24 h	1183	54.5 ± 0.3[b]	86.4 ± 0.4[b]	2.0	8.8	1477.3	21.5	33.5
165 h	1367			6.1	9.7	1564.7	99.4	27.7
P. Sirah late (crushed seeds)								
1 min	1741	28.9 ± 1.2[a]	66.4 ± 0.0[a]	3.8	14.6	1337.2	93.4	15.4
1 h	1820			3.5	11.6	1644.2	101.8	24.1
4 h	1966			4.0	11.4	1463.4	137.6	26.5
24 h	1964	66.4 ± 1.0[c]	89.5 ± 0.0[c]	5.4	11.0	1513.2	173.6	29.6
165 h	2094			5.8	10.6	1344.0	168.0	26.7

[a] Antioxidant activities (% In) are shown as mean values ± SD. Values in the same column followed by the same roman superscript letter are not significantly different at $P < 0.05$. nd, not determined. [b] GAE, gallic acid equivalents. [c] GAE, gallic acid equivalents, average SD = 0.6. [d] CFAE, caffeic acid equivalents, average SD = 0.2. [e] ME, Malvin equivalents, average SD = 7.7. [f] CCE, catechin equivalents, average SD = 1.5. [g] RUE, rutin equivalents, average SD = 0.1.

both grape varieties extracted for 1 min. With longer extraction times, however, *S*-glutathionylcaftaric acid was the main hydroxycinnamic compound found in the extracts, presumably due to oxidation of caftaric acid to the quinone followed by reaction with glutathione.

When compared at equimolar total phenol concentrations, antioxidant activities of extracts toward LDL oxidation varied considerably with crushing of seeds as well as with extraction time (Table 3). The inhibition of LDL oxidation ranged from 31% to 66% at 10 μM GAE and from 66% to 93% at 20 μM GAE. At 10 μM GAE samples prepared by the longer extraction time (24 h) showed significantly higher antioxidant activity than samples prepared by the short extraction time (1 min). Likewise, crushing of seeds before extraction gave higher antioxidant activities. However, the samples of Petite Sirah late extracted for 1 min with intact seeds showed the same antioxidant activity (32%) as the extract from grapes with crused seeds (29%) (Table 3).

Correlation coefficients, *r*, between the relative LDL antioxidant activity at 10 μM GAE with the different phenol constituents were 0.86 ($p < 0.01$) for flavan-3-ols, 0.79 ($p < 0.05$) for total phenols as GAE, and 0.77 ($p < 0.05$) for hydroxybenzoates.

On the basis of these correlation coefficients, the antioxidative capabilities toward LDL of grape phenolics

again seemed to be highly correlated with the total phenol content. Further, the antioxidant activity appeared to be more significantly related to the flavan-3-ol and hydroxybenzoate (benzoic acid) content than with other phenolic classes identified in the red wine grape extracts.

DISCUSSION

In red wines, the dominant components catechin, epicatechin, and gallic acid were particularly active as antioxidants in inhibiting LDL oxidation *in vitro* (Frankel et al., 1995). Catechin oligomers and procyanidin dimers (B_2, B_3, B_4, B_6, B_8) and trimers (C_1, C_2) extracted from grape seeds were also shown to possess significant antioxidant activity toward LDL oxidation *in vitro* (Teissedre et al., 1996).

The present study showed that phenolic compounds extracted from fresh grapes were able to inhibit significantly the *in vitro* LDL oxidation at micromolar phenol concentrations. The antioxidant potency of grape extracts in inhibiting LDL oxidation was in the same order as that of wines. Thus, our extracts of fresh grapes exerted between 22% and 60% inhibition at 10 μM total phenols as GAE. Wines tested under the same conditions had antioxidant activities ranging from 27% to 65% inhibition at 10 μM GAE (Frankel et al., 1995).

For nutritional considerations whole grapes with intact seeds were evaluated for their antioxidant activity by using a short 1 min extraction procedure to minimize oxidation of phenolic constituents. By this extraction procedure, HPLC profiles of red grapes were dominated by anthocyanins, that contributed 80%−99% of the phenolics as detected by HPLC depending on the grape variety (Table 2). The relative LDL antioxidant activity at the same concentration correlated significantly with the anthocyanin content. The total phenol content was consistently higher in extracts of red grape varieties, hence, except for Sauvignon Blanc, Chardonnay, and Red Malaga, the trend was that red grape varieties had higher antioxidant potency compared to white and blush varieties (Figure 1 and Table 2). The latter result is in agreement with previously published data for wines, where the relative inhibition of LDL oxidation was found to be significantly higher for red wines as compared to white wines (Frankel et al., 1995).

Longer extraction time and crushing of seeds prior to extraction led to significantly higher recoveries of hydroxybenzoates and flavan-3-ols from the two red wine grape varieties investigated (Table 3). The relative LDL antioxidant activity of these extracts were highly correlated with the levels of both hydroxybenzoates and flavan-3-ols. These results are in good agreement with previous findings for wines (Frankel et al., 1995). Although deliberate crushing of seeds may not be nutritionally relevant when fresh grapes are consumed, our observation that longer extraction and seed crushing release significant amounts of potent antioxidants may have technological applications in grape juice processing and in the preparation of antioxidant concentrates from byproducts of the wine industry. Grape seed extract, referred to as oligomeric procyanidins ("OPC"), is already widely sold as a nutritional supplement.

In the present study hydroxycinnamates were identified in all grape extracts investigated, but the relative antioxidant activity toward LDL did not correlate with the hydroxycinnamate content. This result may be attributable to the finding that hydroxycinnamates occurred mainly as *S*-glutathionylcaftaric acid in our

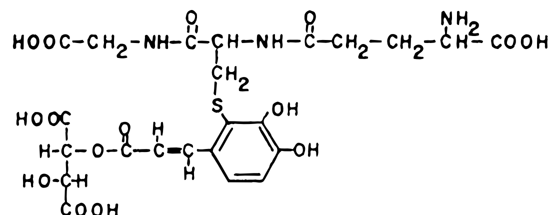

Figure 2. *S*-Glutathionylcaftaric acid (adapted from Cheynier et al., 1986).

extracts. *trans*-Caftaric acid (*trans*-caffeoyl-L-(+)-tartaric acid) is the major hydroxycinnamic compound in grapes (Singleton et al., 1985). *S*-Glutathionylcaftaric acid is derived from enzymatic oxidation of caftaric acid immediately when grapes are crushed. This oxidation reaction can also take place during normal grape juice and wine processing operations (Singleton et al., 1985). The reaction sequence involves conversion of the *o*-diphenol of caftaric acid to the corresponding *o*-quinone, which reacts spontaneously with glutathione (Cheynier and Moutounet, 1992; Singleton et al., 1985). In the presence of glutathione the caftaric quinone ring is reduced to the hydroquinone form to yield *S*-glutathionylcaftaric acid (Figure 2) (Singleton et al., 1985). Glutathione is a well-known radical scavenger, and caffeic acid (the phenol constituent of caftaric acid) was recently shown to inhibit copper-induced LDL oxidation *in vitro* (Nardini et al., 1995). On this basis, *S*-glutathionylcaftaric acid would be expected to be active as an antioxidant. At present, the mechanism of inhibition of LDL oxidation is not clear and more work is needed to test the antioxidant activity of individual phenolic compounds in grapes. Teissedre et al. (1996) suggested that antioxidant action toward LDL may not only be due to radical scavenging and/or metal chelation by phenolic compounds but may also be due to their ability to bind the apoprotein B in the LDL particles. If the protein binding properties of *S*-glutathionylcaftaric acid are significantly different from those of caftaric (and caffeic) acid, the antioxidant efficacy might be affected. This could explain in part the lack of correlation between cinnamate content and relative inhibition of LDL oxidation found in this study.

In all grape extracts investigated in this work, we were able to identify several of the individual compounds in each phenolic class by spectral evaluation of HPLC data (results not shown). However, the available data do not permit us to draw any further conclusions about the significance of differences in individual compound patterns between grape varieties. Previously published work on wine phenolics (Frankel et al., 1995) furthermore indicates that the antioxidant activity of wines is distributed widely among different phenolic substances belonging to several different classes. Our data with grapes largely support this conclusion. However, more detailed studies are needed on the antioxidant interaction of different phenolic grape compounds toward LDL oxidation to clarify any potential synergistic and antagonistic effects of grape phenolics.

Several antioxidative phenolic compounds of fresh grapes are glycosylated as compared to the corresponding phenols found in wine (Singleton, 1982, 1987). Although we found the antioxidant activities of grape phenolics comparable to wine it can be speculated that glycosylation may change the antioxidant activity toward LDL by differences in solubility and partition or ability to bind the apoprotein B. In a recent study on absorbtion of quercetin and quercetin glucosides Holl-

man et al. (1995) conluded that humans absorb appreciable amounts of quercetin and that absorbtion is enhanced by conjugation with glucose but not with rutinose. More research is needed to improve our knowledge on the antioxidant and physiological significance of phenol glycosylation. Thus, it is important to compare both the dietary intake, the absorption and the antioxidant effects of glycosylated phenolic compounds versus their corresponding aglycones. Such data may provide a better understanding of the antioxidant mechanism of fruit phenolics and their potential nutritional benefits.

ACKNOWLEDGMENT

We are grateful to M. A. Walker and E. Hall for their help in selecting the grape varieties for this study.

LITERATURE CITED

Berry, D. A.; Lindgren, B. W. *Statistics: Theory and methods*, 2nd ed.; Duxbury Press at Wadsworth Publ. Co.: Belmont, CA, 1996; pp 608−609.

Bourzeix, M.; Weyland, D.; Heredia, N. A study of catechins and procyanidins of grape clusters, the wine and other by-products of the vine. *Bull. O.I.V.* **1986**, *59*, 1171−1254 (transl. from French).

Cheynier, V.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-*S*-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34*, 217−221.

Cheynier, V.; Moutounet, M. Oxidative reactions of caffeic acid in model systems containing polyphenol oxidase. *J. Agric. Food Chem.* **1992**, *40*, 2038−2044.

Criqui, M. H.; Ringel, B. L. Does diet or alcohol explain the French paradox? *Lancet* **1994**, *344*, 1719−1723.

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

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

Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. L. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J. Agric. Food Chem.* **1995**, *43*, 890−894.

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

Hertog, M. G. L.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A., Nedeljkovic, S.; Pekkarinen, M.; Simic, B. S.; Toshima, H.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381−386.

Hollman, P. C. H.; de Vries, J. H. M.; van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276−1282.

Kanner, J.; Frankel, E.; Granit, R.; German, B.; Kinsella, J. E. Natural antioxidants in grapes and wines. *J. Agric. Food Chem.* **1994**, *42*, 64−69.

Kinsella, J. E.; Frankel, E.; German, B.; Kanner, J. Possible mechanisms for the protective role of antioxidants in wine and plant foods. *Food Technol.* **1993**, *47*, 85−89.

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

Lamuela-Raventos, R. M.; Waterhouse, A. L. A direct HPLC separation of wine phenolics. *Am. J. Enol. Vitic.* **1994**, *45*, 1−5.

Lee, C. Y.; Jaworski A. W. Identification of some phenolics in white grapes. *Am. J. Enol. Vitic.* **1990**, *41*, 87−89.

Montgomery, D. C. *Design and analysis of experiments*, 3rd ed.; John Wiley & Sons: New York, 1991; pp 102−103.

Nardini, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; Di Felice, M.; Scaccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541−552.

Orr, J. R.; Adamson, G. L.; Lindgren, F. T. Preparative ultracentrifugation and analytic ultracentrifugation of plasma lipoproteins. In *Analysis of fats, oils and lipoproteins*; Perkins, E. G., Ed.; American Oil Chemists' Society: Champaign, IL, 1991; pp 524−554.

Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

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

Singleton, V. L. Grape and wine phenolics: background and prospects. In *Proceedings of University of California−Davis, Grape wine centennial symposium*; Webb, A. D., Ed.; Department of Viticulture and Enology, University of California−Davis: Davis, CA, 1982; pp 215−227.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines, and model systems: Observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69−77.

Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

Singleton, V. L.; Salgues, M.; Zaya, J.; Trousdale, E. Caftaric acid disappearance and conversion to products of enzymic oxidation in grape must and wine. *Am. J. Enol. Vitic.* **1985**, *36*, 50−56.

Somers, T. C.; Ziemelis, G. Spectral evaluation of total phenolic components in *Vitis viifera*: Grapes and wines. *J. Sci. Food Agric.* **1985**, *36*, 1275−1284.

Steinberg, D. Metabolism of lipoproteins and their role in the pathogenesis of atherosclerosis. *Atheroscler. Rev.* **1992**, *18*, 1−6.

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

Teissedre, P. L.; Frankel, E. N.; Waterhouse, A. L.; Peleg, H.; German, J. B. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. *J. Sci. Food Agric.* **1996**, *70*, 55−61.

Wang, H.; Cao, G.; Prior, R. L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44*, 701−705.

Received for review September 23, 1996. Revised manuscript received February 7, 1997. Accepted February 20, 1997.[⊗] This research was supported by Research Grant Award No. IS-2260-93RC from BARD, The United States−Israel Agricultural Research and Development Fund.

JF960721A

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1997.