Relative Contributions of Polyphenolic Constituents to the Antioxidant Status of Wines: Development of a Predictive Model

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The concentrations of 17 phenolic constituents in red wine were analyzed by a number of multiple regression models for their contribution to total antioxidant status (TAS). The best model discovered involved a stepwise selection process starting by calculating Mallow's $C(p)$ for each of the 17 subsequent individual models resulting from dropping a single term at a time. The model with the lowest Mallow's $C(p)$ and in which no single step reduced Mallow's $C(p)$ further was chosen as the new model. This model gave excellent correlation between observed and predicted values of TAS. Further analysis of this model with the addition of a nonlinear term, using the 8 predictors discovered from the stepwise selection model, increased the correlation coefficient to 0.961 from 0.930. The equation of this model can predict almost 100% of the TAS by the content of vanillic acid, *trans*-polydatin, catechin, *m*-coumaric acid, epicatechin, quercetin, *cis*-polydatin, and *trans*-resveratrol with a very high degree of confidence. Although syringic and gallic acids were significantly correlated with TAS in a univariate analysis, they do not contribute to a statistical description of this parameter to the extent of the eight constituents already identified. The remaining constituents do not seem to contribute significantly to TAS, nor do they show significant correlation with TAS on univariate analysis.

Keywords: *Wine; antioxidants; flavonoids; phenolic acids; trihydroxystilbenes; gentisic acid; vanillic acid; ferulic acid; p-coumaric acid; m-coumaric acid; caffeic acid; gallic acid; resveratrol; polydatin; catechin; epicatechin; quercetin; morin; syringic acid; α-tocopherol; ascorbic acid; isoquercitrin; Cabernet Franc; Cabernet Sauvignon; Gamay Noir; Merlot; Pinot Noir*

INTRODUCTION

Epidemiologic evidence from surveys of specific and selective populations have overwhelmingly demonstrated a reduced mortality from coronary heart disease (CHD) among moderate alcohol consumers by comparison with abstainers (Rimm *et al.*, 1991; Criqui, 1996; Kannel and Ellison, 1996). About 50% of this protection seems to be due to the well-known ability of ethanol to increase HDL cholesterol (Suh *et al.*, 1992; Gaziano *et al.*, 1993), an important negative risk factor for CHD. Further protection, estimated at around 20−30% of the total, is attributed to reduced blood coagulability. This occurs by two mechanisms: firstly, inhibition of platelet aggregation by ethanol (Renaud *et al.*, 1992; Rubin and Rand, 1994; Renaud and Ruf, 1996); secondly, its ability to increase fibrinolysis by altering the circulatory concentrations of plasminogen together with those of activators and inhibitors responsible for regulating conversion of the latter to active plasmin (Pikaar *et al.*, 1987; Ridker *et al.*, 1994).

An alternative epidemiologic strategy, based upon comparisons among different countries of annual *per capita* beverage consumption with data for incidence of CHD mortality, has revealed a highly significant inverse correlation between the latter and wine intake that was much more dramatic than its relationship with total alcohol consumption or intake of other specific beverages such as spirits or beer (St. Leger *et al.*, 1979; Nanji and French, 1986; Hegsted and Ausman, 1988). Although high intake of dairy fat was positively correlated with CHD mortality in a nearly linear manner, this relationship was not found in countries such as France and Switzerland, whose inhabitants had the highest consumption of wine among those included in the survey; alcohol itself did not seem to offer protection against a high fat diet (Hegsted and Ausman, 1988; Renaud *et al.*, 1992). The superiority of wine over other alcoholic beverages was attributed to its content of biologically active polyphenols, especially those of the flavonoid class. Some investigators were able to generate data from specific population surveys supporting this notion (Rosenberg *et al.*, 1981; Criqui and Ringel, 1994; Gronbaek *et al.*, 1994); others could not confirm the advantage of wine in conferring protection against CHD mortality in studies of this kind (Klatsky and Armstrong, 1993; Maclure, 1993; Rimm *et al.*, 1996).

The health-promoting potential of dietary fruit and vegetables is currently one of the central dogmas in human nutrition (Willet, 1994). This is derived in large measure from the presence in these foodstuffs of a wide range of polyphenolic constituents that have been shown *in vitro* to have potent antioxidant activity (Ho *et al.*, 1991; Hertog *et al.*, 1992; Hubbard *et al.*, 1994). Free-radical damage due to reactive oxygen species is thought to be a fundamental etiological mechanism in heart disease, cancer, and many inflammatory conditions (Ames, 1989; Halliwell, 1994; Grisham, 1994), and it is postulated that dietary antioxidants including (but not limited to) α-tocopherol and flavonoids can limit or abolish this damage (Packer, 1992; Hertog *et al.*, 1993; Mehra *et al.*, 1995; Stampfer and Rimm, 1995). By way of illustration, oxidation of cholesterol-rich low-density lipoprotein (LDL) is considered to be a necessary prelude

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to the unregulated uptake of cholesterol into vascular tissues leading to atherosclerosis (Steinberg *et al.*, 1989; Esterbauer *et al.*, 1992). Oxidation of LDL *in vitro* can be prevented by flavonoids (De Whalley *et al.*, 1990; Mangiapane *et al.*, 1992), and wine polyphenols have been shown to be especially potent in this regard (Frankel *et al.*, 1993).

Taken together, these observations and hypotheses have stimulated considerable interest in the chemical composition of wine, with special emphasis upon its content of polyphenols and antioxidants which, in broad terms, embraces the majority of compounds in these categories present in fruit and vegetables (Singleton and Trousdale, 1983; Roggero *et al.*, 1990; Lamuela-Raventos and Waterhouse, 1994). In parallel, efforts have been initiated to identify techniques whereby specific components, especially resveratrol (3,5,4′-trihydroxystilbene), can be enriched in the must during the wine-making process (Soleas *et al.*, 1995; Jeandet *et al.*, 1995; Kovac *et al.*, 1995). Because not all polyphenols have equal biologic potency and different polyphenols show differences in response to enological manipulations, there are practical advantages in determining their relative contributions to the antioxidant activity of wine, in addition to the theoretical utility of this information which may be equally applicable to these compounds when present as complex mixtures in natural foodstuffs. This objective has been accomplished as a result of our development of chromatographic methods to allow the simultaneous quantitation of multiple polyphenols in the same wine sample (Goldberg *et al.*, 1996; Soleas *et al.*, 1997a) and their application to characterize the polyphenolic composition of a range of monovarietal wines from a circumscribed area of Ontario, the Niagara Penninsula (Soleas *et al.*, 1997b), and is the subject of the present report.

METHODS

TAS Measurement. The total antioxidant status (TAS) was measured using a kit manufactured by Randox Laboratories Ltd., Mississauga, Ontario, Canada (Cat. No. NX2332). Metmyoglobin (a peroxidase) reacts with H_2O_2 to form the radical species ferrylmyoglobin. A chromogen, 2,2′-azinobis-(ethylbenzothiazolinesulfonate) (ABTS), is incubated with the ferrylmyoglobin to produce the radical cation species $ABTS^{\bullet+}$. This has a relatively stable blue-green color, which is measured at 600 nm.

$$HX\text{-}Fe^{3+} + H_2O_2 \rightarrow {}^{\bullet}X\text{-}[Fe^{4+}{=}O] + H_2O$$

$$H_2\text{-}ABTS + {}^{\bullet}X\text{-}[Fe^{4+}{=}O] \rightarrow ABTS^{\bullet+} + HX\text{-}Fe^{3+} + H_2O$$

$HX\text{-}Fe^{3+}$ = metmyoglobin, ${}^{\bullet}X\text{-}[Fe^{4+}{=}O]$ = ferrylmyoglobin, and $ABTS^{\bullet+} + HX\text{-}Fe^{3+}$ = blue-green radical cation.

Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentration. The assay is calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a synthetic form of vitamin E, and results are expressed in mmol/L (TAS units) with a range of 0−2.5 mmol/L.

Assays were performed at 37 °C using the Cobas Fara Bio centrifugal analyzer (Roche Analytical Instruments, Nutley, NJ). This analyzer permits simultaneous monitoring of absorbance in the ultraviolet/visible range of 29 samples and one blank at time intervals as short as 10 s and was ideally suited for following the kinetics of $ABTS^{\bullet+}$ generation. The TAS kit has four components. (1) Buffer: phosphate-buffered saline, 5 mmol/L, pH = 7.4. (2) Lyophilized chromogen: ABTS (610 μmol/L) and metmyoglobin (6.1 μmol/L). (3) Substrate: hydrogen peroxide (250 μmol/L), stabilized. (4) Lyophilized Trolox: (2.50 mmol/L).

The chromogen was reconstituted with 10 mL of buffer, and the standard with 1 mL of deionized water. The Cobas Fara automatically adds 5 μL of sample or standard to 250 μL of substrate and 20 μL of buffered chromogen (total volume 305 μL), by transfer to a set of disposable cuvettes positioned at the periphery of the centrifugal rotor which passes continuously through the light beam set at a wavelength of 600 nm. Absorbance of all 30 cuvettes is automatically recorded at 10 s intervals for 3 min, and the ΔE_{600}/min is calculated over the most linear portion of the progress curve for each sample and converted to mmol/L by reference to the data obtained with three standards in each run. All samples with concentrations greater than 2.5 mmol/L TAS were diluted and reassayed. All red wine samples required a 10-fold dilution with deionized water.

Assay of Polyphenols. Two assay systems were utilized in this investigation. Gas chromatography coupled to mass selective detection (Soleas *et al.*, 1997a) was employed to measure the following polyphenols: gentisic acid, vanillic acid, *m*-coumaric acid, *p*-coumaric acid, gallic acid, caffeic acid, ferulic acid, *cis*-resveratrol, *trans*-resveratrol, epicatechin, catechin, quercetin, *cis*-polydatin, and *trans*-polydatin. In brief, 1 mL of wine diluted 50:50 in water was passed through a Sep-Pak C_8 column and the polyphenols were eluted with 3 mL of ethyl acetate. The eluate was evaporated to dryness and derivatized with 1 mL of 1:1 BSTFA/pyridine at 70 °C for 30 min; 1 μL of the extract was then injected into a MSD-5970. A DB-5HT capillary column temperature-programmed from 80 to 320 °C was used to separate the polyphenol peaks, which were quantitated by selective ion monitoring employing one target and two qualifying ions for each compound. Fisetin served as the internal standard. High-performance liquid chromatography with diode array detection (Goldberg *et al.*, 1996) was used to measure the following: isoquercitrin, syringic acid, and myricetin. All analyses were completed within 24 h of opening the bottles of wine. The relevant data for the concentrations of these constituents in the wines analyzed in this study have already been reported *in extenso* (Soleas *et al.*, 1997b).

Statistical Methods. Determination of univariate and pairwise Pearson correlation, smoothing (Hastie and Tibshirani, 1990), and regression and model selection using Mallow's coefficient $C(p)$ (Sen and Srivastava, 1990) were performed by SAS, Version 6.03 software/Windows release 6.11 (SAS Institute Inc., Cary, NC).

RESULTS

TAS of Individual Phenolics in Pure Solution. The TAS of each of the polyphenols included in this study was measured at four to six concentrations prepared by diluting the pure standard in the appropriate solvent. The standards and solvents were made up as described in Table 1. *cis*-Resveratrol was prepared from the *trans* isomer by UV irradiation (Goldberg *et al.*, 1995a). *trans*-Polydatin was isolated from the dried roots of *Polygonum cuspidatum*, and a portion was converted to the *cis* isomer by UV irradiation (Goldberg *et al.*, 1995b). A range of five dilutions (0−0.5 mmol/L) were used to measure TAS for each polyphenol, and the line relating TAS to concentration was derived by least-squares analysis of the data, examples being given in Figure 1. From the midpoint of each line, the TAS/mmol of compound was calculated. The data, presented in Table 2, show that on a molar basis *trans*-polydatin, epicatechin, isoquercitrin, quercetin, and myricetin had the highest TAS whereas vanillic acid, gentisic acid, syringic acid, ascorbic acid, and α-tocopherol had the lowest.

TAS of Ontario Wines. Thirty-two Ontario wines from various producers were analyzed for their individual polyphenol concentrations and their TAS. The following cultivars were included in this survey: Cab-

Table 1. Source of Phenolic Standards and Dissolving Solvent(s)

compound	supplier	stock std concn (mg/L)	dissolving solvent (v/v)
caffeic acid	Sigma[a]	1013	80% ethyl acetate/20% acetone
(+)-catechin	Sigma	1012	ethyl acetate
m-coumaric acid	Sigma	1076	95% ethyl acetate/5% acetone
p-coumaric acid	Sigma	1013	95% ethyl acetate/5% acetone
(−)-epicatechin	Sigma	1000	20% ethanol/80% acetone
ferulic acid	Sigma	1072	95% ethyl acetate/5% acetone
fisetin	Aldrich[b]	1044	40% acetone/60% ethyl acetate
gallic acid	Sigma	1004	95% ethyl acetate/5% acetone
gentisic acid	Lancaster[c]	1163	ethyl acetate
isoquercitrin	Roth[d]	210	absolute ethanol
myricetin	Sigma	406	absolute ethanol
quercetin	Sigma	1004	methanol
trans-resveratrol	Sigma	4802	absolute ethanol
syringic acid	Sigma	1038	absolute ethanol
vanillic acid	Sigma	1032	95% ethyl acetate/5% acetone

[a] Sigma-Aldrich Canada, Ltd, Mississauga, ON, Canada. [b] Aldrich Chemical Company, Inc., Milwaukee, WI. [c] Lancaster Synthesis Inc., Windham, NH. [d] Roth, Germany.

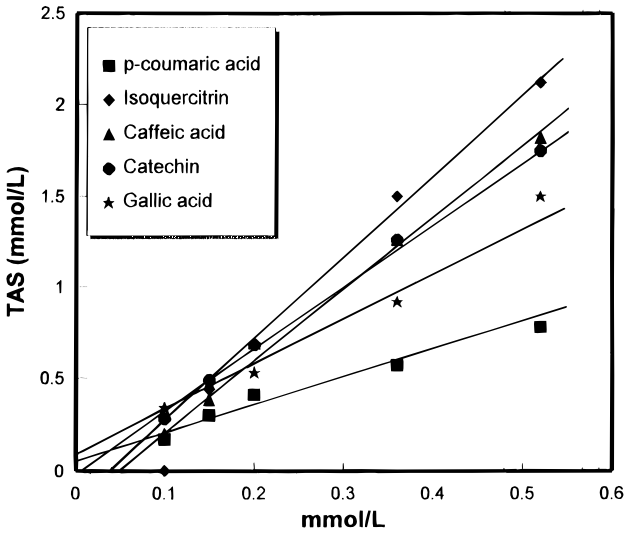

Figure 1. TAS of wine phenolic constituents.

Table 2. TAS of Individual Phenolic Constituents at a Fixed Concentration of 1.0 mmol/L Derived from Calibration Curves of the Type Exhibited in Figure 1

compound	TAS (mmol/L)
trans-polydatin[a]	62.16
(−)-epicatechin	4.96
isoquercitrin	4.24
quercetin	4.24
myricetin	4.04
caffeic acid	3.64
(+)-catechin	3.50
gallic acid	3.00
trans-resveratrol	2.54
ferulic acid	1.84
p-coumaric acid	1.56
vanillic acid	0.92
gentisic acid	0.88
syringic acid	0.76
ascorbic acid	0.76
α-tocopherol	0.90

[a] The TAS for this compound was derived from five concentrations over the range 0.01−0.05 mmol/L, and the values were extrapolated to be consistent with the concentrations used for the other antioxidants as given in this table.

ernet Franc ($n = 7$); Cabernet Sauvignon ($n = 9$); Gamay Noir ($n = 5$); Merlot ($n = 5$); Pinot Noir ($n = 6$) (Table 3). TAS was in the range 6.6−28.6 mmol/L. There was no significant difference in the mean TAS between all the red wines analyzed with the exception of Gamay Noir, where TAS was approximately 50% that of all other red wines tested ($p < 0.05$).

Table 3. Ontario Red Wines Utilized To Study the Relationship between Phenolic Content and TAS ($n = 32$)

varietal	vintage	no.	TAS[a]
Cabernet Franc	1991	1	12.5
	1993	1	18.2
	1994	5	15.4
Cabernet Sauvignon	1993	3	16.8
	1994	6	16.1
Gamay Noir	1993	2	7.5
	1994	3	8.8
Merlot	1993	2	14.2
	1994	3	14.4
Pinot Noir	1991	1	28.6
	1992	1	14.7
	1993	1	9.9
	1994	2	19.3
	1995	1	12.5

[a] Individual values as mmol/L, or mean where more than one sample was analysed

Table 4. Pearson Product Moment Correlation Coefficient for Individual Red Wine Phenolics and TAS Where Value Was Statistically Significant

constituent	*r*	*P*
vanillic acid	0.71	<0.00l
gallic acid	0.62	<0.001
catechin	0.55	0.001
trans-polydatin	0.48	0.005
syringic acid	0.41	0.017
epicatechin	0.37	0.035
quercetin	0.35	0.04

TAS Statistical Analyses for Individual Phenolics in Wine Samples. Multiple regression analyses were performed with TAS as the dependent variable and up to 18 phenolic constituents of wine as the independent variables. The Pearson product moment correlation analysis revealed significant coefficients for seven of these constituents (Table 4), the highest being given by vanillic and gallic acids.

This univariate correlation analysis was followed by pairwise correlations between these constituents; significant relationships ($p < 0.05$) are summarized in Table 5. Positive associations between *cis*- and *trans*-resveratrol, as well as between catechin and epicatechin, were in line with expectations, but there was no relationship between the polydatin isomers, or between quercetin and isoquercitrin. Interestingly, catechin and epicatechin were both significantly correlated with the two polydatin isomers. In a number of instances, significant negative correlations were observed, as between myricetin and epicatechin ($r = -0.39$), gentisic acid and *cis*-polydatin ($r = -0.42$), gentisic acid and

Table 5. Pairwise Pearson Product Moment Correlation Coefficients Relating Various Red Wine Phenolics

	syringic acid	*cis*-resv	*trans*-resv	epicatch	catechin	querct	polydatin *cis*	polydatin *trans*	myricetin	isoquerct	caffeic acid	vanillic acid	gentisic acid	coumaric acid meta	coumaric acid para	gallic acid	ferulic acid
syringic acid	1.00											0.48		−0.41			
cis-resveratrol		1.00	0.58						0.44		0.41				0.59		0.57
trans-resveratrol		0.58	1.00														
epicatechin				1.00	0.92		0.34	0.37	−0.39			0.44				0.42	
catechin				0.92	1.00		0.43	0.37				0.53				0.50	
quercetin						1.00			0.51								
cis-polydatin				0.34	0.43		1.00						−0.42				
trans-polydatin				0.37	0.37			1.00								0.51	−0.37
myricetin		0.44		−0.39		0.51			1.00		0.56				0.57		0.43
isoquercitrin										1.00			−0.41				−0.37
caffeic acid		0.41							0.56		1.00				0.76		0.47
vanillic acid	0.48			0.44	0.53							1.0				0.63	
gentisic acid							−0.42			−0.41			1.00				
m-coumaric acid	−0.41													1.00			
p-coumaric acid		0.59							0.57		0.76				1.00		0.66
gallic acid				0.42	0.50			0.51				0.63				1.00	
ferulic acid		0.57						−0.37	0.43	−0.37	0.47				0.66		1.00

Figure 2. All 18 predictors in a linear model.

isoquercitrin ($r = -0.41$), and syringic acid and *m*-coumaric acid ($r = -0.41$), among others. These negative correlations may be accounted for by competition among precursors for different final pathways of biosynthesis. The correlations between catechin, epicatechin, and the polydatin isomers may reflect similarity in the response of these constituents to stimuli such as sunlight or enological variables such as intensity and duration of skin contact during fermentation. Using all 18 predictors in a linear model enabled TAS to be predicted with a very high correlation to that actually observed in the individual wines (Figure 2).

The full linear model relating the compositional data to TAS can be written as

$$\text{TAS} = b_0 + b_1X_1 + b_2X_2 + \ldots b_{18}X_{18}$$

where $X_1, \ldots, X_{18}$ are the measurements of the phenolics (mmol/L) and $b_1, \ldots, b_{18}$ are the coefficients describing the contribution of the respective phenolics to TAS. If a coefficient for a given phenolic was statistically significantly different from 0, then that phenolic was deemed to make a contribution to TAS.

Contribution of Individual Phenolics to TAS. Further statistical analyses were conducted to reduce the number of variables required for prediction of wine TAS. Model selection was based upon calculation of Mallow's $C(p)$, defined as

$$C(p) = \text{RSS}(p)/s^2 - n + 2p$$

where p is the number of predictors, RSS(p) is the residual sum of squares, s^2 is an estimate of σ^2 in the regression model, and n is the total sample size.

The first model to be tested involved a stepwise selection process starting with calculation of $C(p)$ for each of the 18 subsequent individual models resulting from dropping a single term. The model with the lowest $C(p)$ was chosen as the new model, whereupon candidate models based on dropping one term at a time were chosen on the basis of reduction in the value of $C(p)$. From then on, candidate models were developed by dropping a term in the model or adding a term previously omitted. The final model (model 1) was selected

Table 6. Best Stepwise Model (Model 1) Relating Individual Red Wine Phenolics to TAS Utilizing Mallow's $C(p)$ (r^2 = 0.862)

variable	coefficient	SE[a]	t	P
intercept	1.295	1.481	0.87	0.39
trans-resveratrol	−0.147	0.070	2.10	0.047
epicatechin	−0.048	0.012	4.05	<0.001
catechin	0.026	0.006	4.64	<0.001
quercetin	0.162	0.046	3.52	0.002
cis-polydatin	−0.123	0.046	2.68	0.013
trans-polydatin	0.149	0.031	4.75	<0.001
vanillic acid	0.406	0.086	4.75	<0.001
m-coumaric acid	0.688	0.157	4.38	<0.001

[a] SE = standard error.

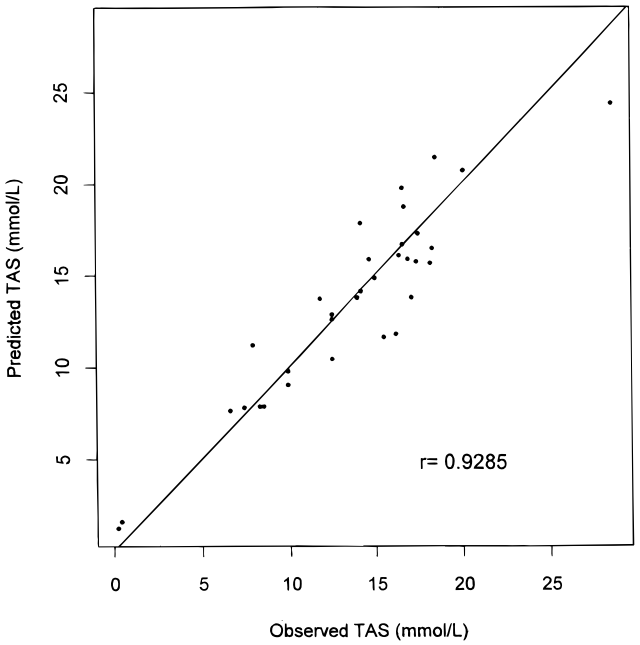

Figure 3. Stepwise selection: the best linear model (1).

Table 7. Best Stepwise Model (Model 2) Relating Individual Red Wine Phenolics to TAS Utilizing Only Statistical Significance of Coefficients (r^2 = 0.676)

variable	parameter estimate	SE[a]	F	P
intercept	2.374	1.720	1.91	0.178
quercetin	0.157	0.054	8.51	0.007
trans-polydatin	0.119	0.041	8.63	0.006
vanillic acid	0.508	0.100	25.71	<0.001

[a] SE = standard error.

on the basis that neither omission nor addition of a single term reduced $C(p)$ further. The constituent polyphenols contributing to this final model which gave a very high value for r^2 (0.862) are exhibited in Table 6. Graphical checks showed no obvious departures from the assumptions of linear regression, and there was excellent correlation between the observed and predicted values of TAS (Figure 3).

Other methods of selection were attempted. Starting with the full model and basing the stepwise selection process only on statistical significance of coefficients instead of $C(p)$, a model (model 2) based upon 3 variables with $r^2 = 0.676$ was derived and is illustrated in Table 7. In another approach, we started with the full model and eventually deleted terms that were not statistically significant (model 3), terminating with a function yielding a value for r^2 of 0.770 and illustrated in Table 8. Finally, starting with no terms and sequentially adding only those that were statistically significant, the same model as presented in Table 7 was obtained. In summary, model 1 provides the most complete description with the highest value for r^2. The other methods discover most of the variables identified by this preferred method, but not *cis*-polydatin or *trans*-resveratrol; furthermore, they do not discover any variables not revealed by model 1, thus providing confidence that the stepwise-selected model is close to optimum.

Table 8. Sequential Deletion from Full Model (Model 3) of Terms That Are Not Statistically Significant (r^2 = 0.770)

variable	parameter estimate	SE[a]	F	P
intercept	0.724	1.651	0.19	0.664
epicatechin	−0.056	0.013	17.91	<0.001
catechin	0.026	0.006	19.06	<0.001
trans-polydatin	0.120	0.037	10.60	0.003
vanillic acid	0.491	0.100	24.02	<0.001
m-coumaric acid	0.573	0.184	9.68	0.004

[a] SE = standard error.

Further Analysis of the Model. With reduction in the number of predictors to 8, it was computationally possible to look for nonlinear relationships by replacing each of the linear terms with a 4 degree-of-freedom smoothing spline. This is a smooth curve placed through the data that acts as a local average. An algorithm was employed to enter each variable linearly or as a smooth term, with calculation of impact upon the $C(p)$. This procedure demonstrated that only *trans*-polydatin was nonlinear ($p = 0.006$). With this substitution, the r^2 for the model, illustrated in Figure 4 (model 4), increased to 0.923. This was higher than the value of $r^2 = 0.901$ which was derived from the initial computation utilizing all 18 variables in a linear model and prior to commencing the modeling process (Figure 2). Thus, 96% of the TAS can be predicted by the content of catechin, *m*-coumaric acid, epicatechin, *cis*-polydatin, *trans*-polydatin, quercetin, *trans*-resveratrol, and vanillic acid present in the wines analyzed. The remaining constituents (caffeic acid, *p*-coumaric acid, ferulic acid, gentisic acid, isoquercitrin, myricetin, *cis*-resveratrol) do not seem to contribute significantly to TAS, nor do they show significant correlation with TAS on univariate analysis. Although gallic and syringic acids are significantly correlated with TAS (Table 4), they do not contribute to a statistical description of this parameter beyond or to the extent of the 8 variables already identified.

DISCUSSION

Despite their growing importance in human nutrition (Willet, 1994) and their apparent association with reduced CHD mortality (Hertog *et al.*, 1993; Criqui and Ringel, 1994), surprisingly little is known about the quantitative content of biologically active polyphenols in different components of the human diet, although

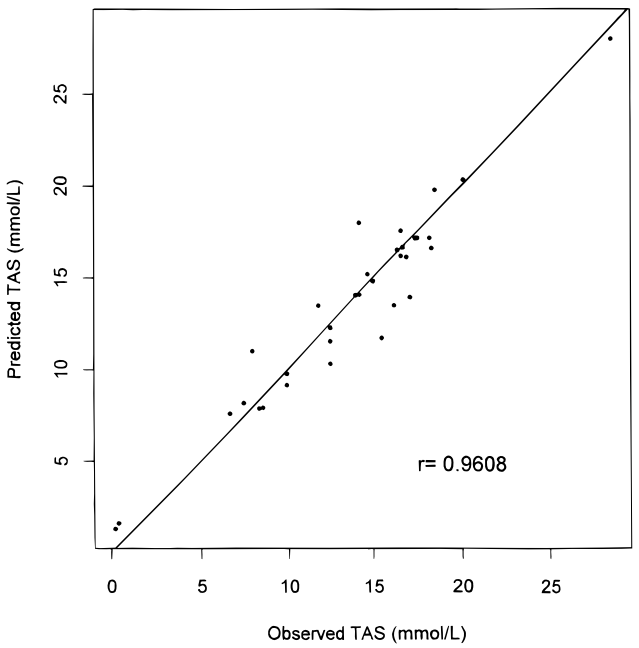

Figure 4. Addition of a nonlinear term: the best model (4).

sporadic and anecdotal information on many plants, often of a qualitative nature, has been published (Namiki, 1990; Ho *et al.*, 1991). An exception is the work of Hertog and associates in the Netherlands. These investigators measured the content of five antioxidants in a large series of fruits and vegetables (Hertog *et al.*, 1992), as well as in wines, teas, and fruit juices (Hertog *et al.*, 1993). Several papers have provided data on the concentration of polyphenolic antioxidants in a range of commercial wines (Roggero *et al.*, 1990; Lamuela-Raventos and Waterhouse, 1994; Goldberg *et al.*, 1996; Frankel *et al.*, 1995), and a broad picture is beginning to emerge about the relative quantitative importance of the various compounds in wine generally, as well as their variance with respect to region, vintage, climate, and cultivar. However, these properties do not indicate their functional significance or their contribution to the actual antioxidant activity of individual wines. Many investigators have examined the structure−function relationships for antioxidant activity of individual wine polyphenols (see Soleas and Goldberg (1997) for review), but to our knowledge this is the first attempt to do so in whole wine as well as to identify those that are most predictive within the framework of a rigorous statistical model.

The most fundamental work in this area to date has come from the group at the University of California, Davis. Beginning with their demonstration of the ability of total wine phenolics to block the copper-mediated oxidation of LDL isolated from healthy human subjects (Frankel *et al.*, 1993), they subsequently compared the relative antioxidant activities of a number of pure phenolic compounds in this system (Frankel *et al.*, 1993) and went on to show that lipid peroxidation by a number of other biological catalysts, including myoglobin and cytochrome *c*, could also be prevented by the total phenols present in grapes and wine (Kanner *et al.*, 1994). Next, they quantitated 11 individual polyphenols in 14 red and 6 white Californian wines and correlated these values with the total antioxidant activity of the wines measured as inhibition of *in vitro* LDL oxidation (Frankel *et al.*, 1995). The highest correlation was found for gallic acid ($r = 0.92$), followed by catechin, myricetin, quercetin, caffeic acid, rutin, epicatechin, cyanidin, malvidin 3-glucoside, and the isomers of resveratrol, respectively, in descending order. Most recently, they fractionated the phenolics of a single wine (Teissedre *et al.*, 1996) and reported that, using the same criterion for antioxidant activity, the dimers of catechin (procyanidins) together with catechin, myricetin, epicatechin, rutin, gallic acid, and quercetin were the most potent, all being at least twice as active as α-tocopherol at a concentration of 5 μmol/L.

It should be kept in mind that inhibition of LDL oxidation of the type measured by these investigators depends upon entry of the antioxidant into the lipid core. Oxidation of the surface apo B protein may be more significant in promoting atherosclerosis because it triggers an immune response (Craig, 1995; Kiener *et al.*, 1995) and inflammatory response (Berliner *et al.*, 1993), and it may also be important in the cytotoxic effects of oxidized LDL (Thorin *et al.*, 1994) as well as induction of smooth muscle proliferation (Auge *et al.*, 1995), all of which contribute to the pathological processes in the arterial subendothelial layers that progress to atherosclerosis. Thus, the antioxidant functions of polyphenols in an aqueous environment are at least equally important in that they are likely to protect the protein component of LDL and to scavenge oxygen free radicals that otherwise may accumulate and initiate inflammatory reactions leading to chronic disease processes, and DNA damage ultimately resulting in cancer. This antioxidant function is measured by the present assay. Further, the antioxidant activity of pure compounds tested in isolation may not necessarily reflect their relative activities when they are present in combination, and in a matrix as chemically complex as wine. It should be emphasized that free-radical generation from H_2O_2 has been implicated as an important etiologic mechanism in inflammatory bowel disease and possibly in related conditions (McKenzie *et al.*, 1996; Jijon and Parsons, 1997), with antioxidants abrogating this H_2O_2-mediated damage. The present TAS assay thus represents a biologically meaningful determination of antioxidant activity.

Although useful insights can be derived from single correlations of the type calculated by Frankel *et al.* (1995), a more complete description requires the multiple regression techniques utilized in this paper. It is also puzzling that, in an assay similar to that employed by Frankel *et al.* (1995), other investigators reported that white wines averaging 10% of the total phenolic content of red wines were more effective than the latter in inhibiting *in vitro* LDL oxidation (Vinson and Hontz, 1995). Moreover, it appears from Table 2 of the paper by Teissedre *et al.* (1996) that, unlike the present assay, which gives excellent linearity with aqueous samples although certain problems have been noted in human blood due to enzyme interference (Schofield and Braganza, 1996), the method of Frankel *et al.* (1995) shows serious departure from linearity.

The utility of combining these approaches is well illustrated by the results now presented. When pure solutions of the individual compounds were employed, the highest antioxidant status was demonstrated by *trans*-polydatin (Table 2). On the basis of single analysis of each phenolic in wine matrix, only seven were significantly correlated with TAS of the wine sample, the highest values for *r* being given by vanillic and gallic acids (Table 4). With statistical modeling utilizing both linear and nonlinear approaches to predict the TAS of wine samples from their polyphenol content, the best

results were obtained with only eight compounds (Table 6), of which only four appear in Table 4, gallic acid being notably absent, arguably because it is highly correlated with four of the variables already included (catechin, epicatechin, *trans*-polydatin, and vanillic acid, Table 5) and therefore does not add further unique information.

One puzzling feature is the presence of negative terms in some of these equations, especially model 1 (Table 6). In some respects, this should come as no surprise given the fact that there are many significant inverse correlations among these polyphenols in wine samples as listed in Table 5, but their meaning and causation require explanation. One thought was that there may be technical interference (or inhibition) of antioxidant activity in general, or in this particular assay, between some of these phenolics. To test this notion, myricetin and epicatechin, which showed a high inverse correlation ($r = -0.39$), were added individually and conjointly to a wine sample, and TAS recovery was calculated. No negative interference between the two compounds was evident, although a more extensive study would be required to rule out this possibility for all pairs demonstrating an inverse correlation.

Another explanation may be the following: TAS probably reflects the overall distribution of polyphenols in the wine, and this is affected by cultivar, climate, region, and many enological processes (Soleas *et al*., 1995; Jeandet *et al*., 1995; Kovac *et al*., 1995). Some of the latter such as fining and oak-aging are likely to influence certain phenolics in a similar direction and promote positive correlation between them. Other environmental factors such as sunlight and botrytis infection (Fritzemeier and Kindl, 1981; Price *et al*., 1995; Jeandet *et al*., 1995), as well as intrinsic gene expression (Fritzemeier and Kindl, 1981), promote or retard the flux of metabolites through various pathways including the shikimate (Hrazdina *et al*., 1984), cinnamate (Fritzemeier and Kindl, 1981), chalcone (Kreuzaler and Hahlbrock, 1975), and stilbene (Melchior and Kindl, 1991) pathways by modulating the activity of enzymes at key branch points. Compounds synthesized by these various pathways are likely to show parallel changes with others within the same pathway but not necessarily with those of other pathways, unless the two pathways are affected in the same way. Opposite effects on two pathways are likely to lead to inversely related concentrations of their products in grape and wine, as seen for *trans*-polydatin and ferrulic acid ($r = -0.37$). However, stimulation of one pathway may lead to competition for rate-limiting enzymes or substrates by metabolites common to this pathway based on different affinity constants, so that the production of one compound is favored over that of another. This inequality of competition may explain the inverse correlation between myricetin and epicatechin (−0.39), which share many steps in their biosynthesis. Although speculative, these proposals represent a challenge to plant biochemists which are amenable to investigation.

When the TAS values attributed to the individual phenolics derived from standard curves such as those presented in Figure 1 were summed for all 17 constituents, the values were in the range of 19−54% of the TAS values measured in the Randox assay, with one example being as high as 82%. Clearly, there are important antioxidants present in wine such as gallate esters of the catechins (Salah *et al.*, 1995) and the procyanidins that are produced by the biosynthetic polymerization of catechins (Da Silva *et al.*, 1991). It appears that, as a consequence of the multiple inter-relationships between these polyphenols, including their biosynthesis from common metabolic pathways and precursor−product status as already discussed, the actual assay of relatively few taken together with the statistically derived coefficients utilized in our models can *predict* the total antioxidant status of wine samples. The corollary of this outcome is that TAS as measured in the Randox assay is an accurate reflection of the antioxidant activity of mixtures of polyphenols in aquous media since there is excellent agreement between the activity as measured and that predicted on the basis of the chemical composition of the mixture.

In parallel with other contemporary efforts to define and characterize the antioxidant activity of polyphenols in general (Elford and Van't Riet, 1991; Salah *et al.*, 1995; Tillement *et al.*, 1996) and those of plants and vegetables in particular (Chen and Ho, 1995; Vinson *et al.*, 1995; Rice-Evans *et al.*, 1995; Rice-Evans, 1995), identification of those phenolics which are most important in endowing wine with its antioxidant activity should help to focus attention on these compounds as especially worthy of enrichment during the wine-making process. It will be necessary to extend the present study beyond the narrow regional confines to which it was restricted to ensure that the conclusions are generally applicable. Other mathematical treatments such as principle components analysis used to classify grape varietals on the basis of their most characteristic polyphenolic constituent (Mattivi *et al.*, 1996) can also be employed in these endeavors. The quantitative relationship between wine phenolics and antioxidant activity, which can now be explored by means of recent technological advances, is a valid quest in the endeavor to bring to the consuming public products with the best potential for improved health.

ABBREVIATIONS USED

HDL, high-density lipoprotein; LDL, low-density lipoprotein; CHD, coronary heart disease; TAS, total antioxidant status.

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