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Unravelling the Total Antioxidant Capacity of Pinotage Wines: Contribution of Phenolic Compounds

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The total antioxidant capacity (TAC) and phenolic composition of 139 Pinotage wines (2002 and 2003 vintages) were determined using the 2,2′-azino-di(3-ethylbenzo-thialozine-sulfonic acid) scavenging assay and high-performance liquid chromatography, respectively. The contribution of individually quantified phenolic compounds to the wine TAC was calculated using their concentrations and Trolox equivalent antioxidant capacity (TEAC) values. The TEAC values of quercetin-3-galactoside, isorhamnetin, and peonidin-3-glucoside are reported for the first time. Between 11 and 24% of the measured TAC of Pinotage wines was explained by the sum of the calculated contributions of their quantified phenolic compounds comprising monomeric phenolic compounds and procyanidin B1. Ultrafiltration was carried out to attempt separation of monomeric and polymeric phenolic compounds. Analysis of ultrafiltration permeates and retentates enabled estimation of the TAC contribution of large molecular weight (MW) unknown compounds (46%) (>50 kDa), including oligomeric and polymeric phenolic compounds and small MW unknown compounds (34%) (<50 kDa). Three mixtures, containing 12 phenolic compounds in typical concentrations expected in Pinotage wines, exhibited 16−23% synergistic antioxidant activity. This suggests that synergy between phenolic compounds does play a role in the wine TAC but that it does not explain the large discrepancy between measured and calculated TAC values.

KEYWORDS: ABTS radical cation; antioxidant; free radical scavenging; HPLC; monomeric phenolic compounds; Pinotage; polymeric phenolic compounds; red wine; synergy; total antioxidant capacity; ultrafiltration

INTRODUCTION

Enhancement of red wine antioxidant capacity, while retaining sensory quality, is a challenge facing the wine industry. An increasing phenolic concentration will increase the antioxidant capacity of wines but can also negatively affect their sensory qualities. A wide range of variables such as cultivar, viticultural practices, and vinification techniques can affect the phenolic composition of red wines. A recent study reported that the unique South African cultivar wine, Pinotage (1998 vintage commercial wines), had an average total antioxidant capacity (TAC) of 15.3 mM Trolox equivalents (TEs) as measured using the 2,2′-azino-di(3-ethylbenzo-thialozine-sulfonic acid) ($ABTS^{\bullet+}$) scavenging assay (*1*). This was comparable with that of other commercial cultivar wines of the same vintage produced in South Africa. Other studies (*2*−*5*) highlighted the unique phenolic composition of Pinotage wines, especially with regard to very high caffeoyltartaric or caffeic acid levels.

The $ABTS^{\bullet+}$ scavenging assay offers an easy and rapid method to screen large numbers of samples. The contribution of individual compounds with regard to the TAC of antioxidant mixtures, such as wine, is important especially where optimization of TAC is a goal. Previous studies on wine estimated the importance of individual compounds by determining their correlation with the TAC (*6*−*8*). Such an approach uses statistical correlations to indicate whether a compound has a relationship with the TAC. Although this gives valuable information, correlations do not prove a causal relationship between the content of a specific compound and the TAC, nor do they give an indication of the relative contributions of individual compounds to the TAC of a complex mixture. Soleas et al. (*6*) reported that 96% of wine TAC could be predicted using only eight individual monomeric phenolic compounds based on a linear multiple regression model.

A different approach is to use the content and the antioxidant potency of individual compounds to calculate their contribution to the TAC (*9*−*11*). Rice-Evans et al. (*11*) found that only 25% of the TAC of a red wine could be estimated from 10 quantified phenolic compounds. The antioxidant potency of many wine

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phenolic compounds in terms of their Trolox equivalent antioxidant capacity (TEAC) values has been reported previously (*12−17*). Whereas these values are valuable in determining the relative importance of the respective compounds, published data cannot be used to calculate the contribution of individual compounds to the TAC of a specific wine. Differences in the protocols and calculation methods will lead to different TEAC values for the respective phenolic compounds. TEAC values of pure reference standards should, therefore, be measured using the same assay protocol as used for determining the TAC of the wines.

Polymeric phenolic compounds, present in wine at levels between 65 and 85% of the total phenolic content, when measured using normal phase high-performance liquid chromatography (HPLC), depending on its age and origin (*18*), may represent a sizable portion of the TAC of wine. TEAC values for polymeric compounds in the wines can, however, not be determined, although proanthocyanidin oligomers up to six units have a higher antioxidant activity than their monomeric counterparts (*19−22*). Ultrafiltration may be used to separate the monomeric and polymeric phenolic compounds in wine in order to estimate the TAC contribution of the large molecular weight (MW) compounds, including polymeric phenolic compounds. However, synergy between phenolic compounds may also possibly influence the TAC of wines (*23−26*).

Knowledge of the antioxidant activity of wine phenolic compounds and their contribution to wine TAC is essential to evaluate which phenolic compounds to manipulate in order to achieve an increase in TAC without detrimental effects to the sensory quality. The aim of the study was to determine the relative contribution of individual phenolic compounds in Pinotage wine to its TAC. This information is needed to prepare guidelines for manipulating the phenolic composition of these wines to obtain increased TAC. The contribution of polymeric phenolic compounds was estimated, and the possible role of synergy between phenolic compounds was investigated.

MATERIALS AND METHODS

Wines. A series of 139 Pinotage wines (63 wines from the 2002 vintage and 76 wines from the 2003 vintage), made from grapes (*Vitis vinifera*) originating from different climatic areas in the Western Cape (South Africa), were selected. Grapes were harvested at approximately 24 °B. The wines were prepared using 20−30 kg of grapes for each wine according to a standard procedure with no wood contact in the experimental cellar of ARC Infruitec-Nietvoorbij (Stellenbosch, South Africa): After crushing, diammoniumfosfate (50 g/HL), SO_2 (50 mg/L), and *Saccharomyces cerevisiae* strain VIN 13 (30 g/HL) were added. Fermentation was carried out at 25 °C, and the cap was punched down three times per day. The skins were separated from the juice using a pneumatic press as soon as the sugar content dropped to <5 °B. Pressed juice was added to the free-run juice and fermented dry at 25 °C. After fermentation, the total SO_2 content was adjusted to 35 mg/L, and 50 g/HL bentonite (ProteaChem, Cape Town, South Africa) was added. The wines were cold-stabilized for 2 weeks at 0 °C, filtered using diatomous earth filter sheets (ProteaChem), sterile-filtered using 0.45 μm nitrocellulose membrane filters (Millipore, Bedford, MA), and bottled in N_2-filled bottles at room temperature, after adjustment of the total SO_2 content to 40 mg/L. The bottled wines were stored at 15 °C for 8 months when aliquots were collected and frozen at −20 °C to prevent further phenolic changes and defrosted immediately before analysis.

Chemicals and Phenolic Reference Standards. ABTS was obtained from Boehringer Mannheim GmbH (Mannheim, Germany), and HPLC grade acetonitrile and phosphoric acid were from Riedel-de Haën (Seelze, Germany) and Fluka (Buchs, Switzerland), respectively. Potassium persulfate ($K_2S_2O_8$) was obtained from Sigma Chemical Co. (St. Louis, MO), and 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) was from Aldrich Chemical Co. (Gillingham, United Kingdom). Phenolic reference standards included gallic acid, (+)-catechin, (−)-epicatechin, quercetin-3-galactoside (gal), and quercetin-3-rhamnoside (rham) from Sigma; caffeoyltartaric acid from Chromadex (Santa Ana, CA); caffeic acid, quercetin, and kaempferol from Fluka; procyanidin B1, quercetin-3-glucoside (glc), and myricetin from Extrasynthese (Genay, France); and delphinidin-3-glc, peonidin-3-glc, petunidin-3-glc, and malvidin-3-glc from Polyphenols AS (Sandnes, Norway). Water used in the experiments was purified and deionized with a Modulab water purification system (Separations, Cape Town, South Africa), except for preparation of HPLC mobile phases where deionized water was further treated using a Milli-Q academic water purifier (Millipore).

HPLC Analysis of Phenolic Composition. The individual phenolic compounds were quantified in duplicate using a reversed-phase HPLC method adapted from Peng et al. (*27*). The HPLC apparatus used was a Waters LC Module I equipped with a Waters 2996 photodiode array detector using Millenium[32] version 4.0 software (Waters, Milford, MA). Separation was achieved on a PRP1 column (250 mm × 4.1 mm, 100 Å pore size, 5 μm particle size) from Hamilton (Reno, NV). A guard cartridge (20 mm × 2.3 mm) packed with the same material and a PEEK PAT frit (5 μm) were used to protect the analytical column. Wines were filtered using 0.45 μm Millex-HV hydrophilic PVDF 33 mm syringe-tip filter devices (Millipore) before automated duplicate injections of 20 μL each. The column was held at 30 °C during the run, and the flow rate was 0.9 mL/min. Data were obtained in the wavelength range of 250−600 nm. The mobile phases used were 1.5% (v/v) aqueous phosphoric acid (A) and 1.5% (v/v) phosphoric acid in acetonitrile/water (80/20) (B). The gradient program was as follows: from 94 to 69% solvent A in the first 73 min, reduced from 69 to 38% from 73 to 78 min, held isocratic at 38% from 78 to 86 min, increased from 38 to 94% from 86 to 90 min, and equilibration at 94% for 20 min.

Compounds were identified by comparison of their retention times and spectral data to those of pure reference standards except in the following cases: The anthocyanin acetate (ac) esters, malvidin-3-glc-coumarate (malvidin-3-glc-coum), and vitisin A were identified from their spectra, which are similar to that of malvidin-3-glc, and their retention times relative to the other anthocyanin compounds (*27*). *p*-Coumaroyltartaric acid was identified from its spectrum (*28*), which is similar to that of caffeic acid, and its retention time relative to that of caffeic acid. (−)-Epicatechin and procyanidin B2 were not quantified due to coelution with peonidin-3-glc and malvidin-3-glc, respectively. Initially, calibration curves for each phenolic reference standard were set up. For each phenolic compound, the response ratio between it and the representative standard for their phenolic group at the optimal wavelength (gallic acid for benzoic acids at 280 nm; (+)-catechin for flavan-3-ols at 280 nm; caffeic acid for hydroxycinnamic acids and their derivatives at 316 nm; rutin for flavonols at 360 nm; and malvidin-3-glc for anthocyanins at 520 nm) was calculated. For subsequent analyses, each group of analyses was started with calibration standards including gallic acid (5−50 mg/L), (+)-catechin (10−150 mg/L), caffeic acid (5−150 mg/L), rutin (3−100 mg/L), and malvidin-3-glc (10−400 mg/L). The response ratios were used to calculate the content of the phenolic compounds in mg/L in the wines. The anthocyanin ac and coum esters were quantified as mg of the corresponding anthocyanin-3-glc equivalents/L, while *p*-coumaroyltartaric acid was quantified as mg *p*-coumaric acid equivalents/L.

The polymeric content, expressed as mg (+)-catechin equivalents/L, was quantified from the area of the broad peak eluting between 80 and 85 min. Peng et al. (*27*) showed that this peak contains mainly polymeric compounds using ultrafiltration, protein binding, and SO_2 bleaching.

$ABTS^{\bullet+}$ Scavenging Assay. The TAC of wines and the TEAC of phenolic reference standards were determined in triplicate using the $ABTS^{\bullet+}$ scavenging assay (*16*). An ABTS solution (7 mM) in water was preincubated for at least 12 h with 2.45 mM (final concentration) $K_2S_2O_8$ to produce the radical cation. The $ABTS^{\bullet+}$ solution was then diluted with ethanol to an absorbance of approximately 0.7 (±0.02) at 734 nm. In the reaction mixture, 1 mL of $ABTS^{\bullet+}$ solution was added to 50 μL of wine sample (50 times diluted with 10% ethanol), standard

Trolox solution (0−400 μM in ethanol), or 10% ethanol (control) and the absorbance was determined after exactly 4 min of incubation at 37 °C. Spectrophotometric measurements were made in disposable polystyrene 2.5 mL macrocuvettes (Brand Gmbh & Co Kg, Wertheim, Germany) with 1 cm path length using a Beckman DU-65 UV/vis spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The concentration of $ABTS^{•+}$ in the control and samples was calculated using the absorbance readings and the molar extinction coefficient of $ABTS^{•+}$, $\epsilon = 16000$ (*16*). A plot of remaining $ABTS^{•+}$ concentration against the Trolox concentration in the standard samples was used to calculate the TAC of the wines expressed as mM TE.

The TEAC value of a compound is the concentration of Trolox in mM needed to achieve the same amount of $ABTS^{•+}$ scavenging as a 1 mM solution of that compound. A concentration range (4−8 concentrations) of each phenolic reference standard, dissolved and diluted in 10% ethanol, was analyzed using the $ABTS^{•+}$ scavenging assay on two separate days in order to determine their TEAC values. Quercetin-3-gal, quercetin, myricetin, kaempferol, and isorhamnetin were dissolved in dimethyl sulfoxide and diluted with water and 10% ethanol to contain 90% water as for compounds dissolved in 10% ethanol. The slopes of the dose−response curves (concentration vs nmol of $ABTS^{•+}$ scavenged) of the test compounds were compared to that of Trolox to determine the TEAC values (mM):

$$\mathrm{TEAC} = \frac{\text{slope (test compound)}}{\text{slope (Trolox)}} \tag{1}$$

The TAC contribution (mM TE) of individual phenolic compounds to the wine TAC was calculated from their content (mg/L) and TEAC values (mM):

$$\text{TAC contribution} = [\text{compound}] \times \mathrm{TEAC} \tag{2}$$

Ultrafiltration of Wines. Two wines with a similar phenolic composition were selected for ultrafiltration. Ultrafiltration of each wine was performed in duplicate using Vivaspin 4 mL centrifuge devices (Vivascience, Hanover, Germany) with polyethersulfone membranes with nominal MW cutoff of 10, 30, and 50 kDa. Centrifugal ultrafiltration of 2 mL of wine, diluted with 1 mL of 10% ethanol, was performed at a speed of 5000 rpm and a temperature of 20 °C, using a Sorvall RC-3B refrigerated centrifuge (Sorvall Instruments, Newtown, PA) until approximately 100 μL of retentate was left. Then, 1 mL of 10% ethanol was added to the retentate and centrifugation was resumed until approximately 100 μL of retentate was left. Both the pooled permeate and the retentate were diluted to the total volume (4 mL) with 10% ethanol and stored at −20 °C until HPLC and antioxidant analyses. The original wine, diluted (1:1) with 10% ethanol, was also stored at −20 °C and analyzed. The tannin content of ultrafiltration retentates was determined in duplicate using a protein precipitation assay (*29*), to confirm the presence or absence of polymeric phenolic compounds.

Synergy between Phenolic Compounds. Three mixtures containing 12 phenolic compounds in typical concentrations in the range as measured in Pinotage wines in this study were prepared in 10% ethanol and analyzed, using the $ABTS^{•+}$ scavenging assay. The TAC of the mixtures was estimated by calculation using the concentration and TEAC values of the phenolic compounds ($TAC_{calculated}$ in mM) and by measurement with the $ABTS^{•+}$ scavenging assay ($TAC_{measured}$ in mM). The percent synergy was calculated as follows:

$$\%\ \text{synergy} = \frac{\mathrm{TAC_{measured}} - \mathrm{TAC_{calculated}}}{\mathrm{TAC_{calculated}}} \times 100 \tag{3}$$

Statistical Analysis. Statistical analyses were carried out using the SAS version 8 software. Analysis of variance analysis was performed on the means of duplicate ultrafiltration samples, and statistical comparisons between means were made using the Student's *t*-LSD test ($P < 0.05$).

RESULTS AND DISCUSSION

Phenolic Composition of Wines. The phenolic composition of a large selection of Pinotage wines, in terms of 23 monomeric compounds, procyanidin B1 (dimer) (see **Figure 1** for representative chromatograms at different wavelengths and **Figure 2** for compound structures), and their polymeric contents are summarized in **Table 1**. Qualitative and quantitative differences were observed. Compounds such as quercetin-3-gal, myricetin, kaempferol, and isorhamnetin were only detected in some wines. Pinotin A, a reaction product of malvidin-3-glc and caffeic acid, which has recently been isolated and identified in Pinotage wines (*3*, *5*), was not detected in the wines. This is possibly due to relatively low levels of caffeic acid in these wines and the fact that they were very young. Several other monomeric phenolic compounds that have been shown to occur in red wine, such as (−)-epicatechin, flavan-3-ol gallate esters, *S*-glutathionylcaftaric acid, stilbenes, stilbene glcs, and pyranoanthocyanins other than vitisin A, have not been detected and/or quantified. These, and possibly others, could well be present in the Pinotage wines, although not detectable and/or quantifiable using the current HPLC methodology. On the other hand, the polymers quantified should not include other dimers; possibly even trimers may be excluded.

The most abundant phenolic compounds were malvidin-3-glc (115.9−297.9 mg/L) and caffeoyltartaric acid (109.3−260.1 mg/L). Other phenolic compounds occurring in average concentrations of >15 mg/L were procyanidin B1, (+)-catechin, *p*-coumaroyltartaric acid, delphinidin-3-glc, petunidin-3-glc, malvidin-3-glc-ac, and malvidin-3-glc-coum. The polymers were present at an average content of 150.4 mg/L (21.5−274.8 mg/L). The total concentration of the quantified compounds was between 567.8 and 1174.1 mg/L (average = 820.1 mg/L). The polymer content observed was much lower than that reported previously using normal phase HPLC (*18*). The reversed phase method used in the present study only gives an estimation of the relative polymer content as only a 60% recovery of polymeric phenolic content from the column has been reported (*27*).

TEAC of Phenolic Reference Standards. The TEAC values of pure standard compounds were between 0.88 and 2.79 mM (**Table 1**) with kaempferol and gallic acid exhibiting the lowest and highest TEAC values, respectively. *p*-Coumaric acid exhibited no $ABTS^{•+}$ scavenging activity (TEAC < 0.01 mM). The TEAC values of quercetin-3-gal (0.96 mM), isorhamnetin (0.95 mM), and peonidin-3-glc (1.49 mM), measured using the $ABTS^{•+}$ scavenging assay, are reported here for the first time to the best of the authors' knowledge. Gallic acid (2.79 mM) exhibited the highest TEAC value of the phenolic acids due to its vicinal trihydroxyl group. Esterification of caffeic acid with tartaric acid, i.e., caffeoyltartaric acid, caused a slight decrease in TEAC value from 0.98 to 0.90 mM. Among the nonglycosylated flavonols, myricetin (2.67 mM) had the highest TEAC value due to the vicinal trihydroxylation of the B ring. The glycosides of quercetin, namely, quercetin-3-glc (0.92 mM), quercetin-3-rham (0.91 mM), and quercetin-3-gal (0.96 mM), had substantially lower TEAC values than the aglycone (1.75 mM). When considering the anthocyanins, delphinidin-3-glc (2.40 mM) had the highest TEAC value due to its three hydroxyl groups on the B ring. Malvidin-3-glc (1.46 mM), on the other hand, had the lowest TEAC value of the anthocyanins due to having only one hydroxyl group and two electron-withdrawing methoxyl groups on the B ring. The procyanidin dimer B1 [(−)-epicatechin 4$\beta \rightarrow$8 (+)-catechin] exhibited a TEAC value (2.30 mM) slightly higher than would be expected from the doubling in available hydroxyl groups as in (−)-epicatechin (1.25 ± 0.02 mM; data not shown) and (+)-catechin (0.96 mM). Oligomers (2−6 units) have been reported to have higher antioxidant

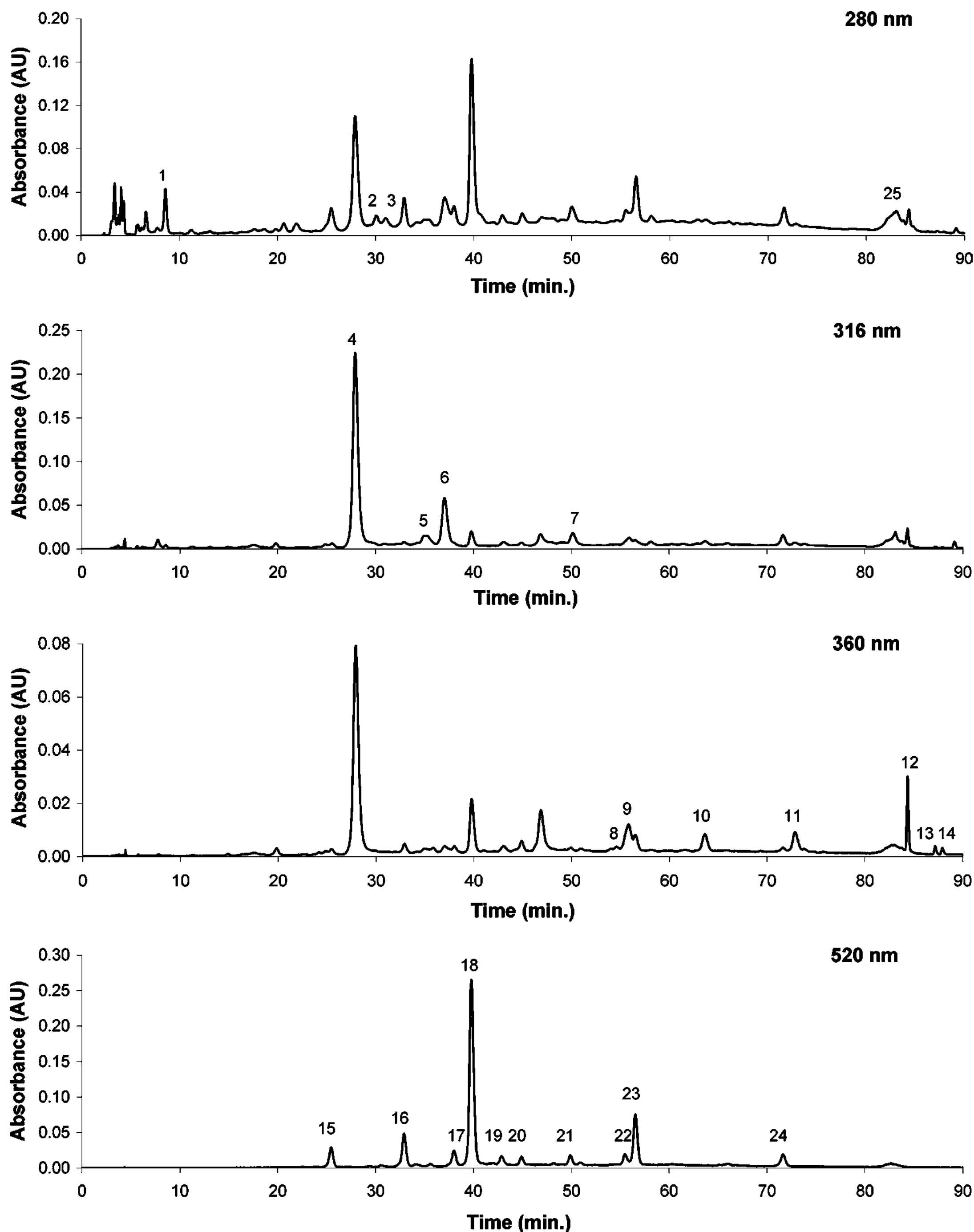


Figure 1. Typical HPLC chromatograms of Pinotage wine recorded at 280, 316, 360, and 520 nm (see **Table 1** for peak identification).

activity in a variety of antioxidant assays than their corresponding monomeric phenols (*19*−*22*). Some authors (*15*, *30*) reported that the antioxidant activity of oligomers per monomer subunit is even higher than that of the respective monomer subunits in the $ABTS^{\bullet+}$ assay. This phenomenon is ascribed to larger areas available for charge delocalization. However, when the degree of polymerization exceeds a critical value, the increased molecular complexity is likely to promote a decrease in antioxidant activity due to steric hindrance reducing the availability of hydroxyl groups (*15*).

TEAC values observed for gallic acid, caffeoyltartaric acid, caffeic acid, and kaempferol are consistent with values reported by Re et al. (*16*) and Baderschneider and Winterhalter (*31*), while TEAC values observed for other compounds are much lower than those reported previously (*12*, *14*−*17*, *31*). It is important to note that published TEAC values also differ between sources. Differences in values observed can be due to differences in radical generation in the presence or absence of the antioxidant molecules (*32*), reaction time (*33*), and reaction medium (*33*−*35*). Differences in calculation methods between authors can also affect the TEAC values. For this reason, the same protocol was used to analyze the wine and phenolic compounds.

Contribution of Individual Phenolic Compounds to TAC of Wine. The measured TAC values for the series of 139 experimental wines varied between 9.04 and 18.89 mM TE (average = 12.84 mM TE) (**Table 1**), which were similar to TAC values [average = 15.29 mM TE; standard deviation (SD) = 2.96 mM TE] previously obtained for commercial Pinotage wines (*1*). The SD for TAC of individual wines was between 0.02 and 0.29 mM TE (average = 0.11 mM TE) corresponding to relative SDs of less than 3% in all cases. The sum of TAC

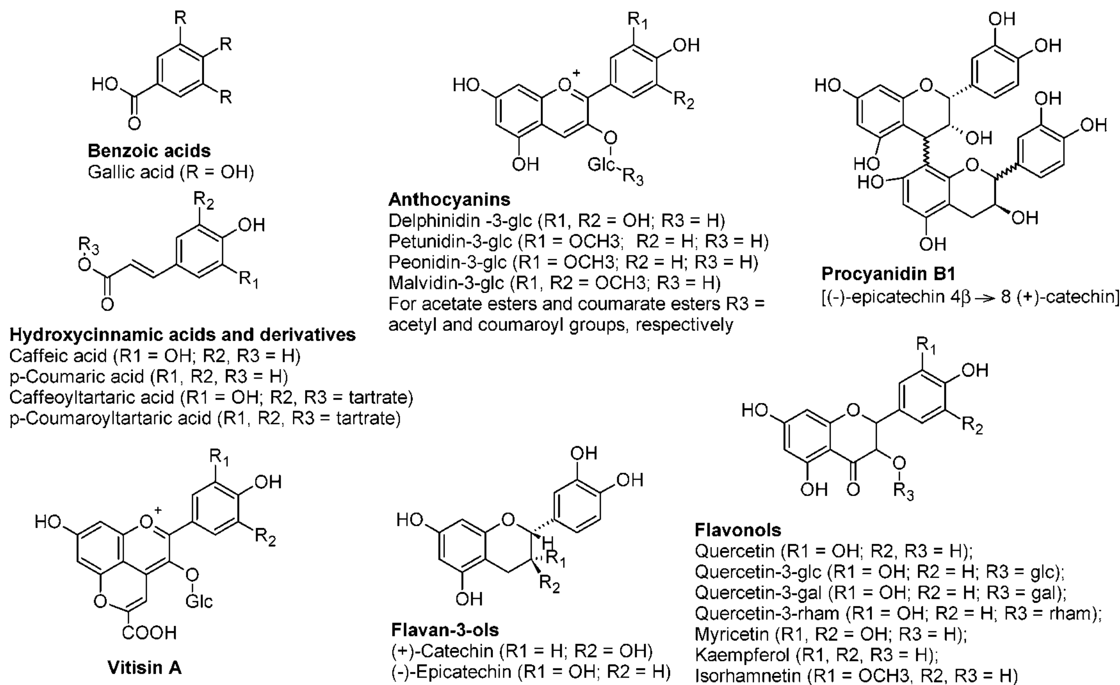


Figure 2. Structures of phenolic compounds identified and quantified in Pinotage wines.

Table 1. Content and TAC Contribution of Individual Phenolic Compounds in Pinotage Wines ($n = 139$)

			content[c]				TAC contribution[d]			
compound	M_r[a]	TEAC[b]	min	max	avg	SD	min	max	avg	SD
1. gallic acid	170.1	2.79 ± 0.04	5.2	43.0	11.9	5.4	0.09	0.70	0.20	0.09
2. procyanidin B1	578.5	2.30 ± 0.06	8.5	59.4	21.6	11.3	0.03	0.24	0.09	0.05
3. (+)-catechin	290.3	0.96 ± 0.05	5.4	39.6	15.1	8.0	0.02	0.13	0.05	0.03
4. caffeoyltartaric acid	312.2	0.90 ± 0.02	109.3	260.1	178.2	31.4	0.32	0.75	0.51	0.09
5. caffeic acid	180.2	0.98 ± 0.08	ND	10.2	3.4	2.6	0.00	0.06	0.02	0.01
6. *p*-coumaroyltartaric acid[e]	296.2	NA	10.5	27.6	17.2	3.5	NA	NA	NA	NA
7. *p*-coumaric acid	164.25	<0.01	0.3	5.7	1.7	1.1	*f*	*f*	*f*	*f*
8. quercetin-3-gal	464.4	0.96 ± 0.01	ND	4.8	2.9	0.9	0.00	0.01	0.01	0.00
9. quercetin-3-glc	464.4	0.92 ± 0.04	5.8	38.3	14.2	5.4	0.01	0.08	0.03	0.01
10. quercetin-3-rham	448.4	0.91 ± 0.03	3.9	16.2	8.8	2.4	0.01	0.03	0.02	0.01
11. myricetin	318.2	2.67 ± 0.22	ND	8.2	2.6	1.7	0.00	0.07	0.02	0.01
12. quercetin	302.2	1.75 ± 0.04	0.6	10.7	3.9	1.9	0.00	0.06	0.02	0.01
13. kaempferol	286.2	0.88 ± 0.07	ND	2.4	0.8	0.4	0.00	0.01	0.00	0.00
14. isorhamnetin	316.3	0.95 ± 0.02	ND	1.3	0.6	0.3	0.00	0.00	0.00	0.00
15. delphinidin-3-glc	465.4	2.40 ± 0.02	5.1	27.3	15.0	5.0	0.02	0.13	0.07	0.02
16. petunidin-3-glc	479.4	2.06 ± 0.23	11.3	35.6	22.6	5.2	0.05	0.14	0.09	0.02
17. peonidin-3-glc	463.4	1.49 ± 0.09	0.9	17.1	7.5	3.5	0.00	0.05	0.02	0.01
18. malvidin-3-glc	493.4	1.46 ± 0.03	115.9	297.9	221.3	31.5	0.32	0.82	0.61	0.09
19. delphinidin-3-glc-ac[h]		NA	1.9	10.1	5.3	1.7	0.01	0.05	0.03	0.01
20. vitisin A[h]		NA	0.5	14.7	5.7	3.0	0.00	0.04	0.02	0.01
21. petunidin-3-glc-ac[h]		NA	2.0	10.2	5.5	1.7	0.01	0.04	0.02	0.01
22. peonidin-3-glc-ac[h]		NA	2.3	9.4	5.1	1.7	0.01	0.03	0.02	0.01
23. malvidin-3-glc-ac[h]		NA	20.5	100.9	59.4	15.2	0.06	0.28	0.16	0.04
24. malvidin-3-glc-coum[h]		NA	6.9	41.4	21.2	6.9	0.02	0.11	0.06	0.02
25. polymers[i]	NA	NA	21.5	274.8	150.4	54.1	NA	NA	NA	NA
total			567.8	1174.1	820.1	97.6	1.59	2.79	2.04	0.20
TAC measured[c]							9.04	18.89	13.15	1.98
% TAC accounted[j]							11.1	23.7	16.1	2.4

[a] MW in g/mol. [b] TEAC in mM ± SD. [c] mg/L except where otherwise noted. [d] TAC expressed as mM TEs. [e] mg *p*-coumaric acid equivalents/L. [f] Neglible. [g] mg rutin equivalents/L. [h] mg corresponding anthocyanin-3-glc equivalents/L. [i] mg (+)-catechin equivalents/L. [j] % TAC accounted for = TAC calculated × 100/TAC measured. Abbreviations: ND, not detected; NA, not available.

contributions (calculated TAC) for the quantified individual phenolic compounds was only between 1.59 and 2.79 mM TE (average = 2.04 mM TE), accounting for between 11 and 24% (average = 16%) of the measured TAC of the experimental wines. The calculated TAC obtained here is somewhat lower than that estimated by Rice-Evans et al. (*11*) from the average TAC of several red wines and the phenolic composition of a red wine as reported by Frankel et al. (*36*).

The TAC contribution of individual phenolic compounds varied according to their TEAC values and concentration in the wines (**Table 1**). The largest TAC contributions were observed for malvidin-3-glc (0.32−0.82 mM TE; average = 0.61 mM

Table 2. Average Phenolic Composition[a] and TAC Values of Ultrafiltration Permeates and Retentates of Two Pinotage Wines

		10 kDa membrane		30 kDa membrane		50 kDa membrane	
	wine	permeate	retentate	permeate	retentate	permeate	retentate
gallic acid	15.3 a[b]	14.6 a (96%)[c]	1.0 c (7%)	14.5 a (95%)	0.8 cd (5%)	13.4 b (88%)	0.0 d (0%)
procyanidin B1	19.9 a	6.8 cd (34%)	4.8 d (24%)	9.5 bc (48%)	3.5 de (18%)	12.7 b (64%)	0.0 e (0%)
(+)-catechin	14.8 a	11.2 b (76%)	0.0 d (0%)	11.3 b (77%)	0.0 d (0%)	7.7 c (52%)	0.0 d (0%)
caffeoyltartaric acid	280.1 a	230.8 c (82%)	41.4 d (15%)	243.5 b (87%)	28.0 e (10%)	251.9 b (90%)	13.5 f (5%)
caffeic acid	8.6 a	6.9 bc (80%)	0.0 d (0%)	7.5 b (87%)	0.0 d (0%)	6.6 c (77%)	0.0 d (0%)
quercetin-3-glc	18.2 a	7.5 cd (41%)	7.1 de (39%)	8.8 c (48%)	5.8 e (32%)	11.5 b (64%)	3.5 f (19%)
quercetin-3-rham	14.1 a	5.5 cd (39%)	4.4 de (31%)	6.6 c (47%)	3.7 e (26%)	8.2 b (58%)	1.4 f (10%)
delphinidin-3-glc	11.1 a	6.2 cd (56%)	5.2 d (47%)	7.3 bc (66%)	4.4 d (40%)	9.0 b (81%)	1.7 e (15%)
petunidin-3-glc	14.0 a	7.1 cd (51%)	6.3 cd (45%)	8.6 bc (62%)	5.1 de (36%)	10.7 b (76%)	3.1 e (22%)
peonidin-3-glc	6.7 a	3.8 bc (56%)	3.2 cd (48%)	4.4 bc (65%)	1.7 de (26%)	5.3 ab (79%)	1.0 e (16%)
malvidin-3-glc	136.4 a	66.6 cd (49%)	58.5 cd (43%)	81.4 bc (60%)	46.2 de (34%)	102.7 b (75%)	24.5 e (18%)
delphinidin-3-glc-ac[d]	3.9 a	0.6 cd (15%)	2.1 bc (53%)	0.7 cd (18%)	1.3 cd (34%)	3.0 ab (77%)	0.0 e (0%)
vitisin A[d]	9.5 a	4.0 d (42%)	6.9 b (73%)	4.5 cd (47%)	6.2 b (65%)	5.9 bc (62%)	4.7 cd (49%)
petunidin-3-glc-ac[d]	3.3 a	2.2 b (68%)	2.3 b (71%)	2.3 b (71%)	1.1 c (34%)	2.6 ab (78%)	0.0 d (0%)
peonidin-3-glc-ac[d]	2.4 a	0.0 c (0%)	0.0 c (0%)	1.0 b (40%)	0.0 c (0%)	1.0 b (42%)	0.0 c (0%)
malvidin-3-glc-ac[d]	33.6 a	15.9 cd (47%)	15.4 cd (46%)	19.3 bc (58%)	12.5 de (37%)	24.1 b (72%)	7.5 e (22%)
malvidin-3-glc-coum[d]	14.0 a	2.0 e (14%)	6.5 b (47%)	4.8 cd (35%)	5.8 bcd (42%)	6.4 bc (46%)	2.6 cd (19%)
total monomers and procyanidin B1[e]	609.2 a	391.5 c (64%)	165.1 d (27%)	436.0 b (72%)	126.2 e (21%)	482.6 b (79%)	63.5 f (10%)
polymers[f]	83.9 a	0.0 c (0%)	16.0 b (19%)	0.0 c (0%)	14.7 b (18%)	0.0 c (0%)	9.0 b (11%)
tannin[g]		ND		ND		ND	
TAC calculated[h]	2.02 a	1.36 d (67%)	0.50 e (25%)	1.49 c (74%)	0.38 f (19%)	1.61 b (80%)	0.18 g (9%)
TAC measured[h]	17.35 a	3.82 f (22%)	12.66 b (73%)	4.88 e (28%)	11.54 c (67%)	7.55 d (44%)	8.08 d (47%)

[a] Content in mg/L. [b] Different lower case letters in a row indicate significant ($P < 0.05$) differences. [c] % of original content. [d] Content in mg corresponding anthocyanin-3-glc equivalents/L. [e] Sum of monomeric phenolic compounds quantified and procyanidin B1. [f] mg (+)-catechin equivalents/L. [g] mg (+)-catechin equivalents/L measured using the tannin assay. [h] TAC in mM TEs.

TE) and caffeoyltartaric acid (0.32−0.75 mM TE; average = 0.51 mM TE). Although these two compounds had relatively low TEAC values, they were the highest contributors to the TAC due to their very high concentration in the wines. Other important TAC contributors (>0.05 mM TE) were gallic acid (average = 0.20 mM TE), procyanidin B1 (average = 0.09 mM TE), (+)-catechin (average = 0.05 mM TE), delphinidin-3-glc (average = 0.07 mM TE), petunidin-3-glc (average = 0.09 mM TE), malvidin-3-glc-ac (average = 0.16 mM TE), and malvidin-3-glc-coum (average = 0.06 mM TE). In the case of gallic acid and procyanidin B1, their high TEAC values gave rise to the high TAC contributions, although they were present in relatively modest amounts. Quercetin-3-gal, kaempferol, and isorhamnetin contributed the least to the TAC of Pinotage wines (<0.01 mM TE), due to their very low concentrations.

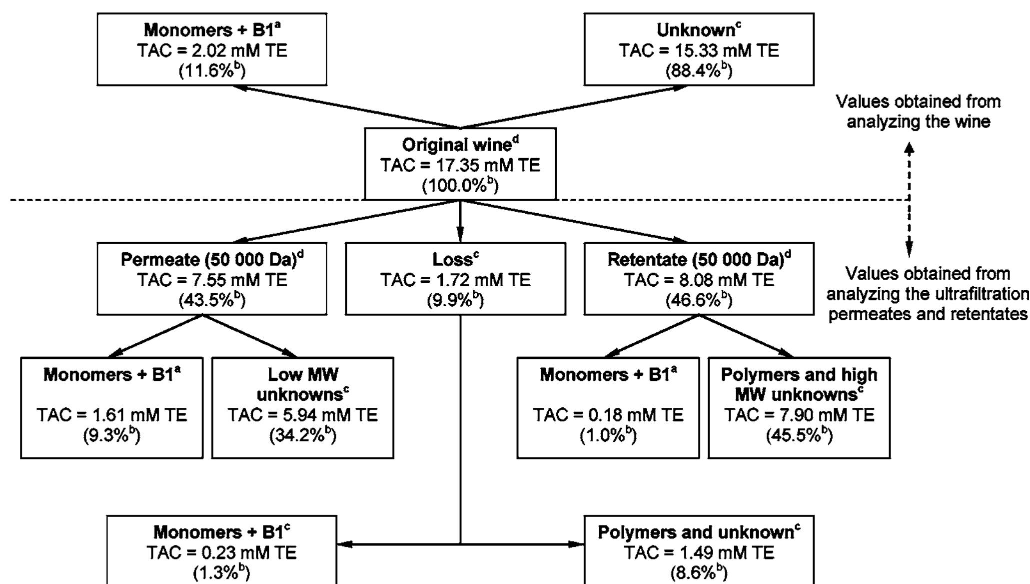
Ultrafiltration. Ultrafiltration was carried out to attempt separation of monomeric and polymeric compounds in order to determine their respective contributions to the TAC of the wine. For this reason, membranes with a range of nominal MW cutoffs (10, 30, and 50 kDa) were tested to determine at which cutoff this separation occurs. Results for the two wines exhibited similar trends (see Supporting Information), and the average phenolic composition and TAC of the wines and ultrafiltration permeates and retentates are presented in **Table 2**.

Recovery of some phenolic compounds after ultrafiltration was not quantitative (**Table 2**). The low recovery of procyanidin B1, (+)-catechin, quercetin-3-rham, and malvidin-3-glc-coum in the permeates and retentates was possibly due to adsorption on the membrane, while that of delphinidin-3-glc-ac and peonidin-3-glc-ac can be ascribed to their low quantities in the original wine, making quantification of even lower concentrations in the permeates and retentates difficult. Myricetin and quercetin were present in very low quantities in the original wine and were not detected in any of the ultrafiltration permeates or retentates (data not shown). Recovery of polymers in the retentates was very low. Adsorption of polymers on the ultrafiltration membrane is a likely source of polymer loss during ultrafiltration. Furthermore, recovery of the retentate from the ultrafiltration device was also not entirely quantitative.

Permeation increased with increasing membrane pore size for most phenolic compounds. Permeation of nearly all of the gallic acid from the original wine was achieved using the 10 kDa membrane (**Table 2**). Other compounds with good permeation through the 10 kDa membrane were (+)-catechin, caffeoyltartaric acid, and caffeic acid, with >70% of the original content detected in the 10 kDa permeate. Most of the phenolic compounds reached levels of 60% of the original level or higher in the 50 kDa permeate. Exceptions were quercetin-3-rham, peonidin-3-glc-ac, and malvidin-3-glc-coum due to low recovery values. The low amount of (+)-catechin observed in the 50 kDa permeate was unexpected, as 76 and 77% of the original (+)-catechin content were observed in the 10 and 30 kDa permeates, respectively. The 50 kDa permeate contained 79% of the total monomer and procyanidin B1 content of the original wine, while no polymers were detected in the 50 kDa permeate, by HPLC or a protein precipitation assay. Many of the individual phenolic compounds, namely, gallic acid, procyanidin B1, (+)-catechin, caffeic acid, delphinidin-3-glc-ac, petunidin-3-glc-ac, and peonidin-3-glc-ac, were not detected in the 50 kDa retentate, while the others were present in very low concentrations. On the basis of these data, it is clear that the 50 kDa ultrafiltration membrane was the most effective for separation of the monomeric and polymeric phenolic compounds.

The calculated TAC for the ultrafiltration permeates was relatively low as compared to the measured TAC as observed for the original wines. The relative contribution of the quantified phenolic content of the permeate to its measured TAC increased with a decrease in membrane pore size, which was 21, 31, and 36% for the 50, 30, and 10 kDa permeates, respectively. This may suggest that more unknown compounds are retained with the smaller membrane size. Another possible explanation may be that as more of the proteins and peptides are excluded with

Scheme 1. Scheme of TAC Contribution of Different Wine Fractions to the TAC of Wine[a]



[a] Calculated from phenolic composition and TEAC values (mM). [b] TAC as % of original wine TAC. [c] Calculated by difference. [d] Measured; B1 = procyanidin B1.

decreasing membrane size, less masking of antioxidant activity of the phenolic compounds occurred. Masking of the antioxidant activity of phenolic compounds by proteins has been reported previously (*37*, *38*).

The TAC of the original wine can be divided into different portions based on the ultrafiltration data using the 50 kDa membrane, if TAC contributions of different compounds and classes of compounds are additive. The contribution of monomeric phenolic compounds and procyanidin B1 to the measured TAC of the original wine, as calculated from their content and TEAC values, was only 12% with 88% of the wine TAC contributed by unidentified compounds and/or factors such as masking or synergy (**Scheme 1**). Using data from the ultrafiltration experiment, the low MW fraction (50 kDa permeate) and high MW fraction (50 kDa retentate) accounted for 44 and 47% of the wine TAC, while 10% of the TAC is lost during ultrafiltration. Some of the TAC not recovered during ultrafiltration is due to loss of monomeric compounds and procyanidin B1 (1% of the original wine TAC). The rest is attributed to loss of unknown compounds and polymers, as well as possible effects of masking and synergy that changed with the modification of the matrix due to physical separation of compounds. The fact that recovery of polymers was very low during ultrafiltration suggests that most of the TAC loss is due to polymers. If ~9% of the original wine TAC is due to polymers, which is a significant contribution as compared to that of the monomers, a large amount of the TAC is not accounted for by the quantified monomers, procyanidin B1, and polymers. After subtracting the amount of TAC due to quantified phenolic compounds in the 50 kDa permeate and the 50 kDa retentate, 34 and 46% of the wine TAC from the low MW and high MW fraction were unexplained, respectively. This is ascribed to low MW unknowns and high MW unknowns, respectively. The high MW unknown fraction includes the polymeric phenolic compounds although other high MW compounds such as proteins, peptides, or polysaccharides could also be present. Fernández-Pachón et al. (*39*) reported that phenolic polymers retained on a C18 SPE cartridge after elution with acetonitrile and ethyl ac contributed 51% of the TAC of red wines.

Synergy and Unknown Compounds Affecting TAC. Because synergy between compounds was considered to contribute to the TAC of wine, this effect was tested, using mixtures of some phenolic compounds in typical concentrations as found in Pinotage wines (**Table 3**). Synergy of between 16 and 23% was observed. This suggests that some, but not all, of the discrepancy between measured and calculated TAC values can be explained by synergy between the phenolic compounds. The situation is, however, more complex as synergy between the phenolic compounds and other wine constituents cannot be ruled out.

Table 3. Mixtures of Phenolic Compounds[a] in Typical Concentrations Found in Pinotage Wines Tested for Synergy

	mixture 1	mixture 2	mixture 3
gallic acid	9.92	29.75	11.90
procyanidin B1	32.34	19.60	19.60
(+)-catechin	22.75	14.48	14.48
caffeoyltartaric acid	180.96	109.04	120.64
caffeic acid	5.78	9.64	7.71
quercetin-3-glc	12.36	8.24	20.60
quercetin-3-rham	7.83	7.83	8.70
quercetin	3.94	3.94	4.92
delphinidin-3-glc	18.08	20.34	9.04
petunidin-3-glc	25.62	25.62	14.64
peonidin-3-glc	9.30	14.88	9.30
malvidin-3-glc	212.16	254.40	149.76
TAC calculated[b]	1.78	1.98	1.34
TAC measured[b]	2.18	2.30	1.60
% synergy[c]	22.5%	16.2%	19.4%

[a] Content in mg/L. [b] TAC in mM TEs. [c] % synergy = (TAC measured − TAC calculated) × 100/TAC calculated.

Sulfur dioxide has the ability to regenerate phenolic compounds from their phenoxyl radicals, causing a synergistic increase in antioxidant activity (*24*). However, at the concentrations normally present in wines, it does not contribute significantly to the free radical scavenging activity of wines against ABTS, DPPH, DMPD, or superoxide radicals (*40*−*42*). Using the same $ABTS^{\bullet+}$ scavenging assay protocol as used in the present study, the addition of sulfur dioxide up to 150 mg/L did not affect the TAC of Pinotage wine (*42*). Phenolic antioxidants are able to recycle ascorbic acid and α-tocopherol in a lipid peroxidation assay (*25*). One study presented data on the regeneration of phenoxyl radicals by phenolic compounds,

indicating that (+)-catechin is able to regenerate quercetin from its phenoxyl radical (*26*). This may be a mechanism for the synergistic effect observed for mixtures. Regeneration of phenoxyl radicals will depend on competing reactions such as disproportionation and dimerization of the phenoxyl radicals, as well as further oxidation of the phenoxyl radical to form a quinone.

Other possibilities include unidentified low MW phenolic compounds of high potency present in concentrations that were too low to quantify or other monomeric phenolic compounds not detected with the current HPLC methodology. Possible candidates include (−)-epicatechin, flavan-3-ol gallate esters, *S*-glutathionylcaftaric acid, stilbenes, stilbene glcs, and pyrano-anthocyanins other than vitisin A. As procyanidin B1 was detected in the ultrafiltration permeates, it is reasonable to expect other dimers such as the procyanidin dimers and anthocyanin-flavan-3-ol condensation products to be present also. Other oligomers (trimers and tetramers) may also be divided between the permeate and the retentate and thereby contribute to the unknown portions of these fractions. These were, however, not expected to be responsible for the 80−90% of unexplained TAC. Other compounds of low or high MW such as proteins (*12*, *37*, *38*), peptides, polysaccharides, and possibly others could also conceivably contribute to the TAC of Pinotage wines.

In conclusion, the present study showed that only a small amount of the TAC of Pinotage wines is contributed by their content of monomeric phenolic compounds and procyanidin B1, with oligomeric and polymeric phenolic compounds, as well as other unknown compounds, largely contributing to the remaining TAC. Simple addition of TAC contributions as calculated in this study may, however, not be appropriate, as synergy between phenolic compounds and possibly other wine constituents and even masking of antioxidant capacity by proteins cannot be ruled out. These findings suggest that by manipulating the monomeric phenolic composition of Pinotage wines the TAC is not likely to be increased substantially as was previously supposed, although some improvement may be possible. More detailed characterization and quantification of the phenolic content of red wines, e.g., using LC-MS techniques, are needed to obtain a clearer picture of the contribution of various types of phenolic compounds. Radical scavenging activity is an important aspect of antioxidant activity, although the in vitro radical scavenging activity of wine components does not necessarily coincide with in vivo antioxidant activity, as bioavailability, metal chelating properties, lipid phase partitioning, and metabolism of individual wine components may differ considerably. Knowledge of the metabolites and their antioxidant activity is required to better evaluate their relative importance. Unabsorbed compounds may also exert protective effects in the gastrointestinal system. These issues need more attention before firm recommendations can be made with regard to which phenolic compounds need to be manipulated in order to obtain a red wine with optimal health benefits.

ABBREVIATIONS USED

ac, acetate; coum, coumarate; gal, galactoside; glc, glucoside; rham, rhamnoside; MW, molecular weight; TAC, total antioxidant capacity; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity.

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Supporting Information Available: Tables containing the ultrafiltration data for the two separate wines analyzed. This material is available free of charge via the Internet at http://pubs.acs.org.

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