JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Compositional Investigation of Phenolic Polymers Isolated from *Vitis vinifera* L. Cv. Pinot Noir during Fermentation

PATRICIA M. ARON AND JAMES A. KENNEDY*

Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331

Phenolic polymer material extracted during commercial red wine fermentations (*Vitis vinifera* L. cv. Pinot noir) was isolated and analyzed to characterize its chemical composition. Phenolic polymer isolates were prepared from samples taken throughout fermentation and isolated by adsorption chromatography. Isolates were subjected to phloroglucinolysis to analyze the proanthocyanidin amount as well as the subunit composition. Results of phloroglucinolysis revealed that the proanthocyanidin content of individual phenolic polymer isolates varied from 27 to 54%. Subsequent analyses were done in an attempt to quantify materials other than known proanthocyanidin subunits. Results of all experiments indicate that up to 82% of the phenolic polymer isolates could be accounted for by mass. While this figure accounts for a significant portion of the polymeric phenolic material, further investigation will be needed to qualify the remaining 18%.

KEYWORDS: Proanthocyanidin; carbohydrate; phloroglucinolysis; pigmented tannin; oxidation; red wine fermentation

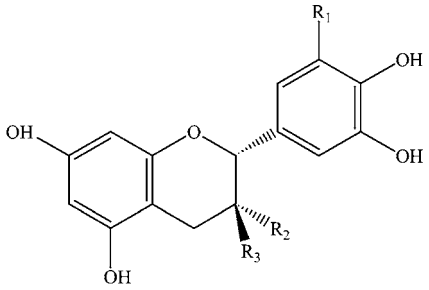
INTRODUCTION

As most wine makers seek to produce wine of the highest quality, it is of incredible importance to understand, even at the most basic level, the chemical structures of all wine components. Of considerable interest in the study of red wine are the polyphenols, as their quantity and structure affect wine quality.

Important red wine polyphenols include several compounds of the flavonoid family with a C6−C3−C6 phenylbenzoypyran ring structure (*1*−*3*). Originating from grape berry skin, seed, and stem tissues, those most prevalent in red wine are the anthocyanins, the flavan-3-ols (**Figure 1**), and their polymerized products. Existing as monoglucosides in *Vitis vinifera* L., the anthocyanins are responsible for the red color of wine. Structural transformation of anthocyanins into pigmented polymers during winemaking affects both color and polyphenol colloidal stability in finished red wines (*4*) and may also modify mouthfeel (*5*−*10*).

Comprising a greater part of grape and red wine polyphenols (*11*), the flavan-3-ols exist in red wine as monomers, oligomers, and polymers (proanthocyanidins or condensed tannins) (*12*). Proanthocyanidin subunits of significance to grapes and red wine include (+)-catechin and (−)-epicatechin, (−)-epigallocatechin, and an esterified derivative of (−)-epicatechin, (−)-epicatechin-3-*O*-gallate (*13*) (**Figure 1**). The proanthocyanidin structure and degree of polymerization are thought to influence apparent astringency and bitterness of red wine (*14*).

Several variables affect the extraction of polyphenols into wine during maceration and fermentation, including time, temperature, alcohol content, and SO_2 concentration (*15*−*20*). Anthocyanin concentrations typically peak early, while proanthocyanidin extraction generally increases to reach a maximum at pressing. However, the localization of phenolics in grape tissue and interaction of phenolics with other grape components limit extraction of total grape polyphenols into wine (*21*).



Flavan-3-ols	R_1	R_2	R_3
(+)-catechin	H	H	OH
(-)-epicatechin	H	OH	H
(-)-epigallocatechin	OH	OH	H
(-)-epicatechin-3-*O*-gallate	H	*O*-G[1]	H

Figure 1. Flavan-3-ol monomeric units in proanthocyanidins. [1]*O*-gallate (G).

Although a considerable amount of the phenolic polymer material extracted into red wine during fermentation can be accounted for and characterized as known compounds, a significant portion of the material remains uncharacterized (*10*). Despite the unknown chemical composition of the extracted material, the extracts are generally considered to be comprised of flavan-3-ols and their polymerized (proanthocyanidins) or chemically modified products. The purpose of this investigation was thus to quantify and characterize the phenolic polymer

* To whom correspondence should be addressed. Tel: +1-541-737-9150. Fax: +1-541-737-1877. E-mail: james.kennedy@oregonstate.edu.

10.1021/jf0704817 CCC: $37.00
Published on Web 06/14/2007

material extracted during commercial red wine fermentations (*V. vinifera* L. cv. Pinot noir).

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were high-performance liquid chromatography (HPLC) grade. Acetone, methanol, and *N*,*N*-dimethylformamide were purchased from Burdick and Jackson (Muskegon, MI). Acetonitrile, hydrochloric acid, gallic acid monohydrate, sodium acetate anhydrous, L-(+)-ascorbic acid, potassium metabisulfite, potassium bitartrate, sodium hydroxide, and lithium chloride were purchased from J. T. Baker (Phillipsburg, NJ). Phloroglucinol, (+)-catechin, (−)-epicatechin, Folin−Ciocalteu (FC) reagent (2 N), acetic acid, and trifluoroacetic acid (99+% spectrophotometric grade) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium carbonate monohydrate was purchased from MultiPharm EMD (Gibbstown, NJ). Ammonium phosphate monobasic and orthophosphoric acid were purchased from Fisher Scientific (Santa Clara, CA).

Instrumentation. An Agilent model 1100 HPLC (Palo Alto, CA) was used with Chemstation software for chromatographic analyses. All spectrophotometric analyses were conducted using a Cary 50 spectrophotometer (Varian, Palo Alto, CA).

Commercial Fermentations. Commercial Pinot noir wines were made at Willakenzie Estate Winery (Oregon). *V. vinifera* L. cv. Pinot noir grapes (clone 777) were harvested from vineyard blocks A and B at 24.4 and 24.8 °Brix and immediately destemmed without crushing. Following a 5 day prefermentation cold soak at 4.4 °C, blocks A and B were fermented in separate (4.54 metric tons) open top, double-jacketed stainless steel tanks using *Saccharomyces cerevisiae* strain RC 212 (Lallemand, Rexdale, Ontario) as described by the manufacturer. Both fermentations received equivalent punch-downs (once daily) from day 5 to day 15 and equivalent pumpovers (once daily) from day 8 to day 16. Maximum temperatures of 27.5 °C (A and B) were recorded on day 13. Finishing at −2.1 °Brix, both tanks were drained and pressed on day 22. Must samples were taken on days 6, 8, 10, 13, and 21 after harvest. Samples were kept frozen at −20 °C until isolation and purification of phenolic polymers.

Recovery Experiment. Prevéraison skin tannins and wine tannins (*V. vinifera* L. cv. Pinot noir) were isolated as per Kennedy and Jones (*22*). Model must was prepared with 25% w/w fructose:glucose (1:1) and 5.0 g/L tartaric acid (pH 3.6 with KOH). Model wine was prepared with 12.0% v/v ethanol and 5.0 g/L tartaric acid (pH 3.6). Prevéraison skin tannin (0.3 g/L) was dissolved in model must. Wine tannin (0.5 g/L) was dissolved in model wine. Tannin solutions were then applied (~18 mL/min) to a glass column (Kimble Kontes Chromaflex, 4.8 cm × 15 cm, Vineland, NJ) containing Toyopearl chromatography resin (HW 40C, Supelco, Bellefonte, PA) to an approximate bed volume of 206 mL following in-line filtration (polypropylene, 400 cm^2 EFA 1.0 μm Polycap 36 HD MAPP, Whatman, Clifton, NJ). The column had previously been equilibrated with water containing 0.05% v/v trifluoroacetic acid (TFA). The applied must was then rinsed with 1.0 L of water containing 0.05% v/v TFA to remove sugars and organic acids. A 1.0 L amount of 1:1 methanol:water containing 0.05% v/v TFA was then used to remove anthocyanins and other low molecular weight phenolics. Finally, 300 mL of 2:1 acetone:water containing 0.05% v/v TFA was used to elute the high molecular weight phenolic fraction. The column was then washed with 500 mL of water, followed by 200 mL of 2:1 acetone:water, again with 500 mL of water, and finally re-equilibrated with 500 mL of water containing 0.05% v/v TFA. The phenolic polymer fraction was concentrated under reduced pressure (35 °C) to remove acetone and lyophilized to a dry powder. Phloroglucinolysis was performed on recovered isolates to determine possible effects of processing (described below).

Isolation and Purification of Phenolic Polymers. A 250 mL amount of must (thawed to room temperature) was centrifuged twice (3398 RCF, 10 min) to remove gross precipitates. Following a second volume measurement, the must was applied to the column and the isolate was collected as per the recovery experiment above (in quadruplicate for each time point of both blocks).

Isolation of Seed and Skin Proanthocyanidin. Seeds and skins were isolated by extraction (2:1 acetone:water, 1 mL per berry) from 50 whole berries from each block in quadruplicate as previously described (*23*).

Acid Catalysis in the Presence of Excess Phloroglucinol (Phloroglucinolysis). Phenolic polymer isolates and grape tissue extracts underwent phloroglucinolysis as previously described (*22*). Three milliliters each of aqueous seed and skin extracts (quadruplicate) and 10 mL of must samples (triplicate) underwent solid-phase extraction using a C_{18} SPE column (1 g Alltech) prior to phloroglucinolysis as previously described (*24*).

A reversed-phase (RP) method described by Kennedy and Taylor (*25*) was used to identify and quantify subunit composition of phloroglucinolysis products (*22*). (+)-Catechin was used as a quantitative standard for determining late-eluting material (LEM). The proportion of seed and skin proanthocyanidin extracted into must during fermentation was determined as previously described (*15*).

RP HPLC Analysis of Phenolic Polymer Isolates. Phenolic monomer and total phenolic contents of the isolates were determined by RP HPLC using a previously described method (*26*). A 5 g/L amount of aqueous phenolic polymer isolate preparations was filtered (0.45 μm, acrodisc CR13, Pall Corp., East Hills, NY) prior to injection. (+)-Catechin and malvidin-3-*O*-glucoside were used as quantitative standards.

Gel Permeation Chromatography (GPC). The GPC method previously described by Kennedy and Taylor (*25*) allowed for size distribution and pigmented polymer determination of the isolates. Malvidin-3-*O*-glucoside and (+)-catechin were used as quantitative standards.

FC Assay. The FC method (*27*) was used to determine the phenolic content of the isolates. Absorbance values were compared to gallic acid and were expressed as a % of gallic acid response (% GA).

Total Phenolics at 280 nm and Total Red Pigments at 520 nm. The methods of Somers and Evans (*12*, *28*) were modified to determine total phenolics and total red pigments of the phenolic polymer isolate solids. Aqueous solutions of phenolic polymer isolates (5 mg/100 mL) were prepared, sonicated (1 min), left at room temperature (3 h), and resonicated (1 min), and the absorbance was measured at 280 nm. To determine total red pigments, 20 mL of the 5 mg/100 mL solution was acidified with 5.0 mL of concentrated HCl to achieve a 1.0 N solution and sonicated (1 min), and the absorbance was measured at 520 nm. Total phenolics and total red pigment values were reported as absorbance of a 1% solution in a 1 cm path length.

Carbohydrate Analysis by GC/MS. Phenolic polymer isolates were subjected to carbohydrate analysis as previously described by Merkle and Poppe (*29*) and York et al. (*30*). GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC interfaced to a 5970 MSD, using an Alltech (Lexington Kentucky) EC-1 fused silica capillary column (30 m × 0.25 mm i.d.). Mass spectral analysis was used to authenticate carbohydrate (fragment ion $m/z = 73$ indicating base fragment for all TMS methyl glycosides, $m/z = 204$ and 217 indicating neutral sugars, $m/z = 173$ indicating amino sugars, and $m/z = 298$ indicating sialic acids).

Monomeric Carbohydrate Analysis. Isolates were analyzed for monomeric carbohydrate content using an Aminex HPX-87 C calcium column (Bio-Rad, Hercules, CA) with guard column and refractive index detector. Chromatographic conditions used were as described by the manufacturer with D-glucose monohydrate and D-fructose used as quantitative standards. A 6 g/L amount of aqueous phenolic polymer isolate preparations was filtered (0.45 μm) prior to injection.

NMR Analysis. NMR spectroscopy was used to characterize the phenolic polymer isolate from day 6 of block A. The sample was deuterium-exchanged by lyophilization from D_2O (99.9996%) and dissolved in 0.7 mL of D_2O (20 mg/mL). Proton, gradient-enhanced correlation spectroscopy, total correlation spectroscopy, gradient-enhanced heteronuclear single quantum coherence, gradient-enhanced heteronuclear multiple-bond correlation, and nuclear Overhauser effect spectrometry NMR spectra were acquired on a Varian Inova-500 MHz spectrometer at 298 K using standard Varian pulse sequences. The proton pulse sequence consisted of 45° pulse length, 3843 Hz spectral width, 16K data points, 2.05 s acquisition time, and a relaxation delay of 1 s with 16 total scans. Internal acetone was used to measure relative chemical shifts ($\delta_H = 2.225$ ppm, $\delta_C = 31.07$ ppm).

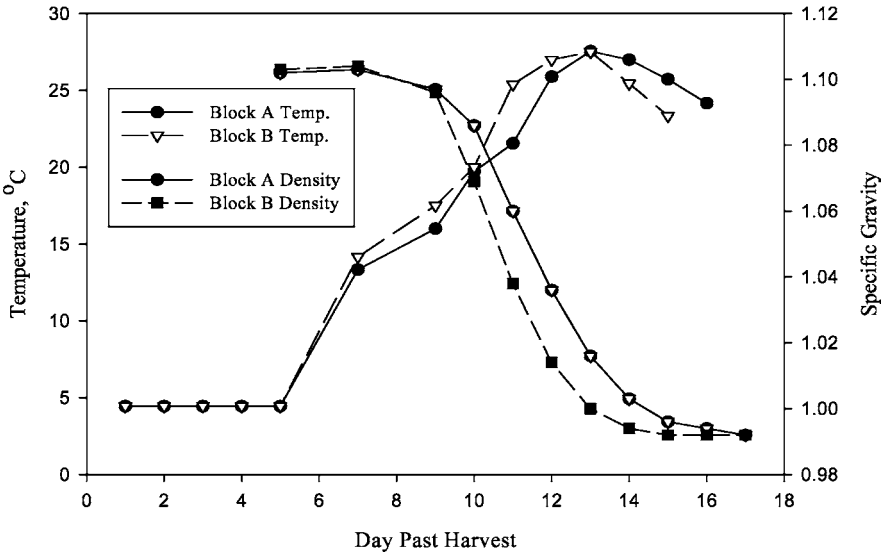


Figure 2. Fermentation temperature and density profiles.

The sample also underwent ultrafiltration (0.5 mL Millipore Microcon centrifugal filter device, 1 or 10 kDa MWCO, Millipore Corp., New Bedford, MA) to obtain a retentate volume of ~50 μL. The retentate was then taken up in 500 μL of D_2O, and the process was repeated once. The retentate and permeate were freeze-dried, and proton NMR spectra were recorded. Proton chemical shifts (δ_H) were identified according to previously published values (*22*, *31*).

RESULTS AND DISCUSSION

The purpose of this investigation was to monitor phenolic polymer extraction and composition during commercial red wine fermentations (*V. vinifera* L. cv. Pinot noir). In this study, phenolic polymer isolates were prepared from berries after destemming, from must during fermentation, and from young wine following the termination of fermentation. Fermentation temperature and density profiles are reported in **Figure 2**. Must samples were collected for phenolic polymer isolation on days 6, 8, 10, 13, and 21 after harvest.

Isolation and Purification of Phenolic Polymers. Phenolic polymers were isolated by adsorption chromatography on Toyopearl resin. This method has been used by others and is generally considered to be an effective phenolic polymer purification method (*22*, *32*−*34*). To determine the effect of filtration and chromatography on the recovery and composition of phenolic polymers, model solutions of prevéraison skin and wine (*V. vinifera* L. cv. Pinot noir) phenolic polymer isolates were applied to the column and eluted with the same procedure used for must phenolic polymer isolation and purification. The polymeric isolates used in this method optimization were isolated using similar chromatography on Toyopearl resin (*22*) with applied concentrations based upon previous yields from must/wine extraction of Pinot noir. Prevéraison skin phenolic polymers were chosen over seed phenolic polymers due to their chemical composition that includes both procyanidins and prodelphinidins. Prevéraison skin phenolic polymers in model must (0.3 g/L) applied to the column resulted in 97.5% mass recovery. Wine phenolic polymers in model wine at 0.5 g/L applied to column yielded a 95.7% mass recovery.

Results of subsequent phloroglucinolysis on these extracts revealed that processing decreased the mass conversion yield from 76.4 to 70.4% in skin phenolic polymers and from 66.3 to 61.4% in wine phenolic polymers. Despite a small decrease in mass per subunit, processing minimally affected subunit proportional composition.

Table 1. Phenolic Polymer Isolate Mass Recovery and mDP ($N = 4 \pm$ SEM)

block	day[a]	recovery[b]	mDP[c]	MM[d]
A	6	0.22 ± 0.01	3.89 ± 0.05	1540
	8	0.35 ± 0.02	3.73 ± 0.11	1640
	10	0.42 ± 0.01	5.66 ± 0.22	2160
	13	0.62 ± 0.01	7.22 ± 0.18	2820
	21	0.99 ± 0.02	5.89 ± 0.28	2380
	wine	1.10 ± 0.10[e]	5.99 ± 0.22	2430
B	6	0.17 ± 0.01	3.08 ± 0.15	1140
	8	0.28 ± 0.01	3.99 ± 0.06	1140
	10	0.48 ± 0.03	6.52 ± 0.14	1990
	13	0.72 ± 0.01	6.58 ± 0.16	2780
	21	0.88 ± 0.01	6.10 ± 0.09	2070
	wine	1.30 ± 0.18[e]	5.04 ± 0.60	2470

[a] Sample day following harvest. [b] Recovery is expressed as grams phenolic polymer powder recovered per liter of must. [c] Apparent mDP based on known phloroglucinol subunit composition. [d] MM based on 50% elution volume of phenolic polymer isolates from GPC; not replicated. [e] Extraction was run in duplicate.

The effect of cold storage (−20 °C, 8 months) on phloroglucinolysis conversion yield of prevéraison skin and seed tannin was also investigated. Cold storage did not significantly affect phloroglucinolysis conversion yield in either prevéraison skin or seed phenolic polymers; skin conversion yield decreased by 0.66% while seed increased by 0.84%. Subunit composition was also not altered due to prolonged cold storage. Despite the conversion yield decreases seen due to phenolic polymer extract processing, the method was considered to be adequate for the experiment due to its reproducibility as seen in the determination of gravimetric yield and during phloroglucinolysis.

Following successful method optimization, phenolic polymers were isolated from must and young wine samples collected throughout fermentation. Isolation and purification of phenolic polymers and their subsequent gravimetric determination confirmed that total extraction increased throughout fermentation, reaching roughly a five-fold increase from day 6 to day 21 (**Table 1** and **Figure 3**). Extraction of phenolic polymers followed a linear trend in both blocks with linear regression r^2 values of A = 0.99 and B = 0.89 (*the* r^2 value does not include young wine mass recovery). These results are in agreement with previously published work that indicates total phenolic extraction increases during red wine maceration (*15*, *18*, *19*, *35*, *36*).

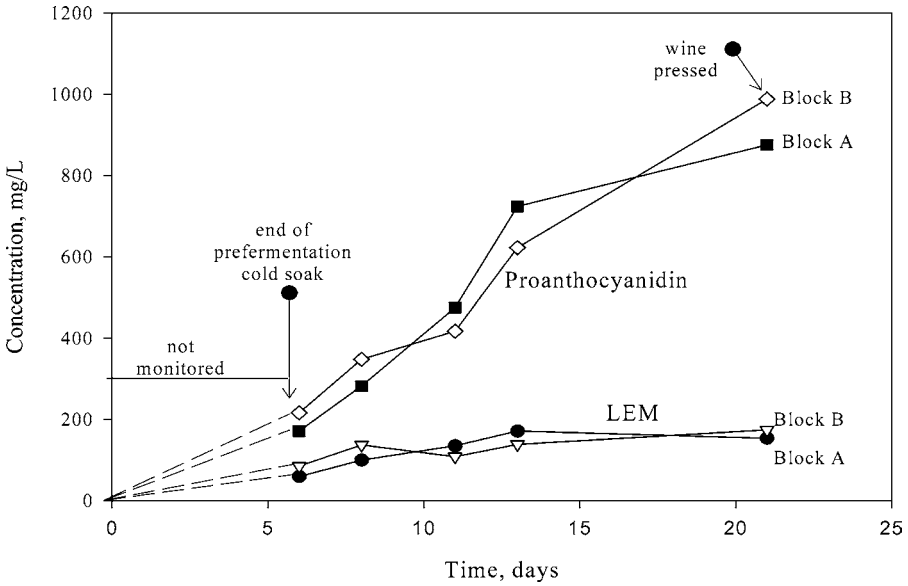


Figure 3. Fermentation extraction profile with extracted proanthocyanidin concentration (determined by phloroglucinolysis) and LEM indicated.

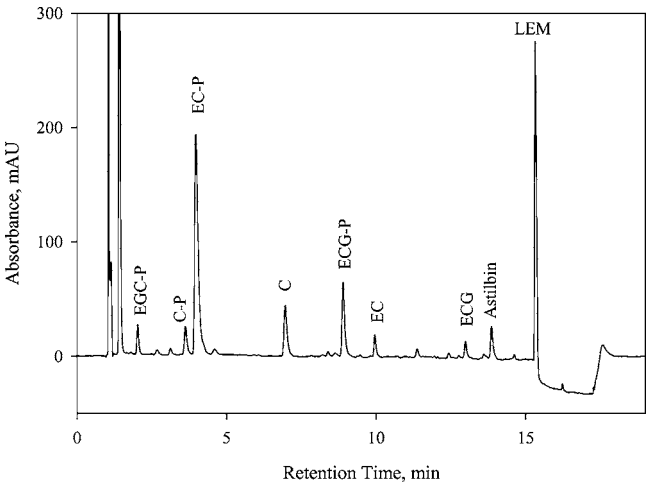


Figure 4. Phloroglucinolysis chromatogram showing flavan-3-ol subunits, astilbin, and LEM.

Proanthocyanidin Content of Phenolic Polymers. To attest that phenolic polymers are chiefly composed of proanthocyanidin material, phloroglucinolysis was applied to analyze proanthocyanidin quantity and subunit composition of the phenolic polymer isolates. Seven major phloroglucinolysis products were observed (*22*) that were further classified as extension [phloroglucinol adducts of (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate] or terminal [free monomers of (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-*O*-gallate] proanthocyanidin subunits (**Figure 4**). Relative molar subunit proportions are reported in **Table 2**.

Phloroglucinolysis allowed for the determination of polymeric polyphenol isolate apparent mean degree of polymerization (mDP) (**Table 1**). The mDP increased to reach a maximum at day 13 and then decreased by day 21 (both blocks). An increase in mDP from prefermentation cold maceration to postalcoholic fermentation has previously been reported (*37*). It is suggested that the increase in mDP is due to progressive extraction of oligomers during fermentation.

GPC has been used reliably in polymer analysis for several decades, including the direct analysis of proanthocyanidins (*25*). GPC results allowed for phenolic polymer size distribution determination. The 50% elution volume of the replicate containing the least low molecular weight contaminant (discussion to follow) for each sample was used to calculate average molecular mass (MM) data for each phenolic polymer extract (**Table 1**, error not reported). MM increased from sample day 6 until day 10 and then decreased by day 21 for both blocks, consistent with observed mDP values from phloroglucinolysis.

GPC results also revealed an increasingly more bimodal molecular weight distribution with progressive fermentation; larger polymers extracted later in fermentation along with an increased quantity of smaller polymers (**Figure 5**). Similar bimodal elution patterns for tannin and pigmented tannin have previously been reported with the distribution variance attributed to the differences in skin and seed phenolic polymer size distribution (*38*). In this study, the proportion of skin proanthocyanidin (see discussion below) increased during fermentation; thus, the bimodal distribution here could be due to earlier extraction of lower molecular weight skin-derived polymers followed by an increased proportion of larger molecular weight skin-derived polymers coupled with extraction of lower molecular weight seed-derived polymers. A comparison of phloroglucinol mDP with GPC MM revealed a linear correlation between the two analytical methods (A-r^2 = 0.93, and B-r^2 = 0.84).

Given that (−)-epigallocatechin subunits derive solely from the skins of grapes, investigation of changes in its molar quantity throughout fermentation can give an idea of the amount of skin tannin extracted over time. In phloroglucinolysis, (−)-epigallocatechin is detected as a phloroglucinol adduct. (−)-Epigallocatechin-phloroglucinol adduct molar concentrations decreased from day 6 (A = 0.14 mol %, and B = 0.11 mol %) to day 8 (A and B = 0.08 mol %), increased until day 13 (A and B = 0.18 mol %), to finally decrease by day 21 (A and B = 0.14 mol %) (**Table 2**). An increase in skin proanthocyanidin concentration during alcoholic fermentation with low-temperature prefermentation maceration has been previously reported; however, the initial decrease is inconsistent with reported trends (*15*).

Quantification of skin proanthocyanidin from whole berry isolates (commercially ripe fruit) allowed for the determination of phenolic polymers skin proanthocyanidin proportion. The total molar quantities of (−)-epigallocatechin and (−)-epicatechin extension subunits extracted from the grape skin isolates were calculated for both blocks and compared as previously

Table 2. Phenolic Polymer Isolate Proanthocyanidin Composition by Phloroglucinolysis ($N = 4 \pm$ SEM)

		extension[a]				terminal		
block	day	% EGC-P	% C-P	% EC-P	% ECG-P	% C	% EC	% ECG
A	6	14.2 ± 0.4	6.2 ± 0.1	52.7 ± 0.2	1.2 ± 0.1	18.2 ± 0.4	2.1 ± 0.1	5.4 ± 0.2
	8	8.0 ± 0.7	6.9 ± 0.1	56.7 ± 1.0	1.4 ± 0.1	20.9 ± 0.6	2.8 ± 0.2	3.2 ± 0.4
	11	16.8 ± 0.1	5.1 ± 0.1	58.1 ± 0.8	2.2 ± 0.1	14.9 ± 0.7	2.1 ± 0.1	0.8 ± 0.1
	13	17.6 ± 0.1	5.0 ± 0.1	60.1 ± 0.4	3.5 ± 0.1	11.1 ± 0.3	2.0 ± 0.1	0.9 ± 0.1
	21	14.3 ± 0.3	6.1 ± 0.1	57.6 ± 0.6	4.7 ± 0.1	12.7 ± 0.8	3.2 ± 0.1	1.4 ± 0.1
	wine	13.3 ± 0.1	6.1 ± 0.2	59.5 ± 0.8	4.3 ± 0.1	11.9 ± 0.5	3.8 ± 0.1	1.1 ± 0.3
B	6	10.7 ± 0.6	6.4 ± 0.2	49.0 ± 1.3	0.9 ± 0.1	23.0 ± 0.9	3.9 ± 1.4	6.1 ± 0.1
	8	8.0 ± 0.3	6.5 ± 0.1	58.9 ± 0.5	1.5 ± 0.1	21.4 ± 0.4	2.5 ± 0.2	1.2 ± 0.1
	11	17.4 ± 0.3	4.5 ± 0.1	60.3 ± 0.4	2.4 ± 0.4	12.9 ± 0.4	1.9 ± 0.1	0.6 ± 0.1
	13	17.9 ± 0.2	5.4 ± 0.1	57.8 ± 0.3	3.7 ± 0.1	11.7 ± 0.3	2.5 ± 0.1	1.1 ± 0.1
	21	14.2 ± 0.2	5.9 ± 0.1	58.8 ± 0.3	4.6 ± 0.8	11.7 ± 0.2	3.2 ± 0.1	1.5 ± 0.1
	wine	12.6 ± 0.5	5.6 ± 0.2	56.2 ± 1.7	4.1 ± 0.2	15.3 ± 2.2	4.9 ± 0.4	1.3 ± 0.1

[a] Percent composition of proanthocyanidin subunits (in moles) and with the following subunit abbreviations: –P, phloroglucinol adduct of extension subunit; EGC, (–)-epigallocatechin; C, (+)-catechin; EC, (–)-epicatechin; and ECG, (–)-epicatechin-3-*O*-gallate.

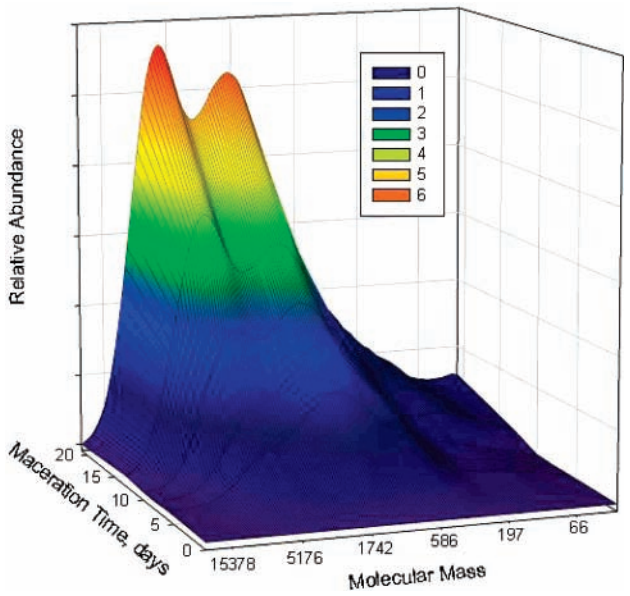


Figure 5. MM profile of phenolic polymer isolates determined by GPC and with peak areas adjusted to reflect gravimetric recovery (block A).

described (*15*). The ratio of (−)-epigallocatechin to (−)-epicatechin molar proportions in skin proanthocyanidin isolates neared 0.70 for both blocks: A = 0.67, and B = 0.68. Previously reported ratios in Pinot noir range from as low as 0.33 (*24*) to as high as 0.56 (*23*). Variation in ratios of EGC to EC may be due to variation in grape maturity at time of harvest (*39*), wine-making practice (*15*), climatic conditions during the growing season (*23*), vineyard management practice, vineyard site topography, and physical and chemical soil characteristics (*24*).

The skin proanthocyanidin proportion of phenolic polymers (**Table 3**) decreased from day 6 to day 8, more than doubled from day 8 to day 13, and decreased by day 21. The increase in extracted skin proanthocyanidin proportion seen from day 8 to 13 conflicts with previously reported data in which skin proanthocyanidin proportion decreased from day 1 to day 8 of fermentation. To provide additional information on the accuracy of data from day 6, solid-phase extraction was used to isolate phenolic polymer fractions directly from must (must isolates). Phloroglucinolysis of the must isolates resulted in similar results to those obtained for phenolic polymer isolates. Phenolic polymer must isolates from day 21 were not prepared due to inadequate sample quantity. The low skin proportion of phenolic

Table 3. Phenolic Polymer Isolate Proanthocyanidin Proportion

		proanthocyanidin	skin proanthocyanidin		pigmented polymer	
block	day	% yield[a]	% skin[b]	% skin[c]	% MDE[d,e]	MDE % w/w[d,f]
A	6	35.7 ± 1.5	40.5 ± 1.2	21.1 ± 1.3	2.67	2.17
	8	29.0 ± 2.4	21.5 ± 2.1	23.8 ± 1.6	3.00	3.30
	10	39.8 ± 1.1	43.5 ± 0.8	40.5 ± 0.1	2.88	3.37
	13	53.0 ± 1.3	43.9 ± 0.4	49.1 ± 0.7	1.67	2.05
	21	51.0 ± 1.1	37.3 ± 0.7		1.46	2.12
	wine	46.1 ± 1.6	33.5 ± 0.8		2.38	
B	6	31.0 ± 1.2	31.9 ± 1.6	25.1 ± 1.9	3.59	2.86
	8	27.0 ± 1.0	20.0 ± 0.9	19.1 ± 1.6	3.81	3.40
	10	42.9 ± 1.5	42.2 ± 0.8	42.6 ± 0.3	3.61	3.81
	13	53.8 ± 0.8	45.2 ± 0.6	45.6 ± 4.0	1.72	2.30
	21	53.2 ± 0.7	35.4 ± 0.6		1.70	2.58
	wine	41.3 ± 0.7	32.9 ± 0.5		3.82	

[a] Percent conversion yield (m/m) resultant from conversion of phenolic polymer isolates to known proanthocyanidin subunits. [b] Proportion of skin tannin in known proanthocyanidin fraction of phenolic polymer isolates. [c] Proportion of skin tannin in known proanthocyanidin fraction of must isolates. [d] Purest replicate per sample day was chosen for calculations (no error is reported). [e] Pigmented polymer per mg expressed as % malvidin-3-*O*-glucoside equivalents by mass (%MDE) as calculated by GPC. [f] Pigmented polymer expressed as % malvidin-3-*O*-glucoside equivalents by mass as calculated from SA.

polymers extracted early during fermentation could be explained by the possible effects of oxidation on proanthocyanidins that could lead to formation of new compounds that are uncharacterizable via phloroglucinolysis methods used in this experiment (see further discussion below) (*15*, *40*−*42*).

Phenolic polymer isolate conversion yields were calculated based on phloroglucinolysis results (**Table 3**). Conversion yields were lowest for both blocks on day 8 (A = 29.0%, and B = 27.0%), increased in a linear fashion (A-r^2 = 0.99, and B-r^2 = 0.95) up to day 13 (A = 53.0%, and B = 53.8%), and then slightly decreased on day 21 (A = 51.0%, and B = 53.2%). Conversion yield continued to decrease postfermentation as seen in the young wine samples (A = 46.1%, and B = 41.3%). According to Kennedy et al. (*43*), phenolic polymers isolated from prevéraison grape seeds average a conversion yield of 88%; however, following véraison, conversion yield decreases to as low as 69% at commercial grape maturity. Furthermore, Kennedy and Jones (*22*) report conversion yields as low as 56.4% for Shiraz berry skin proanthocyanidins extracted using similar chromatography (Toyopearl HW 40F). However, the phenolic polymer isolates of samples from day 6 and day 8 possess the lowest conversion yields, leaving up to 73% of block

A and 71% of block B sample material unknown by mass (day 8). This observation led to speculation that early extracted phenolic polymers would consist of proanthocyanidins modified with other grape berry components (e.g., carbohydrate or protein), a plausible explanation in that proanthocyanidins contained within the plant vacuole could be allowed to react with other cellular constituents due to compromised cellular integrity of grape cells during ripening or processing. Flavanols and proanthocyanidins have been observed to be associated with cell wall polysaccharides as seen in apples (*44*) and flower petals (*45*), contained within cell vacuoles (in free solution or linked to protein matrices), and associated with cell nuclei as seen in various tree and tea flower species (*46*). Because the diffusional barriers to extraction in these situations would be reduced, it was hypothesized that this material would be the first material to be extracted during red wine fermentation. The next portion of this investigation focused on identifying the nonproanthocyanidin-containing portion of the phenolic polymer isolates.

Phenolic Polymer Hydration. While it was considered that low molecular weight impurities made up a small proportion of the phenolic polymer isolates, additional analyses were undertaken to provide substantiating evidence.

One source of uncharacterized mass could have been due to hydration of lyophilized powders. Prior research reported up to 3.0 mol water of hydration per mol of proanthocyanidin monomer unit, representing 12−15% of overall sample weight (*47*). Dehydration experiments conducted on phenolic polymer isolates prepared in the same way (four samples in duplicate, *V. vinifera* L. cv. Merlot), drying (3 days) over P_2O_5 (2×10^{-1} Pa), resulted in 0.00−0.20% mass loss (average 0.050 ± 0.025%). Excess mass due to hydration was therefore not considered a significant source of uncharacterized mass in this experiment.

Low Molecular Weight Phenolics. Although RP HPLC results indicated that isolates contained negligible phenolic monomer contaminants, phloroglucinolysis revealed the presence of two monomeric impurities (280 nm). While the earlier eluting compound was only present in trace amounts (based upon peak area) or not present at all in some samples, the later eluting compound, based upon peak area, appeared to be a significant contaminant with $\lambda_{max} = 292$ nm. Subsequent analysis using mass spectrometry (negative ionization mode) revealed a molecular ion peak at *m*/*z* 899.3. The late-eluting compound was thus determined to be astilbin (dihydroquercetin-3-*O*-rhamnoside), a bioactive flavanonol thought to provide antimicrobial, antibacterial, cardiopreventative, and possibly chemopreventative effects in humans (*48*−*52*). Although astilbin appeared to be a significant monomeric contaminant, it was not present in all phenolic polymer extracts or in all replicates of extracts. Further investigation revealed that care in sample processing allowed for its exclusion. Calculation of astilbin's contribution to sample mass, based on its response factor at 280 nm (astilbin, 1820 peak area/μg; catechin, 1430 peak area/μg) (*52*) revealed that astilbin was not a significant contaminant, nor could it account for a major portion of the uncharacterized mass; however, its extraction did appear to be linear throughout fermentation (A-$r^2 = 0.95$, and B-$r^2 = 0.97$). The other eluting peak was not characterized; however, because of its similar UV spectrum to astilbin and $\lambda_{max} = 292$ nm, it is thought to either be an unidentified flavanonol or perhaps the 3-epimer isoastilbin (*49*).

Total Color at 520 nm and Pigmented Polymer. Two analyses were used to estimate anthocyanin and/or pigmented polymer contents of the isolates (**Table 3**). The total color was determined via the methods of Somers and Evans (*12*, *28*); however, the replicates used for GPC MM data were chosen for analysis due to sample size limitation (error not reported). The total color content was based upon the molar absorptivity of malvidin-3-*O*-glucoside ($\epsilon = 26455$; *12*) and reported in % w/w malvidin-3-*O*-glucoside equivalents ($MDE_{\%w/w}$). $MDE_{\%w/w}$ increased from sample day 6 to peak at day 10 in both blocks (A = 3.36%, and B = 3.80%). $MDE_{\%w/w}$ decreased from day 10 to 13 and then slightly increased until day 21 in both blocks. A plot of $MDE_{\%w/w}$ values to conversion yield data did not indicate a strong correlation between total color and low conversion yield (A-$r^2 = 0.46$, and B-$r^2 = 0.31$). Because the majority of anthocyanin monomers were eluted with water and methanol during phenolic polymer isolation, the color of these extracts is thought more likely to be due to the presence of pigmented polymer. A calculation of pigmented polymer extracted throughout fermentation revealed that the extraction of pigment follows a logarithmic pattern. Similar extraction of pigmented polymer throughout fermentation has previously been reported (*53*).

Table 4. Approximate Mass Balance (% w/w) for Phenolic Polymer Isolates of Block A

day	% PA[a]	% LEM[b]	% MDE[c]	% carb[d]	% total
6	35.7	34.6	2.7	6.6	79.6
8	28.9	35.3	3.0	12.8	80.0
10	39.8	28.4	2.9	4.9	76.0
13	53.0	23.7	1.7	3.6	82.0
21	51.0	17.6	1.5	4.4	74.5

[a] Percent proanthocyanidin content as determined from known subunit composition by phloroglucinolysis (PA). [b] Phloroglucinolysis LEM. [c] Pigmented polymer expressed as % malvidin-3-*O*-glucoside by GPC (% MDE). [d] Carbohydrate by GC/MS.

To more accurately determine if pigmented polymer comprised a portion of uncharacterized material, GPC was used to quantify total absorbance of phenolic polymer isolates at 520 nm. With the exception of sample day 8 (A = 3.00%, and B = 3.81% w/w), trends for pigmented polymer content assessed by GPC agree with patterns obtained from Somers assay (SA) results. A comparison of % MDE vs conversion yield indicates that lower conversion yields correlate to higher pigmented polymer content per mg of phenolic polymer extract (A-$r^2 = 0.82$, and B-$r^2 = 0.71$). Data indicating that pigmented polymer may comprise a portion of uncharacterized material from phloroglucinolysis have previously been reported (*38*). Pigment polymerization has been attributed to the logarithmic decrease of anthocyanin content during skin fermentation, a process that may in part be due to enzymatic oxidation during the early stages of fermentation (*53*). The increase in pigmented polymer observed in phenolic polymer extracts of the young wine is most likely due to transformations of anthocyanin structure that lead to formation of more stable pigmented compounds during wine aging (*7*, *8*, *54*).

Carbohydrate Analysis by GC/MS. Isolates were subjected to GC/MS carbohydrate analysis as previously described by Merkle and Poppe (*29*) and York et al. (*30*). Total carbohydrates in the phenolic polymer extract samples ranged from 3.6 to 12.8% w/w (**Table 4**). Glucose (Glc) was the major constituent along with trace amounts of arabinose (Ara), rhamnose (Rha), xylose (Xyl), glucuronic acid (GlcA), mannose (Man), and galactose (Gal). Sample days 6 and 10 had trace amounts of fucose (Fuc), C_{16} and C_{18} saturated fatty acids, and C_{18} unsaturated fatty acids, while sample day 13 had only C_{18} saturated fatty acids. Relative amounts of glycosyl residues also

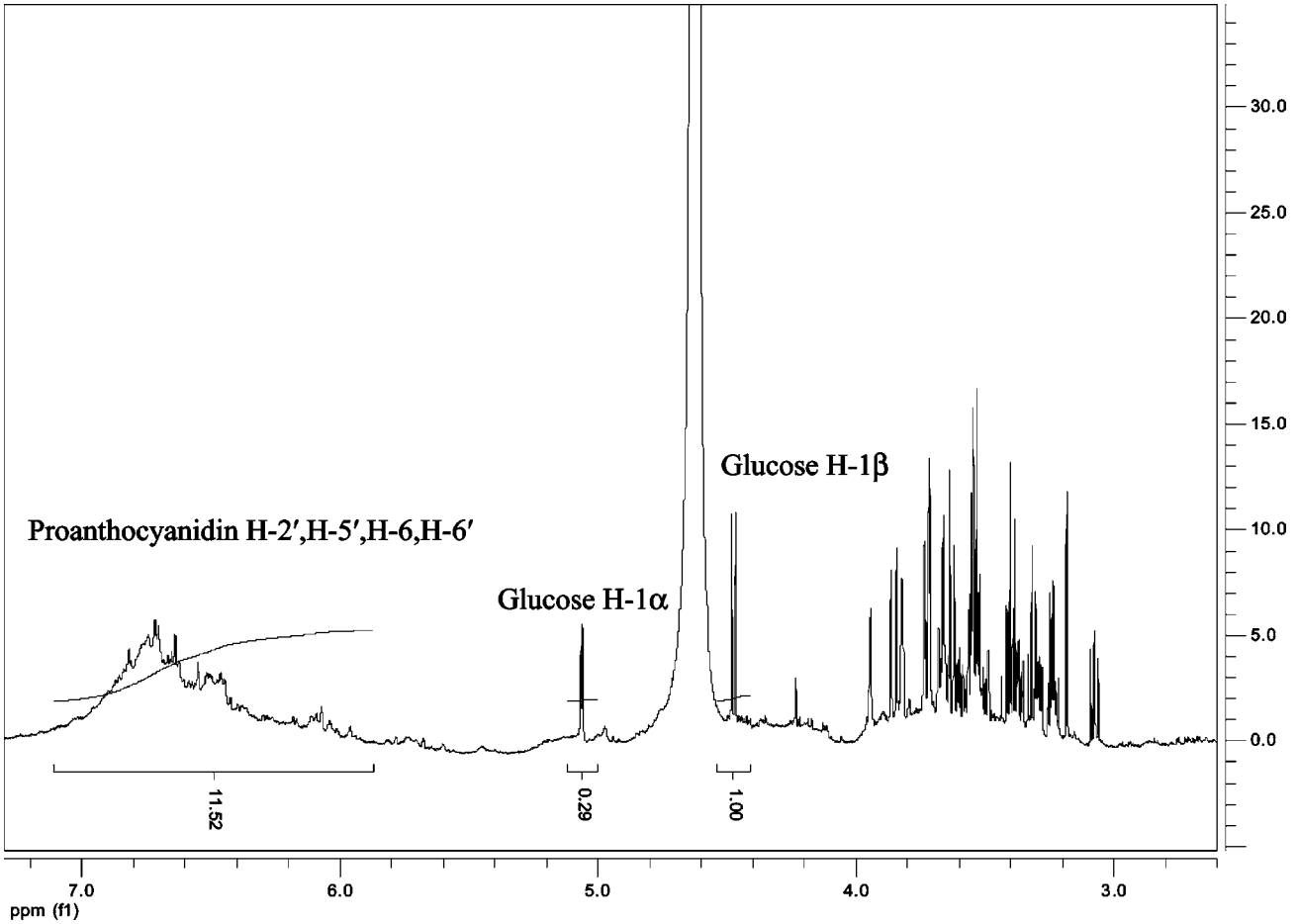


Figure 6. Phenolic polymer isolate ^{1}H NMR (block A, day 6).

Table 5. Carbohydrate ^{1}H and ^{13}C NMR Chemical Shifts

		chemical shift (ppm)[a]						
residue		1	2	3	4	5	6	6′
α-Glc	^{1}H	5.24 (5.23)	3.54 (3.54)	3.73 (3.72)	3.42 (3.42)	3.84 (3.84)	3.84 (3.84)	3.73 (3.76)
	^{13}C	92.8 (93.0)	72.0 (72.4)	73.6 (73.7)	70.6 (70.7)	72.2 (72.3)	61.3 (61.8)	
β-Glc	^{1}H	4.66 (4.64)	3.26 (3.25)	3.50 (3.50)	3.41 (3.42)	3.46 (3.46)	3.72 (3.72)	3.91 (3.90)
	^{13}C	96.6 (96.8)	74.8 (75.2)	76.6 (76.7)	70.4 (70.7)	76.6 (76.7)	61.1 (61.8)	

[a] Literature values in parentheses; ref *31*.

varied per sample and did not suggest any trends related to extraction. Results indicate that carbohydrate residues from grape cell walls (Ara, Xyl, Glc A, Gal, and Fuc), yeast autolysis (Man and fatty acids), and degradation of glycosylated flavonoids (Rha and Glc) were all present in the phenolic polymer extracts. Despite the generally low and variable glycosyl residue concentration within samples, GC/MS results indeed confirmed the presence of carbohydrate in the phenolic polymer isolates.

Monomeric Carbohydrate Analysis by HPLC. Ion exchange chromatography was also used to estimate the monomeric sugar content of block A isolates. Upon the basis of GC/MS results, sample day 8 of block A (12.8% w/w carbohydrate) and sample day 21 of block A (4.4% w/w) were chosen for representative analysis. Results indicated that the monomeric carbohydrate content for sample day 21 was below detectable concentration. Comparison of sample day 8 of block A to the glucose and fructose standards revealed the presence of both monomers at 7.7 and 6.7% by mass, respectively, indicating that the total contribution of monomeric carbohydrate to sample day 8 of block A could be as high as 14.5%. To further explore the potential contribution of carbohydrates to the phenolic polymer structure, NMR spectroscopy was utilized.

NMR Spectroscopy. Following confirmation of glycosyl residue presence in the isolates by GC/MS, NMR spectroscopy was used on an early sample for structural elucidation of oligosaccharides and glycosides that it might contain (**Figure 6**). The untreated phenolic polymer isolate of day 6 (block A) exhibited several broad peaks thought to arise from proanthocyanidin (H-2′, H-5′, H-6, and H-6′). Proton and chemical shifts for both proanthocyanidins and carbohydrate peaks are in agreement with previously reported values (*22*, *31*) (**Table 5**).

Results of the two-dimensional experiments provided information that enabled complete assignment of the carbohydrate peaks. Free glucose appeared to represent a majority of the carbohydrate peaks, with proton and chemical shifts consistent with α- and β-forms of glucose. The signals between 3 and 4 ppm are consistent with the nonanomeric protons of α- and β-glucose. The downfield proanthocyanidin H-3 signal can be explained by the presence of the acyl substituted (−)-epicat-

Table 6. Analysis of Total Phenolics Using Various Analytical Methods

		GPC[a]				
block	day	CE/mL wine	CE/mg	E_{280}[b,c]	E_{280}/L[b,d]	% GA[e]
A	6	0.26 ± 0.01	1.2 ± 0.1	126.1	27.3	69.0 ± 2.1
	8	0.45 ± 0.01	1.3 ± 0.1	146.7	51.1	69.8 ± 1.8
	10	0.52 ± 0.01	1.3 ± 0.1	155.2	64.8	79.5 ± 0.4
	13	0.81 ± 0.02	1.3 ± 0.1	141.4	88.0	82.5 ± 0.9
	21	1.3 ± 0.03	1.4 ± 0.1	145.7	144.1	83.9 ± 0.6
	wine	1.5 ± 0.02	1.3 ± 0.1			81.9 ± 1.7
B	6	0.21 ± 0.01	1.2 ± 0.1	147.3	25.2	68.9 ± 0.8
	8	0.37 ± 0.01	1.3 ± 0.1	168.6	47.6	69.9 ± 1.9
	10	0.58 ± 0.02	1.2 ± 0.1	147.7	70.2	76.2 ± 1.9
	13	0.93 ± 0.01	1.3 ± 0.1	141.4	102.3	83.7 ± 0.6
	21	1.1 ± 0.02	1.3 ± 0.1	148.8	139.5	82.9 ± 0.6
	wine	1.7 ± 0.03	1.3 ± 0.1			75.7 ± 0.6

[a] (+)-Catechin equivalents (CE) at 280 nm minus monomeric contaminants. [b] Replicate containing the least amount of low molecular weight phenolic material was selected for each sample day (no error is reported). [c] Absorbance of aqueous solutions of phenolic polymer isolates expressed as a 1% w/v solution. [d] Absorbance of aqueous solutions of phenolic polymer isolates expressed as a 1% w/v solution per liter of must (E_{280}/L). [e] FC phenolic polymer isolate absorbance at 765 converted to % of gallic acid response.

echin-3-*O*-gallate and appears to contribute to a minor fraction of the sample, consistent with phloroglucinolysis results. Minor peaks at 1 (singlet), 1.1 (singlet), and between 2 and 2.6 ppm (multiplets) are consistent with amino acids or fatty acids. Small amounts of amino acids could also possibly be underneath the nonanomeric proton peaks of glucose, but no conclusive evidence confirmed this. Integration of H-2′, H-5′, H-6, H-6′, H-1 α-, and H-1 β-glucose protons estimated a mass ratio of 3.5:1 (proanthocyanidin:glucose) for the polymer isolate of day 6 (block A). Generally, quantitative analysis of samples may be accomplished through integration of proton peak intensities of pure samples. However, while integrated intensities of proton resonances are proportional to proton number within a molecule, as complexity (heterogeneity) of a sample increases, the accurate integration of one-dimensional (1D) NMR signals requires careful optimization of several data acquisition parameters (*55*). The optimization of 1D NMR parameters during this experiment was adjusted for qualitative analysis and not quantitative analysis; thus, the resultant integration ratios found in polymer isolate from day 6 (block A) overestimate the glucose contribution to the sample (*56*).

To further explore the association of carbohydrate material with the phenolic polymer, ultrafiltration in combination with NMR was employed. Ultrafiltration (10 kDa MWCO) further fractionated the sample material into retentate (MW above 10 kDa) and permeate (MW below 10 kDa) fractions, giving confirmation that the majority of the carbohydrate present was of low molecular weight.

Subsequent ultrafiltration of the low molecular weight fraction (1000 MWCO) was done in an attempt to separate tannin-bound glucose from free glucose. Results of this experiment failed to provide conclusive evidence of covalent linkage between carbohydrate and proanthocyanidin.

The association of polysaccharide−proanthocyanidin covalent linkage has previously been evidenced from data acquired via electrospray ionization mass spectrometry (ESI-MS) (*39*). Although the results of dialysis did not exclude the possibility that a small amount of proanthocyanidin-bound carbohydrate was present, it was apparent that other methods would be necessary to isolate the conjugates, if present.

General Phenolic Determination (Table 6). If not due to the presence of pigmented polymer, low molecular weight phenolic and carbohydrate contaminants, or hydration, low phloroglucinolysis conversion yields could also result from the presence of phenolic oxidation products. Although oxidation products would not be directly characterizable via phloroglucinolysis (*57*), they may be detectable via analytical methods that simply measure total phenolic content. To determine whether or not a portion of the uncharacterized material of the phenolic polymer isolates was indeed phenolic, four different analyses were conducted to measure total phenolics: RP HPLC, GPC, SA, and the FC assay.

RP HPLC analysis indicated that total phenolics at 280 nm per mg of isolate increased from day 6 to day 8 in both blocks and then remained relatively unchanged throughout the remainder of fermentation, suggesting the samples from day 8 contain more phenolic material by mass than samples from day 6 and that the samples from day 8 to day 21 contain nearly equivalent proanthocyanidin concentration by mass (data not shown).

GPC results (**Table 6**) revealed that total phenolics at 280 nm per mg [(+)-catechin equivalents/mg] of phenolic polymer extract was lowest for sample day 6, increased by sample day 8, decreased by day 10, and increased again until day 21. Despite having the lowest conversion yields, both GPC and RP results indicate that sample day 8 isolates of both blocks contain more phenolic material by mass than other sample days.

The absorbance of the phenolic polymers was determined by UV/vis spectrophotometry at 280 nm (*12*, *28*). Total phenolic values were reported as absorbance of a 1% solution at 280 nm (E_{280}, 1 cm optical path length). The replicates used for GPC MM data were chosen for analysis due to sample size limitations. Upon the basis of overall absorbance, the phenolics per mL of must or wine increased linearly from day 6 to day 21 in both blocks (A-$r^2 = 0.99$, and B-$r^2 = 0.95$), which is consistent with mass recovery, GPC, and RP data. However, E_{280} values did not increase in a linear fashion from day 6 to day 21. E_{280} values increased from day 6 ($E_{280} = 126$, and $E_{280} = 147$) to day 8 in both blocks; yet, trends from day 8 to day 10 were inconsistent by block. Block A E_{280} remained somewhat constant from day 8 until day 21 ($E_{280} = 147$, and $E_{280} = 146$), while block B E_{280} decreased from day 8 to day 10 ($E_{280} = 169$ to $E_{280} = 148$) and then remained somewhat constant until day 21 ($E_{280} = 149$). The E_{280} values from this experiment were compared to Czochanska et al. (*47*). According to Czochanska et al., a plot of E_{280} (1% solution, 1 cm optical path length) vs mole fraction of procyanidin or prodelphinidin should result in a straight line to yield values of E_{280} of 130 and 62 for the λ_{max} band of 270−280 nm for pure procyanidin and prodelphinidin polymers, respectively. The procyanidin mole fraction for each sample date was calculated based on the results of phloroglucinolysis. Expected E_{280} values were then estimated based on these values and compared to experimental E_{280} values. All but one sample point (day 8, block B) fell within the range of variance recorded by Czochanska, suggesting that phenolic polymer isolates were mostly phenolic in nature. Results of total phenolics at 280 nm also corroborated both GPC and RP results, suggesting that sample day 8 phenolic polymer isolates contain more phenolic material by mass despite having lower conversion yields than sample day 6 isolates.

The FC assay was used to gain further insight regarding phenolic polymer isolate composition (*27*, *58*). FC results indicated that the material response to the FC reagent increased linearly from day 8 (A = 69.8% GA, and B = 69.9% GA) to day 13 (A-$r^2 = 0.84$, and B-$r^2 = 0.99$) and then plateaued until

Table 7. Phloroglucinol LEM

block	day	% LEM[a]	block	day	% LEM[a]
A	6	34.6 ± 1.4	B	6	39.3 ± 7.6
	8	35.3 ± 0.8		8	39.4 ± 1.4
	10	28.4 ± 0.3		10	25.9 ± 2.6
	13	23.7 ± 0.6		13	22.2 ± 0.6
	21	17.6 ± 0.3		21	17.6 ± 0.1
	wine	22.0 ± 0.4		wine	20.5 ± 0.9

[a] Percent LEM (% LEM) by weight of phenolic polymer extract.

the end of fermentation in both blocks (A = 83.9% GA, and B = 83.0% GA), data that are inconsistent with GPC, RP, and SA results.

A comparison of % GA to conversion yield indicated a strong correlation between low conversion yield and low % GA (A-r^2 = 0.85, and B-r^2 = 0.97) in the block A and B phenolic polymer isolates, whereas very low correlation was found between low conversion yield and results of the other total phenols assays. This correlation may be due to the nature of the FC assay, in that it is a phenolic assay whose basic mechanism is an oxidation/reduction reaction in which the phosphotungstic/phosphomolybdic reagent oxidizes phenols. As such, the presence of oxidized proanthocyanidins in the phenolic polymer isolates would thus be expected to result in their containing less observed "total phenolic" material by mass (*59*−*61*).

Proposed Proanthocyanidin Oxidation. Under the assumption that modification of phenolic polymer extracts by oxidation would result in an increase in cleavage resistant material, LEM was monitored and its area was quantified (**Figure 6** and **Table 7**). This was based upon results from a previous study (*57*) that indicated that LEM increased dramatically following controlled proanthocyanidin oxidation.

Values of LEM peak area were then determined in (+)-catechin equivalents and then compared to the original powder and expressed as % LEM (w/w). Consistent with phloroglucinolysis conversion yield, % LEM was highest on day 8 (A = 35.3% LEM, and B = 39.4% LEM), then decreased until day 21 for both blocks (A = 17.6% LEM, and B = 17.6% LEM). A comparison of conversion yield to % LEM revealed that low conversion yield correlates well with higher % LEM (A-r^2 = 0.84, and B-r^2 = 0.94).

Calculation of LEM contribution to total mass extraction per liter of must or wine during fermentation confirmed that although this material increased throughout fermentation, the relative proportion that it contributed to the overall must or wine decreases, suggesting that the majority of material comprising the LEM is extracted or produced early during maceration (**Figure 3**). A proportional decrease in LEM would be expected with progressive fermentation due to CO_2 evolution and/or the accumulation of ethanol that hinders enzymatic oxidation (*62*).

Unpublished work (Kennedy) indicates that material comprising the LEM may result from oxidation of catechols to *o*-quinones within the berry. Here, grape seed procyanidin isolated from grapes (*V. vinifera* L. cv. Shiraz) at commercial harvest underwent phloroglucinolysis (*22*). The material resistant to acid-catalyzed cleavage was purified and reacted with *o*-phenylenediamine (*41*). *o*-Phenylenediamine reacts with *o*-quinones to produce phenazines measurable at 450 nm (*41*, *63*). The reaction of resistant seed material with *o*-phenylenediamine produced an increase at 450 nm indicating the presence of *o*-quinones.

Evidence consistent with oxidation suggests that grape proanthocyanidin oxidation may occur in musts or berries that have begun to deteriorate due to infection, physiological changes (*64*), and enzymatic and chemical oxidation incurred during wine processing (*62*, *65*, *66*). Proanthocyanidins are key substrates for oxygen in musts and wine (*66*), with proanthocyanidin structure determining ease of oxidation (*65*). It could thus be postulated that (−)-epigallocatechin, being more susceptible to oxidation than other subunits, becomes oxidized early during fermentation to result in lower detectable molar quantities by phloroglucinolysis, and thus would explain the low apparent proportion of skin tannin in the early samples as measured by phloroglucinolysis.

Although quinonic forms found in wine may be produced via either chemical or enzymatic oxidation, those formed via enzymatic oxidation of grapes or must early in winemaking could have more time to polymerize and react to produce new compounds such as those potentially comprising the LEM (*42*, *62*, *66*). The role of enzymatic oxidation in the formation of novel fruit-derived phenolics has been reported by others (*41*, *42*, *67*, *68*).

In total, the results of this study indicate that phenolic polymers isolated from wine during fermentation are significantly different than those isolated from exhaustive extraction of grape berry tissues (*22*, *25*, *39*). Summation of accounted for phenolic polymer isolate material indicates that roughly 80% of each sample can be accounted for by mass (**Table 4**). While this figure accounts for a significant portion of the material, further investigation is still needed to more accurately qualify and quantify compounds comprising the LEM. Moreover, despite preliminary evidence in the data that significant oxidation was present early in wine production, further examination is necessary to better ascertain the roles of both chemical and enzymatic oxidation during both fruit ripening and wine production.

ACKNOWLEDGMENT

We recognize Drs. Parastoo Azadi and Christian Heiss of the Complex Carbohydrate Research Center for their analytical services. We also thank Willakenzie Estates Winery of Yamhill, OR, for their collaboration and assistance in the commercial production of the wine used in this study.

LITERATURE CITED

(1) Marais, J. P. J.; Deavours, B.; Dixon, R. A.; Ferreira, D. The stereochemistry of the flavonoids. In *The Science of Flavonoids*; Grotewold, E., Ed.; Springer Science and Business Media, Inc.: Columbus, Ohio, 2006; pp 1−46.

(2) Ribéreau-Gayon, P. *Plant Phenolics*; Hafner Publishing Company: New York, New York, 1972; p 254.

(3) Haslam, E. *Practical Polyphenols: From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, England, 1998; p 422.

(4) Saucier, C.; Bourgeois, G.; Vitry, C.; Roux, D.; Glories, Y. Characterization of (+)-catechin-acetaldehyde polymers: A model for colloidal state of wine polyphenols. *J. Agric. Food Chem.* **1997**, *45*, 1045−1049.

(5) Zimman, A.; Waterhouse, A. L. Incorporation of malvidin-3-glucoside into high molecular weight polyphenols during fermentation and wine aging. *Am. J. Enol. Vitic.* **2004**, *55*, 139−146.

(6) Boulton, R. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *Am. J. Enol. Vitic.* **2001**, *52*, 67−87.

(7) Bakker, J.; Timberlake, C. F. Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.* **1997**, *45*, 35−43.

(8) Hayasaka, Y.; Asenstorfer, R. E. Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 756−761.
(9) Dallas, C.; Ricardo-da-Silva, J. M.; Laureano, O. Products formed in model wine solutions involving anthocyanins, procyanidin B2, and acetaldehyde. *J. Agric. Food Chem.* **1996**, *44*, 2402−2407.
(10) Cheynier, V. Polyphenols in foods are more complex than often thought. *Am. J. Clin. Nutr.* **2005**, *81*, 223S−229S.
(11) Gagne, S.; Saucier, C.; Geny, L. Composition and cellular localization of tannins in Cabernet Sauvignon skins during growth. *J. Agric. Food Chem.* **2006**, *54*, 9465−9471.
(12) Somers, T. C.; Evans, M. E. Spectral evaluation of young red wines: Anthocyanin equilibria, total phenolics, free and molecular SO_2,"Chemical age". *J. Sci. Food Agric.* **1977**, *28*, 279−287.
(13) Gawel, R. Red wine astringency: A review. *Aust. J. Grape Wine Res.* **1998**, *4*, 74−95.
(14) Singleton, V. L. Tannins and the qualities of wines. In *Plant Polyphenols: Synthesis Properties Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum: New York, 1992; Vol. 59.
(15) Peyrot des Gachons, C.; Kennedy, J. A. Direct method for determining seed and skin proanthocyanidin extraction into red wine. *J. Agric. Food Chem.* **2003**, *51*, 5877−5881.
(16) Wightman, J. D.; Price, S. F.; Watson, B. T.; Wrolstad, R. E. Effect of processing enzymes on anthocyanins and phenolics in pinot noir and cabernet sauvignon wines. *Am. J. Enol. Vitic.* **1995**, *46*, 404.
(17) Sacchi, K. L.; Bisson, L. F.; Adams, D. O. A review of the effect of winemaking techniques on phenolic extraction in red wines. *Am. J. Enol. Vitic.* **2005**, *56*, 197−206.
(18) Ribéreau-Gayon, P.; Glories, Y. Phenolics in grapes and wines. *Proceedings of Sixth Australian Wine Industry Technical Conference, 1986*; Lee, T., Ed.; AIP: Adelaide, South Australia, 1986; pp 247−256.
(19) Ribéreau-Gayon, P.; Sudraud, P.; Milhe, J. C.; Canbas, A. Recherches technologiques sur les composes phenoliques des vins rouges. *Connaissance Vigne Vin.* **1970**, *4*, 133−144.
(20) Ribéreau-Gayon, P.; Dubourdieu, D.; Doneche, B.; Lonvaud, A. *The Handbook of Enology. The Microbiology of Wine and Vinifications*, 2nd ed.; John Wiley and Sons Ltd.: West Sussex, England, 2006; Vol. 1, p 497.
(21) Singleton, V. L.; Noble, A. C. Wine flavor and phenolic substances. In *Phenolic, Sulfur, and Nitrogen Compounds in Food Flavors*; Charalambous, G., Katz, I., Eds.; American Chemical Society: Washington, DC, 1976; pp 47−70.
(22) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740−1746.
(23) Pastor del Rio, J. L.; Kennedy, J. A. Development of proanthocyanidins in *Vitis vinifera* L. cv. Pinot noir grapes and extraction into wine. *Am. J. Enol. Vitic.* **2006**, *57*, 125−132.
(24) Cortell, J. M.; Halbleib, M.; Gallagher, A. V.; Righetti, T. L.; Kennedy, J. A. Influence of vine vigor on grape (*Vitis vinifera* L. cv. Pinot noir) and wine proanthocyanidins. *J. Agric. Food Chem.* **2005**, *53*, 5798−5808.
(25) Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J. Chromatogr. A* **2003**, *995*, 99−107.
(26) Lamuela-Raventos, R. M.; Waterhouse, A. L. A direct HPLC separation of wine phenolics. *Am. J. Enol. Vitic.* **1994**, *45*, 1−5.
(27) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
(28) Somers, T. C.; Evans, M. E. Wine quality: Correlations with colour density and anthocyanin equilibria in a group of young red wines. *J. Sci. Food Agric.* **1974**, *7*, 203−208.
(29) Merkle, R. K.; Poppe, I. Carbohydrate composition analysis of glycoconjugates by gas-liquid chromatography/mass spectrometry. In *Methods in Enzymology*; Lennarz, W. J., Hart, G. W., Eds.; Academic Press, Inc.: Orlando, FL, 1994; Vol. 230, pp 1−15.
(30) York, W. S.; Darvill, A. G.; McNeil, M.; Stevenson, T. T.; Albersheim, P. Isolation and characterization of plant cell walls and cell wall components. In *Methods in Enzymology*; Academic Press, Inc.: Orlando, Florida, 1986; Vol. 118, pp 3−40.
(31) Agrawal, P. K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* **1992**, *31*, 3307−3330.
(32) Remy, S.; Fulcrand, H.; Labarbe, B.; Cheynier, V.; Moutounet, M. First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *J. Sci. Food Agric.* **2000**, *80*, 745−751.
(33) Iijima, K.; Yoshizumi, M.; Hashimoto, M.; Kim, S.; Eto, M.; Ako, J.; Liang, Y. Q.; Sudoh, N.; Hosoda, K.; Nakahara, K.; Toba, K.; Ouchi, Y. Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin a gene. *Circulation* **2000**, *101*, 805−811.
(34) Souquet, J.-M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.
(35) Bissell, P.; Steans, G.; Ewart, A. A study of colour development in Pinot noir wines. *Aust. Wine Ind. J.* **1989**, *4*, 58−61.
(36) Zoecklein, B. An overview of maceration during red winemaking. *Wine Ind. J.* **1991**, 265−267.
(37) Gonzales-Manzano, S.; Santos-Buelga, C.; Perez-Alonso, J. J.; Rivas-Gonzalo, J. C.; Escribano-Bailon, M. T. Characterization of the mean degree of polymerization of proanthocyanidins in red wines using liquid chromatography-mass spectrometry (LC-MS). *J. Agric. Food Chem.* **2006**, *54*, 4326−4332.
(38) Kennedy, J. A.; Hayasaka, Y. Compositional investigation of pigmented tannin. In *Red Wine Color, Exploring the Mysteries*; Waterhouse, A. L., Kennedy, J. A., Eds.; ACS Division of Agricultural and Food Chemistry: Washington, DC, 2004; pp 247−264.
(39) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348−5355.
(40) Lopez-Serrano, M.; Ros, Barcelo, A. Reversed phase and size exclusion chromatography as useful tools in the resolution of peroxidase-mediated (+)-catechin oxidation products. *J. Chromatogr.* **2001**, *919*, 267−273.
(41) Tanaka, T.; Kondou, K.; Kouno, I. Oxidation and epimerization of epigallocatechin in banana fruits. *Phytochemistry* **2000**, *53*, 311−316.
(42) Lopez-Serrano, M.; Ros Barcelo, A. Comparative study of the products of the peroxidase-catalyzed and the polyphenoloxidase-catalyzed (+)-catechin oxidation. Their possible implications in strawberry (frafaria x ananassa) browning reactions. *J. Agric. Food Chem.* **2002**, *50*, 1218−1224.
(43) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry* **2000**, *55*, 77−85.
(44) Renard, C. M.; Baron, G. C.; Guyot, S.; Drilleau, J. F. Interactions between apple cell walls and native apple polyphenols: Quantification and some consequences. *Int. J. Biol. Macromol.* **2001**, *29*, 115−125.
(45) Markham, K. R.; Gould, K. S.; Ryan, K. G. Cytoplasmic accumulation of flavonoids in flower petals and its relevance to yellow flower colouration. *Phytochemistry* **2001**, *58*, 403−413.
(46) Pinelo, M.; Arnous, A.; Meyer, A. S. Upgrading of grape skins: Significance of plant cell-wall structural components and extraction techniques for phenol release. *Trends Food Sci. Technol.* **2006**, 1−12.
(47) Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J.C.S. Perkin I* **1980**, 2278−2286.

(48) Landrault, N.; Larronde, F.; Delaunay, J.-C.; Castagninon, C.; Vercauteren, J.; Merillon, J.-M.; Gasc, F.; Cros, G.; Teissedre, P.-L. Levels of stilbene oligomers and astilbin in french varietal wines and in grapes during noble rot development. *J. Agric. Food Chem.* **2002**, *50*, 2046−2052.

(49) Du, Q.; Li, L.; Jerz, G. Purification of astilbin and isoastilbin in the extract of smilax glabra rhizome by high-speed counter-current chromatography. *J. Chromatogr. A* **2005**, *1077*, 98−101.

(50) Souquet, J. M.; Labarbe, B.; Le Guerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(51) Vitrac, X.; Castagnino, C.; Waffo-Teguo, P.; Delaunay, J.-C.; Vercauteren, J.; Monti, J.-P.; Deffieux, G.; Merillon, J.-M. Polyphenols newly extracted in red wine from southwestern france by centrifugal partition chromatography. *J. Agric. Food Chem.* **2001**, *49*, 5934−5938.

(52) Singleton, V. L.; Trousdale, E. White wine phenolics: Varietal and processing differences as shown by HPLC. *Am. J. Enol. Vitic.* **1983**, *34*, 27−34.

(53) Bakker, J.; Preston, N. W.; Timberlake, C. F. The determination of anthocyanins in aging red wines: Comparison of HPLC and spectral methods. *Am. J. Enol. Vitic.* **1986**, *37*, 121−126.

(54) Dallas, C.; Ricardo-Da-Silva, J. M.; Laureano, O. Interactions of oligomeric procyanidins in model wine solutions containing malvidin-3-glucoside and acetaldehyde. *J. Sci. Food Agric.* **1996**, *70*, 493−500.

(55) Pauli, G. F.; Jaki, B. U.; Lankin, D. C. Quantitative 1H NMR: Development and potential of a method for natural products analysis. *J. Nat. Prod.* **2005**, *68*, 133−149.

(56) Pauli, G. F. qNMR-A versatile concept for the validation of natural product reference compounds. *Phytochem. Anal.* **2001**, *12*, 28−42.

(57) Jorgensen, E. M.; Marin, A. B.; Kennedy, J. A. Analysis of the oxidative degradation of proanthocyanidins under basic conditions. *J. Agric. Food Chem.* **2004**, *52*, 2292−2296.

(58) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(59) Bors, W.; Michel, C.; Stettmaier, K. Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch. Biochem. Biophys.* **2000**, *374*, 347−355.

(60) Castillo, J.; Benavente-Garcia, O.; Lorente, J.; Alcaraz, M. J.; Redondo, A.; Ortuno, A.; Del Rio, J. A. Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by x-rays of flavan-3-ols (procyanidins) from grape seeds (*Vitis vinifera*): Comparative study versus other phenolic and organic compounds. *J. Agric. Food Chem.* **2000**, *48*, 1738−1745.

(61) Waterhouse, A. L.; Laurie, V. F. Oxidation of wine phenolics: A critical evaluation and hypothesis. *Am. J. Enol. Vitic.* **2006**, *57*, 306−313.

(62) de Gaulejac, N. V.; Vivas, N.; Nonier, M.-F.; Absalon, C.; Bourgeois, G. Study and quantification of monomeric flavan-3-ol and dimeric procyanidin quinonic forms by HPLC/ESI-MS. Application to red wine oxidation. *J. Sci. Food Agric.* **2001**, *81*, 1172−1179.

(63) Witter, R. F.; Snyder, J.; Stotz, E. Colorimetric determination of acetylacetone and related β-diketones. *J. Biol. Chem.* **1948**, 493−500.

(64) Kennedy, J. A.; Troup, G. J.; Pilbrous, J. R.; Hutton, D. R.; Hewitt, D.; Hunter, C. R.; Ristic, R.; Iland, P. G.; Jones, G. P. Development of seed polyphenols in berries from *Vitis vinifera* L. cv. Shiraz. *Aust. J. Grape Wine Res.* **2000**, *6*, 5348−5355.

(65) deFreitas, V. A. P.; Glories, Y.; Laguerre, M. Incidence of molecular structure in oxidation of grape seed procyanidins. *J. Agric. Food Chem.* **1998**, *46*, 376−382.

(66) 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.

(67) Sojo, M. M.; Nunez-Delicado, E.; Garcia-Carmona, F.; Sanchez-Ferrer, A. Partial purification of a banana polyphenol oxidase using triton X-114 and PEG 8000 for removal of polyphenols. *J. Agric. Food Chem.* **1998**, *46*, 4924−4930.

(68) Sanchez-Ferrer, A.; Bru, R.; Valero, E.; Garcia-Carmona, F. Changes in pH-dependent grape polyphenoloxidase activity during maturation. *J. Agric. Food Chem.* **1989**, *37*, 1242−1245.

Received for review February 18, 2007. Revised manuscript received May 10, 2007. Accepted May 10, 2007. We gratefully acknowledge the Northwest Center for Small Fruits Research for financial support.

JF0704817