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New Phenolic Grape Skin Products from *Vitis vinifera* cv. Pinot Noir

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Anthocyanins and their related compounds were extracted from grape skins of Pinot noir, using 50% aqueous methanol, and purified by solid phase extraction chromatography using XAD-7 resin to obtain a pigment-rich fraction. This fraction was subjected to multilayer coil countercurrent chromatography (MLCCC) using a quaternary solvent system consisting of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water acidified with 0.01% trifluoroacetic acid (2:2:0.1−1.8:5) (v/v/v/v) in a step gradient elution to separate anthocyanin oligomers from grape anthocyanins. In the process of the characterization of the MLCCC fractions by electrospray mass spectrometry, two noncolored anthocyanin derivatives were found and characterized on the basis of their mass spectral data. As a result, these compounds have been tentatively identified as coupling products between both hydrated malvidin-3-glucoside and peonidin-3-glucoside, with 2-*S*-glutathionyl caffeoyl tartaric acid (GRP). It is therefore proposed that grape skins contain this new class of coupling product, and a possible chemical pathway for their formation is suggested.

KEYWORDS: Grape skins; Pinot noir; anthocyanins; grape reaction product (GRP); glutathione (GSH); caftaric acid; multilayer coil countercurrent chromatography (MLCCC)

INTRODUCTION

Red wine color is important to its quality, and this is borne out by wine show judges who use color as a quality indicator (*1−5*). The color of a wine is influenced by the anthocyanin content, which is readily extracted from the skins of red grapes. Polyphenol oxidase (PPO) is active in grape skins, and its influence is obvious immediately after crushing grapes in the presence of oxygen (*6*). Caffeoyl and *p*-coumaroyl tartrates are the most abundant hydroxycinnamic derivatives in grapes, and these are good substrates for PPO oxidation (tyrosinase and laccase) (*7*,*8*). Reaction of these enzymes with the hydroxycinnamic derivatives produces quinones which are able to react with glutathione (**1**) (GSH) (**Figure 1**) to furnish colorless 2-*S*-glutathionyl caffeoyl tartaric acid (**2**), commonly referred to as grape reaction product (GRP) (**Figure 1**) (*9*, *10*). These enzymes (laccase in particular) can further react with the GRP to afford higher order quinones, which can also lead to increased color (*11*). Finally, it has been reported that the disappearance of anthocyanins may occur due to interactions with quinones formed by the action of PPO on certain phenolic substrates (*12*). The investigation of GRP in red grapes/wines has received little attention, which is surprising as these products (*11*) are in abundance. Anthocyanins exist in several pH dependent forms. They can act both as electrophiles and as nucleophiles (in the noncolored hemiacetal form) at wine pH (*13*, *14*).

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Multilayer coil countercurrent chromatography (MLCCC) combined with a stepwise gradient elution program has allowed for the separation of the different classes of grape skin anthocyanins (*15*, *16*). After exhaustive elution of the major grape anthocyanins into the mobile phase it has been demonstrated that pigmented materials remain in the stationary phase, which is devoid of grape anthocyanins (monomers) but contains anthocyanin oligomers, dimers and trimers (*16*).

In the present study, we repeated the isolation of anthocyanin oligomers from Pinot Noir grape skin extract using a similar MLCCC method to one in the literature, and report the tentative identification of a new class of compounds using mass spectrometric techniques.

MATERIALS AND METHODS

Solvents. All solvents used were purchased from Merck (Darmstadt, Germany) and were of HPLC grade or higher. The resin XAD-7 was purchased from Sigma (St. Louis, MO, USA). Milli Q water was generated in the laboratory through a Millipore system.

Grapes. *Vitis vinifera* grapes from the cultivar Pinot Noir were sourced from a commercial vineyard at Mt Barker in the Adelaide Hills region, South Australia. The grapes were harvested on March 3, 2007, when total soluble solids (TSS), pH and titratable acidity (TA) were 24.3 °Brix, 3.36 and 9.0 g/L, respectively.

Grape Skins. Twelve kilograms of grapes was used for skin isolation. The grapes were stored at 0 °C and were hand peeled until 600 g of fresh skins had been collected. The fresh skins were stored at −18 °C until needed. A 500 g aliquot of skins was frozen in liquid nitrogen and ground using a ceramic mortar and pestle.

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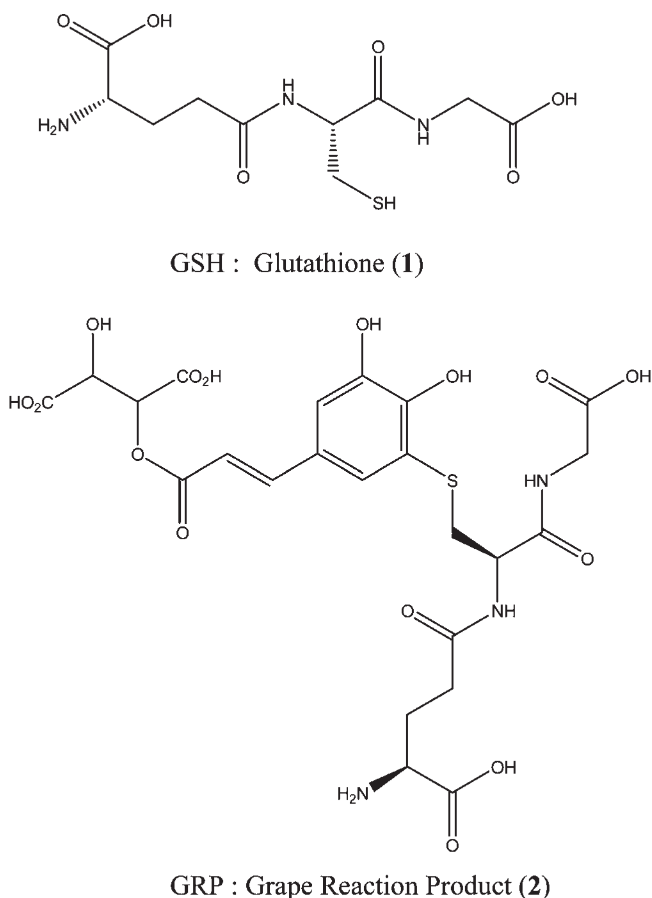

Figure 1. Glutathione thiol (**1**) and grape reaction product (**2**).

Skin Extract Preparation. The powdered skins (250 g) were suspended in 50% aqueous methanol acidified with 2% acetic acid (v/v) (1.0 L). The mixture was mechanically stirred at 200 rpm at ambient temperature for 65 h and then centrifuged at 10,000 rpm for 15 min at 5 °C. The supernatant was decanted and centrifuged again under the same conditions. After the removal of solvent *in vacuo*, the skin extract was lyophilized and then reconstituted with water (250 mL) and loaded onto Amberlite XAD-7 resin (120 mL bed volume) which had been prepared as described by Kreamer-Schafhalter et al. (*17*). The column was eluted with water until the refractive index of the eluant was zero. The remaining materials on the column were eluted with 2 bed volumes (240 mL each) of 25%, 50%, 75% methanol in water and finally 100% methanol. The 100% methanol fraction was lyophilized after removal of methanol *in vacuo*. The resultant red powder was directly used in the MLCCC separation.

MLCCC System. The MLCCC system was a Quattro (Analytical and Environmental Consultancy Services (AECS), Bridgend, S. Wales, United Kingdom) equipped with an analytical set of coils, comprising four coils on two holders (bobbins of 190 mm diameter) with each bobbin composed of two coils (1.6 mm i.d. Teflon tubing) with volume capacity of both 100 mL (outer coil) and 250 mL (inner coil). The temperature of the cabinet containing the coils was controlled by a Ratek circulated cooling device. The whole system was equipped with a pressure relief valve (250 psi), set upstream of the bobbins. The samples were injected into the system through a Rheodyne injection loop and the flow rate was controlled by a Waters 600E pump. The elution was monitored by two detectors, a UPC-900 detector (Amersham Pharmacia Biotech, Sweden) at 546 nm, and a GBC diode array detector at 280 nm. When the coils were not connected in series, those not in use were filled with water as a counter balance for each bobbin.

MLCCC Conditions. Step gradient elution mode described by Vidal et al. (*15*) was applied with some modifications for the elution mode. The quaternary solvent system used consisted of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water, acidified with 0.01% trifluoroacetic acid (2/2/0.1−1.8/5). The lower phase (aqueous layer) was employed as the stationary phase, and as a result a tail to head (T−H) mode was adopted. The skin extract (250 mg) was dissolved in 5 mL of a mixture of the upper and lower phases (1:1) and injected through a 5 mL loop into the 100 mL coil. The flow rate was set at 2 mL/min, and the fraction collector was set to collect 10 mL fractions. The system was operated at 800 rpm, and the temperature was set at 25 °C. The instrument was stopped after 290 min, and the stationary phase was pumped out of the coil by pumping water into the coil at a flow rate of 2 mL/min. The solvent was evaporated *in vacuo*, and residual liquid was lyophilized to a powder.

High Performance Liquid Chromatography (HPLC). Analysis of grape anthocyanins was carried out as described by Cozzolino et al. (*18*) using an Agilent 1100 HPLC system equipped with a quaternary pump, degasser, autosampler, column oven and photodiode array (PDA) detector.

Electrospray Mass Spectrometry (ESI-MS). Mass spectrometric analysis was carried out using a 4000 Q Trap hybrid tandem mass spectrometer equipped with a Turbo ion spray source (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Positive ion mass spectra were recorded in an appropriate mass range. Nitrogen gas was employed for the curtain, nebulizer, turbo and collision gases. The turbo ion source parameters were set at 5500 V for the ion spray voltage, 60 V for the declustering potential and 10 V for the entrance potential. The pressures of the nebulizer, turbo and curtain gases were set at 8, 0, and 10 psi, respectively, for direct infusion experiments and 50, 50, and 15 psi, respectively, for liquid chromatography−tandem mass spectrometry (LC−MS/MS) experiments. The temperature of the turbo gas was set at 500 °C for the LC−MS/MS experiment. For tandem mass spectrometry (MS/MS), the collision potential was optimized in a range of 25−50 V as appropriate. Data acquisition and processing were performed using Analyst, version 1.4.2 (Applied Biosystems/MDS Sciex).

Direct Infusion Electrospray Mass Spectrometric Analysis (Infusion ESI-MS). The lyophilized fractions from the MLCCC separations were dissolved in 50% aqueous acetonitrile containing 0.5% formic acid (v/v). The sample solutions were directly infused into the mass spectrometer at a flow rate of 5 μL/min using a syringe pump (Pump 22, HARVARD, Holliston, MA). Full scan spectra ranging from m/z 200 to 2000 were recorded.

Liquid Chromatography Mass Spectrometry (LC−MS/MS). LC−MS/MS analysis was carried out using the mass spectrometer combined with an Agilent 1200 HPLC system equipped with a binary pump, degasser, autosampler, column oven, and photodiode array (PDA) detector. A 10 μL aliquot of the above sample was injected and chromatographed on a 150 mm × 2 mm (i.d.), 4 μm Synergi Hydro-RP 80A. The column temperature was maintained at 25 °C during the HPLC run. A binary gradient with mobile phase consisting of 1% formic acid/99% water (v/v, solvent A) and 1% formic acid/19% water/80% acetonitrile (v/v/v, solvent B) was employed. The elution conditions were as follows: flow rate of 200 μL/min (solvent A) and (solvent B) with a linear gradient of 0 to 20% solvent B for 35 min followed by 20 to 60% for 7 min.

RESULTS AND DISCUSSION

MLCCC Fractionation of Grape Skin Extract. Stepwise gradient elution coupled to MLCCC was originally developed for the separation of a broad range of anthocyanins present in skins of *Vitis vinifera* grape cultivars (*14*). In this study, 250 mg of Pinot Noir grape skin extract was subjected to MLCCC separation. The MLCCC elution profile of 546 nm absorbing materials exhibited a dominant peak between retention times ranging from 45 to 80 min (data not shown). The peak was confirmed to be coelution of grape anthocyanins (cyanidin (Cy-G), delphinidin (Dp-G), peonidin (Pn-G), petunidin (Pt-G), and malvidin-3-glucoside (Mv-G)) by HPLC (data not shown). Vidal et al. (*15*) reported that grape anthocyanins in Shiraz grape skin extracts were separated based on the respective glycosides; glucoside, acetylglucoside, coumaroylglucoside and caffeoylglucoside using similar MLCCC conditions to our study. The elution profile in our study showed only one major peak which consists of anthocyanidin glucosides due to the fact that Pinot Noir is a variety that only contains glucosides (*19*).

MLCCC Stationary Phase Fractions. After removing the monomeric anthocyanins, the MLCCC experiment was ceased after 290 min and the stationary phase immediately pumped out

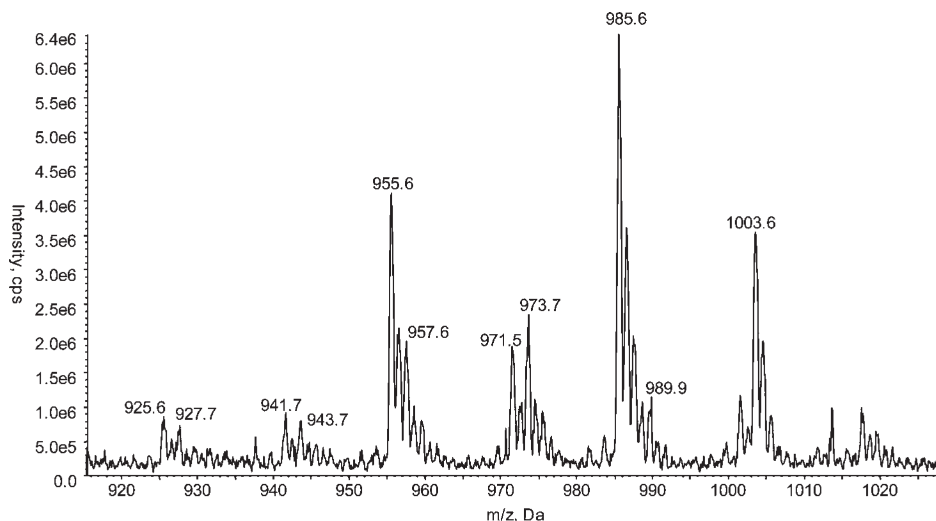

Figure 2. Mass spectrum of anthocyanin dimers in the stationary phase analyzed by infusion ESI-MS.

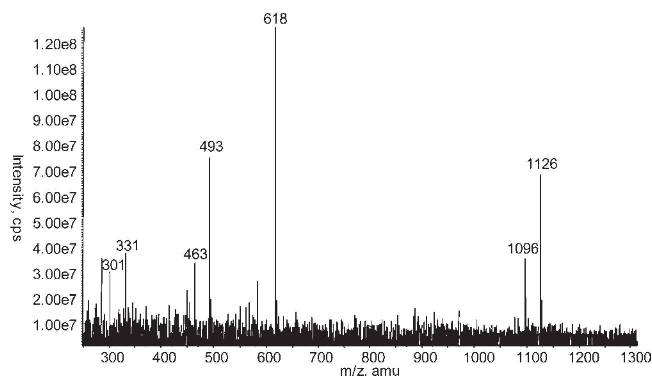

Figure 3. Mass spectrum of Fr-4&5 obtained by infusion ESI-MS.

of the coil to recover remaining materials. The elution profile of the stationary phase starting from 290 min exhibited a number of peaks with significantly lower absorbance than the anthocyanin peak seen in the mobile phase. Since all the colored stationary phase fractions were confirmed to be devoid of anthocyanins by HPLC (data not shown), these peaks most likely represented pigmented materials other than the monomeric anthocyanins (data not shown). Vidal et al. (*15*) reported that pigmented materials remained in the stationary phase after the completion of MLCCC and that some were confirmed to be anthocyanin oligomers. In fact, our study also showed the presence of anthocyanin oligomers in the fractions of the stationary phase (**Figure 2** for anthocyanin dimers).

Compounds other than anthocyanin oligomers were also screened for within the stationary phase fractions by infusion ESI-MS. When investigating the combined fractions 4 and 5 (Fr-4&5), unusual mass ions (*m*/*z* 1096 and 1126) were detected which did not match any expected anthocyanin-related compounds or anthocyanin derivatives previously reported (**Figure 3**). Fr-4&5 also gave interesting ions at *m*/*z* 618, 493, 463, 331, and 301 in addition to *m*/*z* 1096 and 1126. The ions *m*/*z* 463/301 and 493/331 were consistent with Pn-G and Mv-G, respectively. Since Fr-4&5 was confirmed to be devoid of anthocyanins by HPLC (data not shown), the mass spectrum indicated the possible presence of coupling products involving these anthocyanins. The ion *m*/*z* 618 was consistent with the $[M + H]^+$ ion of grape reaction product (GRP) between glutathione (GSH) and caftaric acid. The mass difference between *m*/*z* 1096 and 1126 was 30 Da, which is the same as that between Pn and Mv (301 and 331).

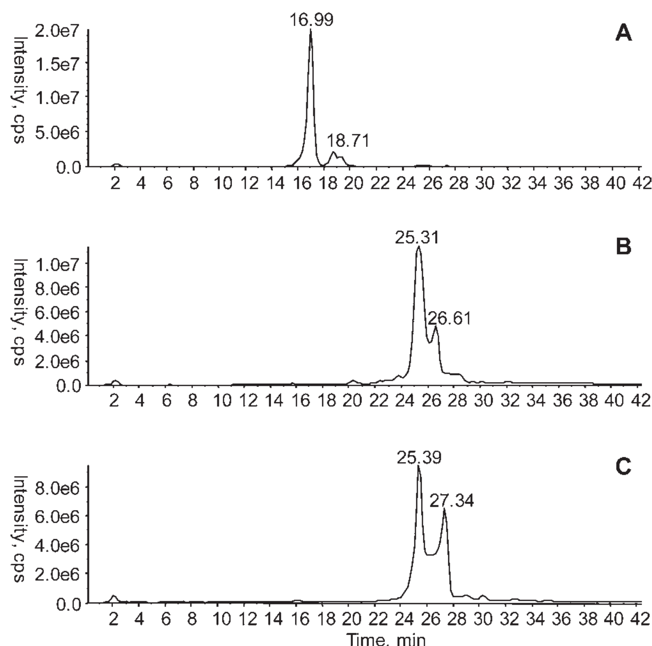

Figure 4. Total ion chromatograms of product ion scans of (**A**) *m*/*z* 618, (**B**) *m*/*z* 1096, and (**C**) *m*/*z* 1126 obtained by LC−MS/MS.

Taking these ions into consideration, *m*/*z* 1096 and 1126 likely represented the compounds composed of GRP with Pn-G and Mv-G as a core structure, respectively. Such coupling products have not been previously identified in grape skins, wine marc or wine itself.

Characterization of *m*/*z* 618, 1096, and 1126 by LC−MS/MS. Fr-4&5 was further characterized by LC−MS/MS in product ion scans of *m*/*z* 618, 1096, and 1126. The product ion scans of *m*/*z* 618 gave a dominant peak at a retention time of 16.99 min followed by a small peak at 18.71 min (**Figure 4A**). Both peaks gave very similar product ion spectra including the major fragment ions at *m*/*z* 543, 489 and 264, (**Figure 5A**), which agreed with those of GRP as reported elsewhere (*20*). The two peaks likely represent GRP isomers due to the presence of *trans*- (major isomer) and *cis*-caftaric acid (*21*, *22*). In the product ion scans of *m*/*z* 1096 and 1126, both ions gave unresolved peaks with two maxima at 25.31/26.61 min and 25.39/27.34 min, respectively (**Figures 4B,C**). The mass spectra obtained throughout the unresolved peaks of *m*/*z* 1096 and1126 showed essentially the

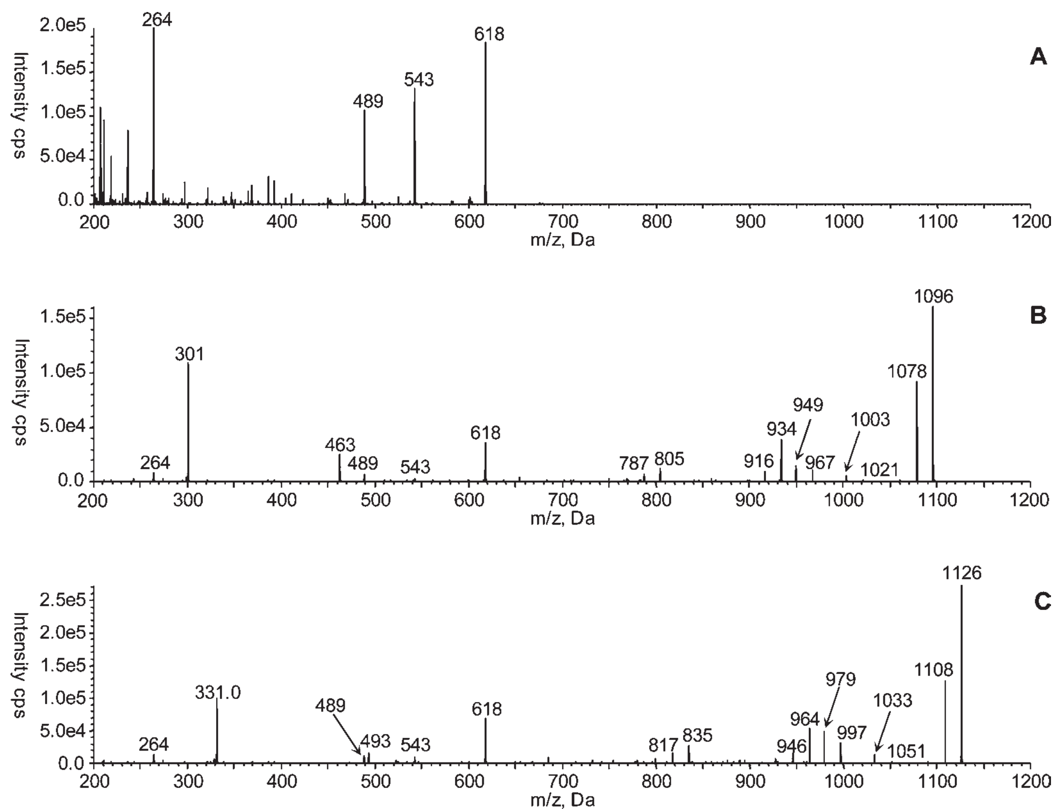

Figure 5. Product ion spectra of (**A**) *m/z* 618, (**B**) *m/z* 1096, and (**C**) *m/z* 1126 obtained by LC−MS/MS.

Glu: glucose, GRP: Grape reaction product , Mv-3-Glu: malvidin-3-glucoside, Pn-3-Glu: peonidin-3-glucoside, GSH: Glutathione, R = H: Pn, R = OCH_3: Mv

Figure 6. Proposed structures of coupling products and expected fragmentations.

same product ion spectra, respectively, indicating the presence of multiple isomers of both compounds (**Figures 5B,C**). Both ions were fragmented in a similar fashion with a mass difference of 30 Da, e.g. fragment ions at *m*/*z* 1078/1108 and 934/964 resulting from losses of 18 (H_2O) and 162 (dehydrated glucose) from the $[M + H]^+$ ions, respectively. In addition, ions at 463/493 (Pn-G and Mv-G, respectively) and 301/331 (Pn and Mv, respectively) were observed (**Figures 5B,C**). On the other hand, *m*/*z* 618, 543, 489, and 264 ions were commonly observed in both spectra and were consistent with the ions derived from GRP (**Figure 5A**). Consequently, the *m*/*z* 1096 and 1126 ions were ascribed to the $[M + H]^+$ ions of the coupling products between GRP and with hydrated Pn-G and Mv-G, respectively (**Figure 6**); this assignment is similarly supported by the infusion ESI-MS experiment. The major fragment ions appeared to be derived by losses, from the $[M + H]^+$ ions, of H_2O (to give *m*/*z* 1078/1108); of glucose (to give *m*/*z* 934/964); and of the entire anthocyanin portions (to give *m*/*z* 618). This last ion can then lose either the glycyl or γ-glutamyl moieties to give ions *m*/*z* 543 and 489, respectively (**Figure 6**).

Formation of New Phenolic Products. The formation of GRP has been well established as proceeding via enzymic oxidation of caftaric acid to the corresponding *o*-quinone by PPO (polyphenol oxidase) in the presence of oxygen, followed by nucleophilic attack of the glutathione thiol on the *o*-quinone (*6*, *9*, *10*, *23*). PPO is a strongly bound enzyme found in grape skins; its activity can differ from year to year and is also variety dependent (*24*−*26*).

The interaction of anthocyanins and caftaric acid *o*-quinone (generated by PPO) (*27*) has been studied in white grape musts with added grape anthocyanins (*28*) and grape must-like model solutions (*29*). This latter study demonstrated that anthocyanins were degraded in the presence of caftaric acid *o*-quinone but that this degradation was totally inhibited by the addition of GSH, while the former study showed that caftaric acid *o*-quinone primarily reacts with GSH to form GRP (*28*). GRP can be

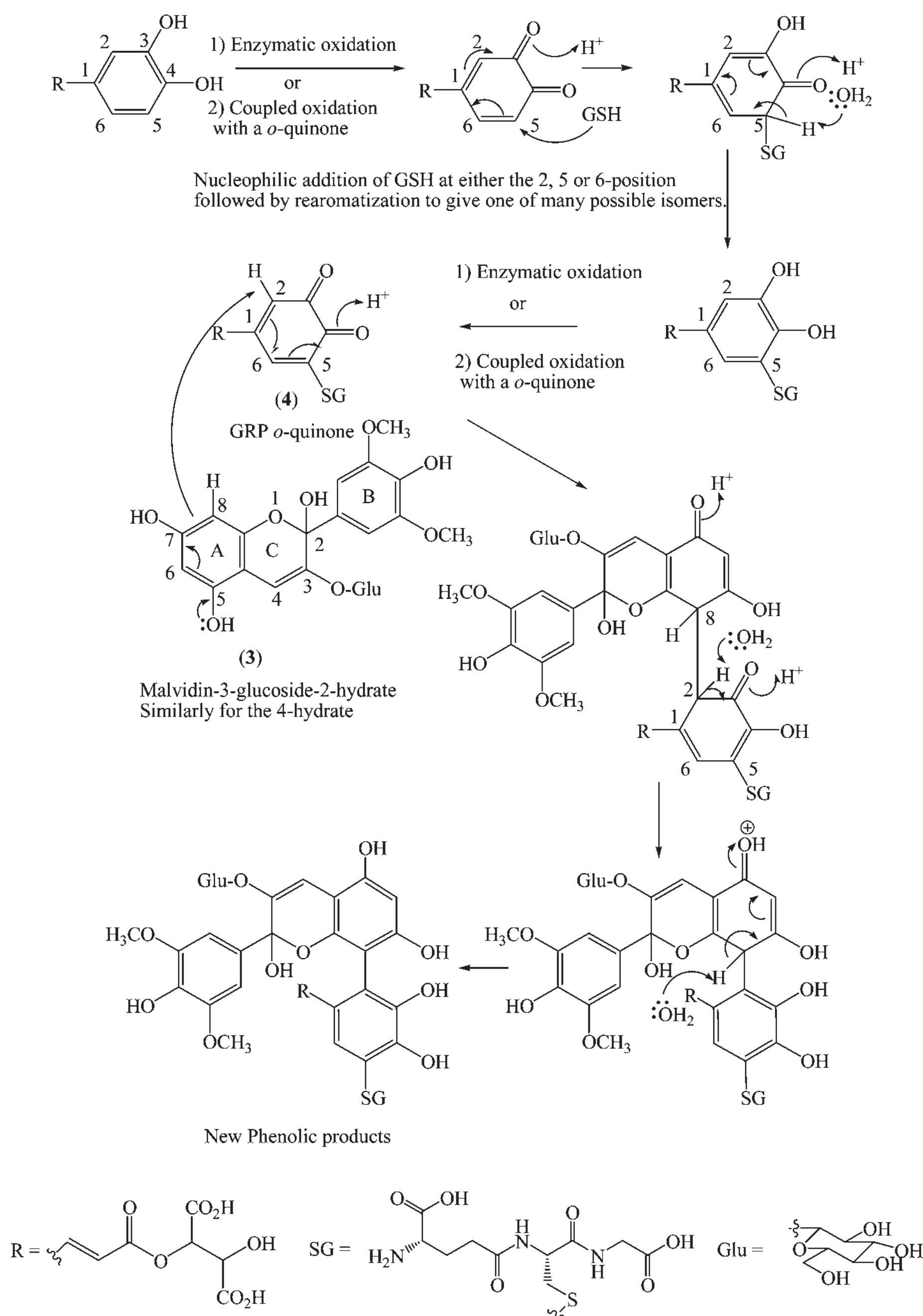

Figure 7. Proposed mechanism for the formation of new phenolic compounds.

oxidized by caftaric acid *o*-quinone (reaction known as coupled oxidation) to form GRP *o*-quinone, which can further react with nucleophiles resulting in the production of copolymers (*11*, *30*). GRP can also be further oxidized in the presence of other enzymes (*laccase*) to yield quinones which are also susceptible to nucleophilic attack as demonstrated by Salgues (*11*). *o*-Quinone of GRP (*11*) in the presence of nucleophiles like the anthocyanin hydrate may form a coupled product. Although this is a possibility, in the absence of *laccase* activity, the GRP and anthocyanins have been found to be rather stable to PPO (*10*, *12*); thus the formation of the *o*-quinone of GRP is unlikely. The coupled products of an anthocyanin, caftaric acid and GSH as proposed by the present study have not yet been reported.

The GRP *o*-quinone can then undergo electrophilic aromatic substitution by the hydrated anthocyanin to give the GRP-anthocyanin adduct. Similarly the *cis*-isomer of caftaric acid can undergo the same series of reactions; its presence in skins is well-known (*22*). Alternatively, oxidation of the GRP can occur in the presence of other *o*-quinones via the coupled oxidation mechanism. Anthocyanins containing an *o*-diphenol moiety, such as Cy-G, Dp-G or Pt-G, can be oxidized to their corresponding *o*-quinone forms by this mechanism, and these can then proceed further to other coupling products (*29*).

On the other hand, anthocyanins lacking this *o*-diphenol arrangement, such as Pn-G and Mv-G, are not subject to such oxidation, and accordingly their hydrated forms can react with GRP *o*-quinone by electrophilic aromatic substitution to give the coupling products identified in this study. This may be the reason why the coupling products are only seen as their hydrates. This coupling reaction can conceivably take place at several positions, at C-6 or C-8 on the anthocyanin component (**3**) or at C-2 or C-6 of the GRP *o*-quinone (**4**) (**Figure 7**). It is also possible that this final coupling step might take place across the enone portion of the caftaric acid. All of the resultant products thus formed by the different possible pathways would be expected to furnish mass spectra very similar to one another, and thus none of them can be excluded at the present time. The total ion chromatograms corresponding to the ions of m/z 1096 and 1126 (**Figures 4 B** and **4C**, respectively) both show broad unresolved peaks with at least two maxima, confirming the presence of multiple isomers due to these different linkage positions.

In summary, this is the first report of such coupling products in grape skins, and their presence leads to several potentially interesting questions: do they survive the fermentation process, and if not, then into what are they converted; and what is their precise structure? Resolution of the final point will no doubt shed valuable light onto the mechanism of their formation. In terms of the wine making process, we know that GRP in white wines can prevent browning, and indeed improve the color quality aspect. This can also occur during red wine making where browning is prevented. Thus, these new GRP anthocyanin derivatives may play a role in inhibiting wine oxidation and, if in equilibrium with their ionized form, may aid in the preservation of color for red wines.

ABBREVIATIONS USED

MLCCC, multilayer coil countercurrent chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; GRP, grape reaction product; GSH, glutathione; PPO, polyphenol oxidase; Mv-G, malvidin 3-glucoside; Pn-G, peonidin 3-glucoside; Pt-G, petunidin 3-glucoside; Dp-G, delphinidin 3-glucoside; Cy-G, cyanidin 3-glucoside.

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