JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Mass Spectrometric Evidence for the Existence of Oligomeric Anthocyanins in Grape Skins

STÉPHANE VIDAL,[†,‡] EMMANUELLE MEUDEC,[§] VÉRONIQUE CHEYNIER,[§] GEORGE SKOUROUMOUNIS,[†] AND YOJI HAYASAKA*,[†]

The Australian Wine Research Institute, Post Office Box 197, Glen Osmond SA 5064, Australia, Cooperative Research Centre for Viticulture, Post Office Box 154, Glen Osmond SA 5064, Australia, and Unité Mixte de Recherche Sciences pour l'Oenologie, INRA, 2, place Viala, 34060 Montpellier cédex, France

The fractionation of a grape skin extract by multilayer countercurrent chromatography coupled with step gradient elution allowed the preparation of a fraction almost devoid of free anthocyanins. This fraction appeared to be almost exclusively polymeric, as judged by liquid chromatographic−mass spectrometric (LC-MS) analysis, color-bleaching tests with sulfur dioxide, and thiolysis. Electrospray mass spectrometric analysis indicated that the pigmented material in this fraction was chiefly composed of direct condensation products of anthocyanin extending up to trimers. With regard to their linkages, the anthocyanin units in the oligomers were possibly linked by either an A-type (by both carbon−carbon and ether bonds) or B-type (by carbon−carbon bond) linkage, like proanthocyanidins. The terminal anthocyanin unit of the oligomers is consistently in the flavylium form but the extension units are in the flavan form for the A-type oligomers and in the flavene form for the B-type oligomers. Although their linkages still need to be defined rigorously, this is the first mass spectrometric evidence confirming the existence of anthocyanin oligomers in the grape skin extract.

KEYWORDS: Grape skin; anthocyanin oligomers; anthocyanins; multilayer coil countercurrent chromatography; electrospray mass spectrometry

INTRODUCTION

Multilayer coil countercurrent chromatography (MLCCC) combined with a stepwise gradient elution program was developed for the separation of the different classes of grape skin anthocyanins (*1*). This method progressively extracted pigmented materials from the stationary phase (mostly water) to the mobile phase (mostly a mixture of organic solvents) and allowed the fractionation of monomeric anthocyanins as a series of glucosides (G), and the corresponding acetylated (AG), *p*-coumaroylated (CG), and caffeoylated derivatives. After the elution of monomeric anthocyanidin glucosides, pigmented material still remained in the stationary phase and was almost devoid of monomeric anthocyanins as confirmed by HPLC (*1*).

On the basis of high-performance liquid chromatography (HPLC) elution profiles (*1*), the remaining polar or highly water-soluble pigments were observed to be structurally diverse and therefore seemed to consist of a complex mixture of polymeric species.

With regard to highly water-soluble pigments in wine, Somers (*2*) reported that considerable color remained in the aqueous wine matrix following the exhaustive extraction of anthocyanins from red wine with isoamyl alcohol. The remaining highly water-soluble pigments exhibited a polymeric nature and therefore were called pigmented polymers. It was estimated that pigmented polymers could account for 50% of color intensity of red wine within the first year (*2*). Since then, the presence and formation of pigmented polymers in red wine has been the subject of many studies and, as a result, there have been two broad mechanisms proposed for their formation in wine. The first is a reaction between anthocyanins and flavanols mediated by the enolic form of acetaldehyde (an acetaldehyde-mediated condensation) (*3*−*5*). The second is a direct condensation of anthocyanin with flavanols (*2*, *6*−*11*). The latter mechanism has recently been supported by a mass spectrometric study of polymeric products isolated from red wine. Their structures were consistent with an anthocyanin linked to a proanthocyanidin containing up to seven subunits (*12*).

On the other hand, relatively few discussions have dealt with the occurrence of pigmented polymers in grapes, and their formation and structures were highly speculative (*13*−*16*).

The purpose of this study was therefore to further investigate the chemical nature of the remaining pigments in the stationary phase (SPskin) after the MLCCC fractionation of the grape skin extract. SPskin was characterized by means of UV−vis spectrometry, thiolysis, and electrospray mass spectrometry.

* Corresponding author: e-mail Yoji.Hayasaka@awri.com.au.
[†] The Australian Wine Research Institute and Cooperative Research Centre for Viticulture.
[‡] Present address: Inter Rhône, 2260 Route du Grès-84100 Orange.
[§] Unité Mixte de Recherche Sciences pour l'Oenologie.

10.1021/jf048939h CCC: $27.50
Published on Web 10/16/2004

MATERIALS AND METHODS

Experimental procedures for the preparation of grape skin extract from grape berries of *Vitis vinifera* cv. Shiraz and the separation of the grape skin extract by MLCCC were described in a previous study (*1*). SPskin subjected to this study was obtained during a previous study (*1*).

Thiolysis. Acid-catalyzed cleavage of SPskin was carried out in the presence of phenylmethanethiol and the resultant reaction mixture was analyzed by HPLC as previously described (*1*).

UV/Visible Spectrophotometry. Solutions of samples (1 mg/mL) were prepared in ethanol/water solution (12:88 v/v) containing 3 g/L tartaric acid buffered at pH 3.5 by addition of concentrated NaOH. Absorbance was measured at 520 nm directly and after addition of 0.015 volume of a 20 g/L sodium metabisulfite solution to 1 volume of sample and incubation for 10 min at ambient temperature.

Mass Spectrometry. Mass spectrometric analysis was carried out with an API-300 triple-quadrupole mass spectrometer equipped with an electrospray (ESI) ion source (MDS−Sciex, Concord, ON, Canada).

Liquid Chromatography−Mass Spectrometry (LC-MS). SPskin was dissolved in 5% formic acid aqueous solution (v/v) at a concentration of 10 mg/mL. A 20 μL aliquot was injected by an autosampler (HP1100, Agilent) and separated on a Synergi 4-μm Hydro-RP 80 Å (2.1 mm × 150 mm; Phenomenex) LC column. The method used a binary gradient with mobile phases containing formic acid/water/acetonitrile, (A) 5:95:0 and (B) 5:15:80 (v/v/v). The elution conditions were as follows: 180 μL/min, linear gradient from 10% to 35% B in 35 min, from 35% to 60% B in 25 min, and 60% B for 1 min. The eluent from the LC was split by use of a T-piece and delivered at 22.5% of the total flow to the mass spectrometer (ESI-MS) and at 77.5% to a UV detector (HP1100, Agilent) with monitoring wavelengths at 280 and 520 nm.

Positive ion mass spectra from *m*/*z* 200 to 3000 were recorded with a step size of 0.2 Da and dwell time of 0.25 ms. The ESI needle, orifice, and ring potentials were set at 5000, 50, and 250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively.

Infusion Electrospray Mass Spectrometry and Tandem Mass Spectrometry (Infusion ESI-MS and ESI-MS/MS). SPskin was dissolved in formic acid/water/acetonitrile solution (5:15:80 v/v/v) at a concentration of 1 mg/mL. The sample solution was infused (5 μL/min) into the ESI source with a syringe pump (Cole-Parmer, Vernon, IL). The ESI conditions were the same as described above except the orifice potential was set to 150 V. Positive ion mass spectra from *m*/*z* 200 to 3000 were recorded with a step size of 0.1 Da and dwell time of 0.2 ms. Twenty mass spectrum scans were consecutively accumulated in the multichannel-acquisition (MCA) mode within the Sample Control software version 1.3 (MDS Sciex).

For the MS/MS experiment, nitrogen gas was used as the collision gas (set at 2 units) and the collision energy was optimized in a range of 30−60 V as appropriate. Product ion spectra were consecutively accumulated until appropriate ion intensity was obtained.

RESULTS AND DISCUSSION

Preliminary Characterization of SPskin. The pigmented materials were collected from the stationary phase (SPskin) following the extraction of monomeric anthocyanins from the skin extract by MLCCC (*1*). SPskin was almost devoid of monomeric anthocyanins and accounted for 8−9% of the total recovered material (w/w) from the MLCCC fractionation of the skin extract (*1*).

When analyzed by LC-MS, the 520-nm-absorbing materials in SPskin eluted primarily as an unresolved broad peak (hump) over 5−45 min (**Figure 1A**), suggesting that the large portion of pigmented materials was polydisperse polymeric material. Monomeric anthocyanin peaks were also observed but their relative areas were minor in comparison with that of the hump.

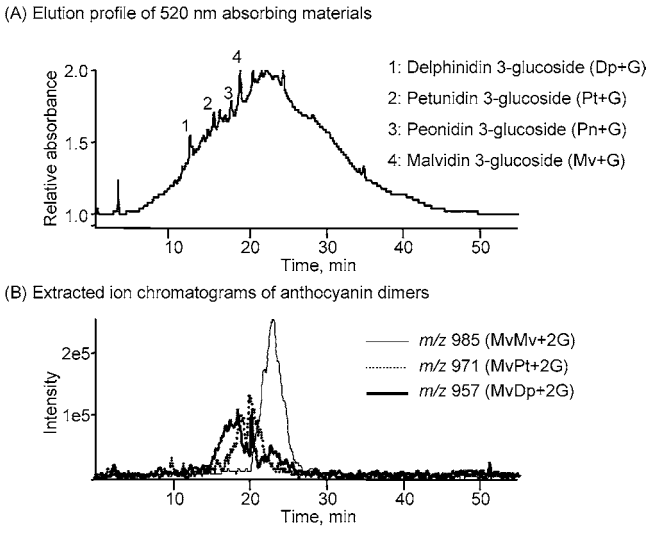

Figure 1. LC-MS analysis of SPskin: (**A**) Elution profile monitored at 520 nm. (**B**) Extracted ion chromatograms of anthocyanin dimers composed of malvidin 3-glucoside with malvidin 3-glucoside (MvMv+2G, *m*/*z* 985), petunidin 3-glucoside (MvPt+2G, *m*/*z* 971), and delphinidin 3-glucoside (MvDp+2G, *m*/*z* 957).

SPskin was also submitted to acid-catalyzed depolymerization in the presence of a thiolytic agent (thiolysis) to confirm the presence of proanthocyanidin-based polyphenolics (proanthocyanidins and derived pigments). This technique allows discrimination between proanthocyanidin terminal units that are released as flavan-3-ol monomers and extension units released as thiol derivatives resulting from the trapping of the cation generated through the acidic cleavage of the interflavanoid linkage by the thiolytic agent (*17*, *18*). Neither monomeric flavanols nor their thiol derivatives were detected, suggesting that pigmented materials in SPskin were not composed of thiolysis-sensitive proanthocyanidin structures. Moreover, the HPLC elution profile of 520-nm-absorbing materials of SPskin after thiolysis was similar to that of SPskin before thiolysis, demonstrating that the majority of pigmented materials were resistant to the acid-catalyzed cleavage.

When SPskin was dissolved in a winelike solution (1 mg/mL) at pH 3.5, its absorbance at 520 nm was intense. A solution of the same concentration of grape anthocyanidin monoglucosides, fractionated by MLCCC (*1*), showed similar absorbance at 520 nm. Although the pigmented materials in SPskin were expected to be larger molecular sizes than monomeric anthocyanin, absorbance at 520 nm was similar to each other at the same concentration. It may result from the fact that the molar extinction coefficient of the pigmented materials is higher than that of monomeric anthocyanins in a winelike solution.

After the addition of sodium metabisulfite to the solution of SPskin, 45% of the absorbance at 520 nm was retained. This resistance to bleaching was not as great as that of pyranoanthocyanins (C-4 substitute anthocyanin) but was clearly distinguishable from that of monomeric anthocyanins (*19*−*21*). Protection against the nucleophilic attack of the bisulfite can indeed occur for pigments with a higher degree of structural complexity, as has been reported for the pigment derived from the acetaldehyde-mediated condensation between catechin and malvidin 3-glucoside (*22*).

Considering the observations above, it is likely that SPskin is composed almost exclusively of polymeric pigmented materials resistant to acid-catalyzed cleavage, and unlike red wine

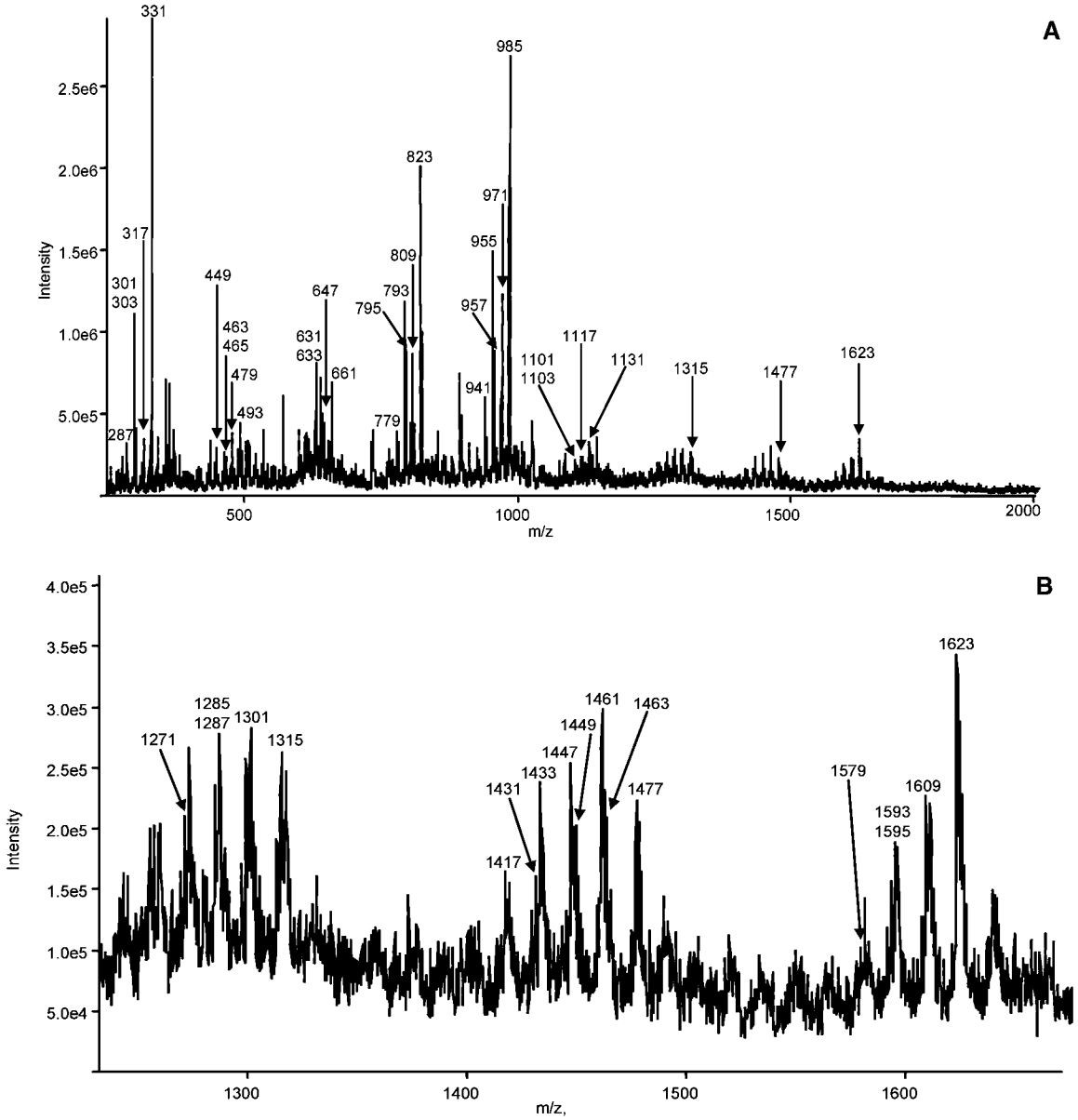

Figure 2. Electrospray mass spectrum of SPskin analyzed by infusion ESI-MS for mass range (**A**) from *m*/*z* 200 to 2000 and (**B**) from *m*/*z* 1230 to 1670. The possible mass assignment of the major ions is shown in **Table 1**.

tannin, proanthocyanidin is not the major building unit of the polymer (*12*).

Mass Spectrometric Characterization of SPskin by Infusion ESI-MS. Direct infusion of SPskin to ESI-MS yielded a complex mass spectrum ranging from *m*/*z* 250 to 1700 (**Figure 2A**). A series of singly charged ions, *m*/*z* 985, 971, 957, 955, and 941, were detected (**Figure 2A**). The most abundant ion, *m*/*z* 985, was consistent with a molecular mass of two malvidin 3-glucoside (Mv+G) units directly linked by either bicyclic carbon−carbon and ether bonds (A-type) or a single carbon−carbon bond (B-type). The terminal Mv+G unit of the malvidin 3-glucoside dimer (MvMv+2G) is consistently in the flavylium form, but the extension unit is in the flavan form for the A-type linkage and in the flavene form for the B-type one (**Figure 3**). Therefore, MvMv+2G (*m*/*z* 985) with either linkage could be detected as a singly charged ion by ESI-MS. The A-type (C4−C8 and C2−O−C7) and B-type (C4−C8) linkages have been reported in proanthocyanidins (*23*) and anthocyanin−procyanidins (*8*−*12*). The other ions at *m*/*z* 971, 957, 955, and 941 were also consistent with molecular masses of the proposed A-type flavan−flavylium or B-type flavene−flavylium dimers comprising one unit of Mv+G and one unit of petunidin 3-glucoside (Pt+G), delphinidin 3-glucoside (Dp+G), peonidin 3-glucoside (Pn+G), and cyanidin 3-glucoside (Cy+G), respectively (**Table 1**).

To a lesser extent, the ions consistent with anthocyanin dimers composed of one glucoside (G) and one *p*-coumaroyl glucoside (CG) were also observed at *m*/*z* 1087, 1101, 1103, 1117, and 1131 for MvCy, MvPn, MvDp, MvPt, and MvMv with G·CG, respectively (**Figure 2A**).

In the higher mass range (>*m*/*z* 1200, **Figure 2B**), two series of ions were observed that were consistent with molecular masses of either the A-type flavan−flavan−flavylium or B-type flavene−flavene−flavylium trimers (**Figure 3**). The possible composition of anthocyanin trimers was MvMvMv+3G for *m*/*z* 1477, MvMvPt+3G for *m*/*z* 1463, MvMvDp+3G for *m*/*z* 1449, MvMvPn+3G for *m*/*z* 1447, and MvMvCy+3G for *m*/*z* 1433. Trimers composed of two glucoside and one *p*-coumaroyl

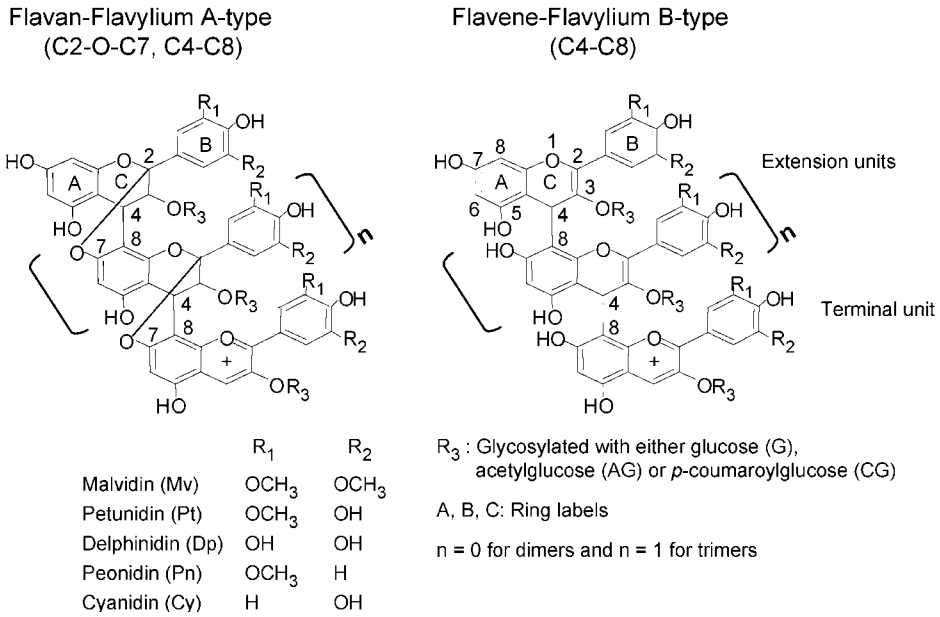

Figure 3. Possible structures of anthocyanin dimers and trimers.

Table 1. Mass Assignment of Ions Found in SPskin[a]

m/z	mass assignment	*m/z*	mass assignment	*m/z*	mass assignment
287[F]	Cy	795[F]	MvDp+G	1315[F]	MvMvMv+2G
301[F]	Pn	809[F]	MvPt+G	1417[F]	MvMvCy+G·CG
303[F]	Dp	823[F]	MvMv+G	1431[F]	MvMvPn+G·CG
317[F]	Pt	941[M]	MvCy+2G	1433[F]	MvMvDp+G·CG
331[F]	Mv	955[M]	MvPn+2G	1433[M]	MvMvCy+3G
449[M]	Cy+G	957[M]	MvDp+2G	1447[F]	MvMvPt+G·CG
463[M]	Pn+G	971[M]	MvPt+2G	1447[M]	MvMvPn+3G
465[M]	Dp+G	985[M]	MvMv+2G	1449[M]	MvMvDp+3G
479[M]	Pt+G	1087[M]	MvCy+G·CG	1461[F]	MvMvMv+G·CG
493[M]	Mv+G	1101[M]	MvPn+G·CG	1463[M]	MvMvPt+3G
617[F]	MvCy	1103[M]	MvDp+G·CG	1477[M]	MvMvMv+3G
631[F]	MvPn	1117[M]	MvPt+G·CG	1579[M]	MvMvCy+2G·CG
633[F]	MvDp	1131[M]	MvMv+G·CG	1593[M]	MvMvPn+2G·CG
647[F]	MvPt	1271[F]	MvMvCy+2G	1595[M]	MvMvDp+2G·CG
661[F]	MvMv	1285[F]	MvMvPn+2G	1609[M]	MvMvPt+2G·CG
779[F]	MvCy+G	1287[F]	MvMvDp+2G	1623[M]	MvMvMv+2G·CG
793[F]	MvPn+G	1301[F]	MvMvPt+2G		

[a] F, fragment ion; M, molecular ion; Cy, cyanidin; Pn, peonidin; Dp, delphinidin; Pt, petunidin; Mv, malvidin; G, glucoside; CG, *p*-coumaroyl glucoside.

glucoside (2G·CG) of anthocyanidins were also observed at *m/z* 1623 for MvMvMv+2G·CG, *m/z* 1609 for MvMvPt+2G·CG, *m/z* 1595 for MvMvDp+2G·CG, *m/z* 1593 for MvMvPn+2G·CG, and *m/z* 1579 for MvMvCy+2G·CG (**Figure 2B** and **Table 1**).

In addition to the ions consistent with molecular masses of the anthocyanin dimers and trimers, their fragment ions were also observed. In the case of MvMv+2G (*m/z* 985), *m/z* 823, 661, 493, and 331 corresponded to the masses of MvMv+G (−162; a loss of glucosyl moiety), MvMv (−324; loss of two glucosyl moieties), Mv+G, and Mv, respectively. The same series of fragment ions were also detected for MvPt+2G, MvDp+2G, MvPn+2G, and MvCy+2G (**Figure 2** and **Table 1**). Similarly, the fragment ions resulting from a loss of a glucosyl (−162; G) or *p*-coumaroylglucosyl moiety (−308; CG) from anthocyanin trimers with 3G or 2G·CG, respectively, were observed at *m/z* 1271, 1285, 1287, 1301, and 1315 for MvMvCy, MvMvPn, MvMvDp, MvMvPt, and MvMvMv with 2G, respectively. In addition, the fragment ions, *m/z* 1417, 1431, 1433, 1447, and 1461 appeared to be derived from MvMvCy, MvMvPn, MvMvDp, MvMvPt, and MvMvMv with 2G·CG, respectively, as a result of the elimination of a glucosyl moiety (**Figure 2B** and **Table 1**).

Formation of Ions Consistent with Anthocyanin Oligomers. Although the ions consistent with anthocyanin oligomers were clearly detected by direct infusion ESI-MS, some ambiguity remains regarding their formation since the ions corresponding to the anthocyanin dimers and trimers may be a product of the electrospray ionization process. When a concentrated Mv+G solution is analyzed by ESI-MS, *m/z* 985 is observed in addition to *m/z* 493, representing the molecular mass of Mv+G. This phenomenon is so-called noncovalent stacking between anthocyanins, possibly one as a flavylium form (493 Da for Mv+G) and the other one as a quinoidal base form (492 Da for Mv+G). However, this possibility was ruled out due to the outcome of LC-MS analysis of SPskin. Individual monomeric anthocyanins were found to be minor components (**Figure 1A**), and Mv+G (peak 4 in **Figure 1A**) eluted earlier than MvMv+2G (*m/z* 985 in **Figure 1B**) with a different peak profile. Anthocyanin dimers were also distinguishable each other (**Figure 1B**) and from the respective monomeric anthocyanins by retention time and peak shape (**Figure 1**). Therefore, the ions consistent with anthocyanin oligomers were not generated as an artifact of the electrospray ionization process.

Characterization of Anthocyanin Oligomers by ESI-MS/MS. To further characterize anthocyanin oligomers, the product ion spectra of the respective ions were obtained by infusion ESI-MS/MS. It is commonly observed that monomeric anthocyanin is simply fragmented into anthocyanidin (aglycon) as a result of a loss of a sugar moiety with a mass of 162 for glucoside or 308 for *p*-coumaroyl glucoside. Those fragment ions were already observed by ESI-MS. Similarly, the successive losses of glucosyl moieties (−162 Da) from anthocyanin dimers were observed at *m/z* 985 → 823 → 661 for MvMv+2G (**Figure 4A**) and *m/z* 955 → 793 → 631 for MvPn+2G (**Figure 4B**). The ions corresponding to Mv (*m/z* 331) and Pn (*m/z* 301) were

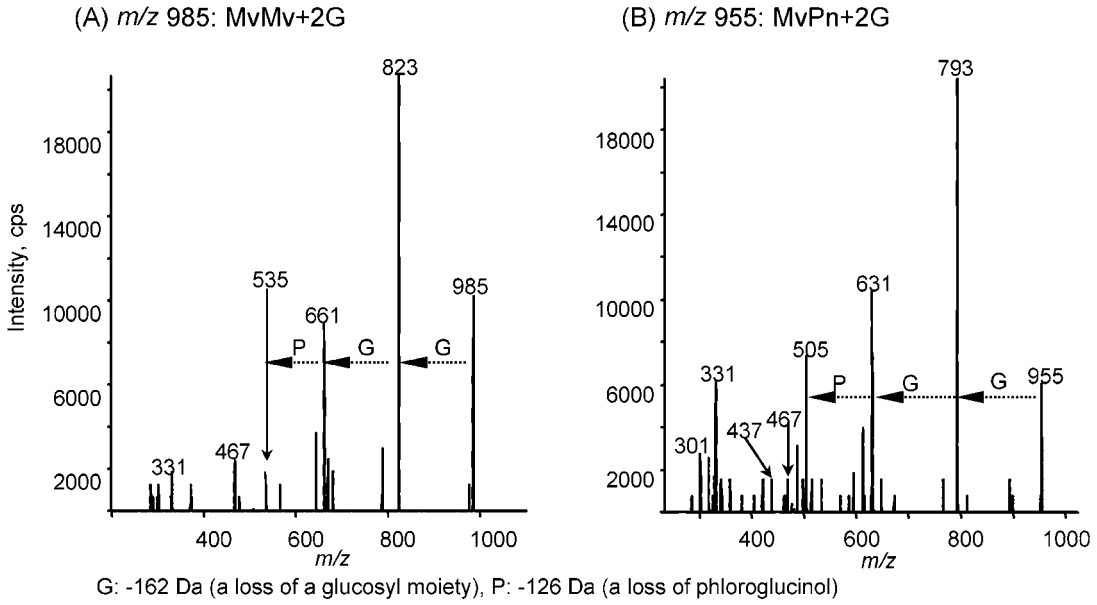

Figure 4. Product ion spectra of anthocyanin dimers composed of malvidin 3-glucoside with (**A**) malvidin 3-glucoside [*m/z* 985, MvMv+2G] and (**B**) peonidin 3-glucoside [*m/z* 955, MvPn+2G], obtained from SPskin analyzed by infusion ESI-MS/MS.

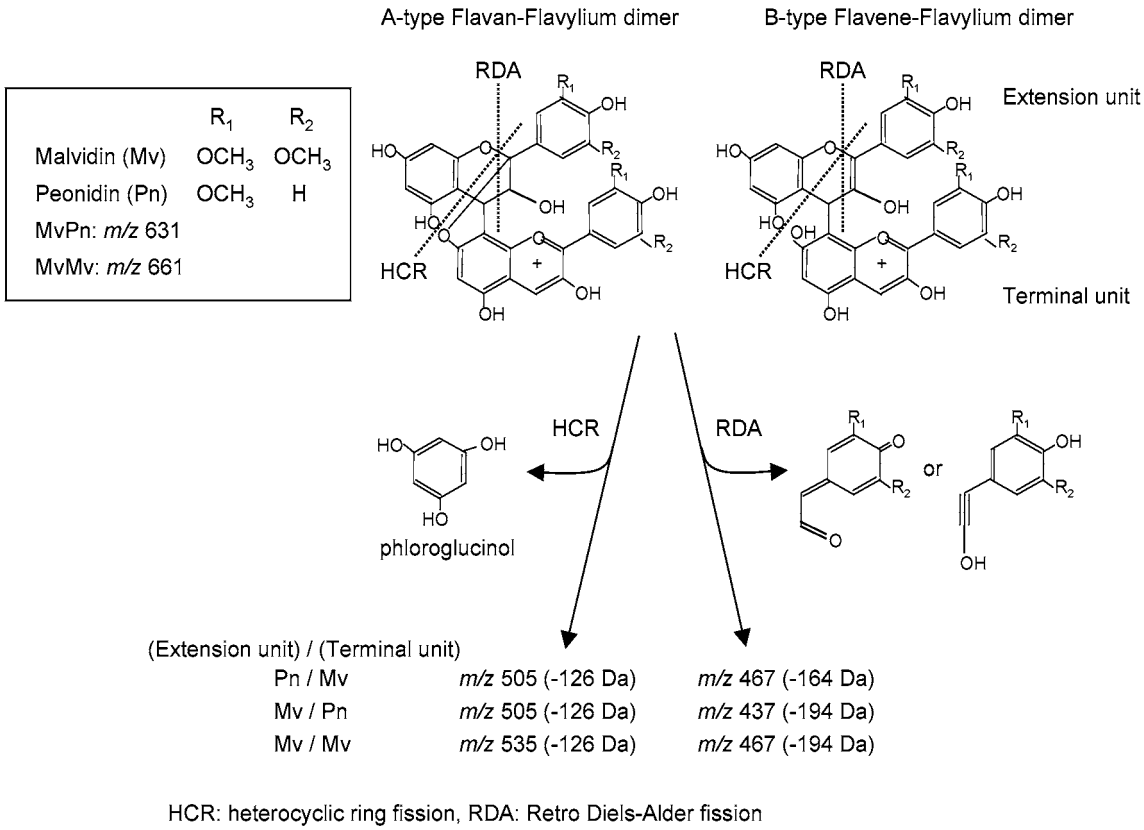

Figure 5. Fragment ions from the anthocyanin dimers composed of malvidin 3-glucoside with malvidin 3-glucoside [*m/z* 985, MvMv+2G] and peonidin 3-glucoside [*m/z* 955, MvPn+2G], obtained from SPskin analyzed by infusion ESI-MS/MS.

found in MvPn+2G (only Mv was found in MvMv+2G), suggesting that anthocyanidin (aglycon) was released resulting from the cleavage of the linkage. Likewise, successive losses of sugar moieties from anthocyanin trimers were observed at *m/z* 1447 → 1285 → 1123 → 961 (−162 Da) for MvMvPn+3G and *m/z* 1609 → 1447 → 1285 (−162 Da) and 1609 → 1301 (−308 Da) for MvMvMv+2G•CG (data not shown). In addition, a loss of 126 Da was commonly observed at *m/z* 661 → 535 for MvMv+2G and *m/z* 631 → 505 for MvPo+2G (**Figure 4**). Those fragment ions are derived by heterocyclic ring (HCR) fission occurring on ring C, resulting in the release of the neutral molecule phloroglucinol (**Figure 5**). This fragment pathway has been already reported for A- and B-type proanthocyanidins in positive and negative ion modes (*24*−*26*), an A-type anthocya-

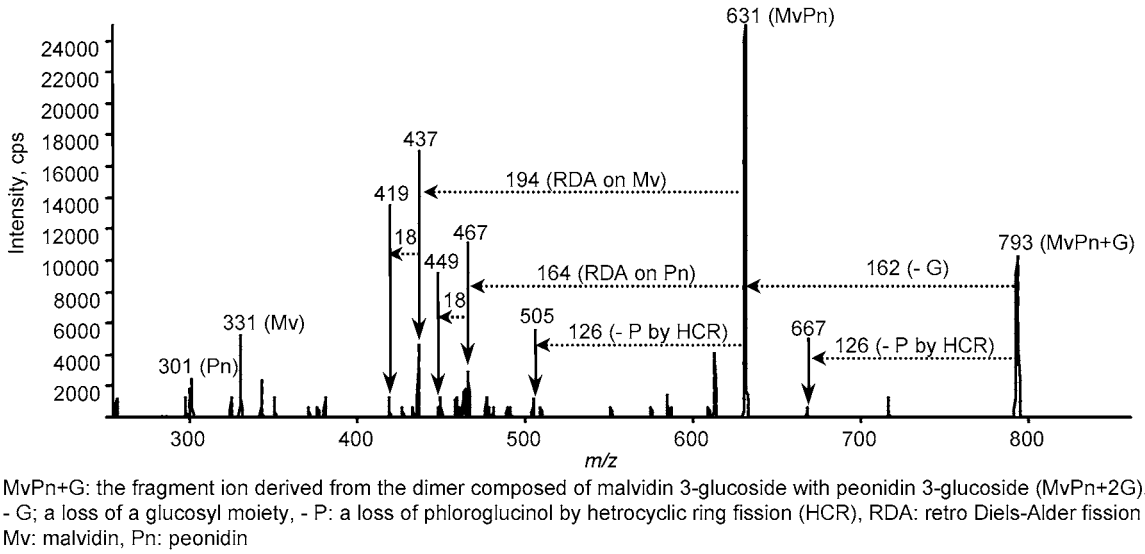

Figure 6. Product ion spectrum of the fragment ion *m*/*z* 793 derived from the anthocyanin dimer composed of malvidin 3-glucoside with peonidin 3-glucoside (MvPn+2G, *m*/*z* 955), obtained from SPskin analyzed by infusion ESI-MS/MS.

nin (flavan form)−flavanol dimer (*9*), and B-type flavanol−anthocyanin (flavylium form) dimer (*10*). Therefore, HCR fission could occur on both the flavan unit of the A-type flavan−flavylium dimer and the flavene unit of the B-type flavene−flavylium dimer (**Figure 5**). The other common fragmentation pathway of proanthocyanidins (*24*−*27*) and direct condensation products of Mv+G and flavanol (*9*, *10*) is retro Diels−Alder (RDA) fission occurring on ring C of a flavan. The ions at *m*/*z* 467 from MvMv+2G (**Figure 4A**) and at *m*/*z* 467 and 437 from MvPn+2G (**Figure 4B**) were observed and were consistent with the ions fragmented by RDA fission (**Figure 5**).

The product ion spectrum of *m*/*z* 793 (MvPn+G; fragment ion from MvPn+2G) exhibited more clearly the fragment ions at *m*/*z* 467 and 437 derived by RDA fission (**Figure 6**). The two ions are possibly derived from the respective MvPn+2G dimers with different anthocyanin configurations (Mv-Pn and Pn-Mv). The terminal anthocyanin unit of both A- and B-type dimers is in the flavylium form, where RDA fission cannot occur. Accordingly, RDA fission should occur on the extension anthocyanin unit, which is either the flavan for the A-type or flavene for the B-type dimer. In the case of the dimer composed of Pn+G positioned at the extension unit and Mv+G at the terminal unit, the ion at *m*/*z* 467 was derived from *m*/*z* 631 after the loss of 164 Da, resulting from RDA fission occurring on Pn. In the opposite configuration of the dimer, the ion at *m*/*z* 437 was derived from *m*/*z* 631 after the loss of 194 Da resulting from RDA fission occurring on Mv (Figures 5 and 6). Both ions were further fragmented to *m*/*z* 449 and 429, respectively, as a result of loss of water (18 Da). Water loss is commonly observed following RDA fission on flavanoid compounds (*24*−*27*). In the case of proanthocyanidins, RDA fission takes place on both A- and B-type linked dimers in positive ion MS/MS (*27*), but a detailed mechanism of RDA fission occurring on the A-type dimer has not been studied. Nevertheless, RDA fission could occur on both the A- and B-type dimers, resulting in the same fragment ion, but the structures of the molecules eliminated from the A- and B-type dimers should be slightly different (**Figure 5**).

Although the purity of the respective product ion spectra is in question due to the possible presence of isomers and isobars, the detection of fragment ions derived by the cleavages of glycosidic bond and the anthocyanin−anthocyanin linkages as well as the HCR and the RDA fissions demonstrates that SPskin is composed of the direct condensation products of anthocyanins shown in **Figure 3**. However, whether the anthocyanin units are linked by the A- or B-type linkage could not be elucidated by the MS/MS data.

Composition of Anthocyanin Oligomers in SPskin. Since *Vitis vinifera* Shiraz contains five different anthocyanidins (Mv, Pt, Dp, Pn, and Cy), each with three major different glycosylated forms (G, AG, and CG), a large number of different anthocyanin dimers and trimers could likely be found in SPskin. Two hundred twenty-five different dimers (15 × 15), ranging in molecular mass from 897 (CyCy+2G) to 1277 (MvMv+2CG), and 3375 (15 × 15 × 15) different trimers, ranging in molecular mass from 1345 (CyCyCy+3G) to 1915 (MvMvMv+3CG), are possible. Mv+G is most abundant among grape-derived anthocyanins; therefore, oligomers composed of Mv+G would be most abundant. In fact, dimers composed of at least one Mv+G and trimers composed of at least either two Mv+G or Mv+G and Mv+CG were clearly detected (**Figure 2** and **Table 1**). The ions corresponding to dimers of MvMv, MvPt, MvDp, MvPn, and MvCy glycosylated with G.AG (*m*/*z* 1027, 1013, 999, 997, and 983, respectively) were also observed but their signals were poorly resolved. Similarly, trimers composed of at least two Mv with 2G.AG (*m*/*z* 1519, 1505, 1491, 1489, and 1475) were detected, but again, they were poorly resolved. Nevertheless, a large number of ions corresponding to anthocyanin oligomers were detected in a wide mass range through to *m*/*z* 2000 and the abundant ions were all derived from Mv-rich oligomers. For that reason, the mass spectrum of SPskin appeared to reflect the abundance distribution of monomeric anthocyanins in grape skin (**Figure 2**).

To our knowledge, this is the first mass spectrometric evidence confirming the existence of anthocyanin dimers and trimers in the skin extract of *Vitis vinifera* cv. Shiraz. The anthocyanin units in the oligomers are possibly linked by either the A- or B-type linkage. The terminal anthocyanin unit of the oligomers is consistently in the flavylium form but the extension units are in the flavan form for the A-type linkage and in the

flavene form for the B-type linkage. The product ion spectra of the anthocyanin dimers could represent both A- and B-type linkages. The anthocyanin oligomers demonstrated resistance to acid-catalyzed cleavage. The A-type anthocyanin−flavanol dimer (*9*) and proanthocyanidins (*28*) are reported to be resistant to acid-catalyzed cleavage. Interestingly, unlike the B-type proanthocyanidins (*15*), the B-type procyanidin−anthocyanin dimer is resistant (*10*). It implies that the linkage of the anthocyanin unit to the anthocyanin or flavanol unit is stable under the acid thiolysis medium regardless of the type of linkage. From the results of the MS/MS and thiolysis experiments, the type of linkage involved in the anthocyanin oligomers remains undetermined.

It should be considered whether the anthocyanin oligomers are originally present in the vacuole of grape skin tissues or are formed after release of monomeric anthocyanins from the vacuole. When the skin extract was analyzed by LC-MS prior to the MLCCC separation, the proposed anthocyanin oligomers were detected (data not shown). In addition, the anthocyanin oligomers, in particular MvMv+2G (*m*/*z* 985), were found in intact commercial red wines (Shiraz and Cabernet Sauvignon) aged less than 3 years when directly analyzed by LC-MS without any sample preparation (data not shown). It suggests that anthocyanin oligomers are unlikely to be formed during the MLCCC separation. The anthocyanin oligomers found in the intact wines could have originated from the vacuole and survived during fermentation and aging. Further observation considering the formation of anthocyanin oligomers was that direct condensation products of anthocyanin with flavanols, which have been found in wine (*11*), were not found in the skin extract. It suggests that anthocyanins and flavanols are probably distributed in different vacuoles and consequently anthocyanin oligomers are formed in vacuoles containing only anthocyanins, whereas in wine the presence of anthocyanins and flavanols together leads to the formation of anthocyanin−flavanol adducts. However, the possibility for their formation in the course of winemaking through the same reaction processes as those taking place during the preparation of the skin extract cannot be ruled out.

The existence of oligomeric anthocyanins in the grape skin extract is of great interest to the wine industry. Their formation, diffusion into wine, and stability in the wine matrix is worthy of investigation to determine their impact on wine color and organoleptic properties.

ACKNOWLEDGMENT

We thank Professor Peter Høj of the Australian Wine Research Institute (AWRI) for his encouragement and critical reading of the manuscript. We also thank Mr. Holger Gockowiack and Ms. Gayle Baldock of AWRI for technical assistance. We thank Professor Isacc Pretorius and Drs. Elizabeth Waters, Markus Herderich, and Mark Sefton of AWRI for their useful suggestions concerning the manuscript.

LITERATURE CITED

(1) Vidal, S.; Hayasaka, Y.; Meudec, E.; Cheynier, V.; Skouroumounis, G. Fractionation of grape anthocyanins into classes using multilayer coil countercurrent chromatography with step gradient elution. *J. Agric. Food Chem.* **2004**, *52*, 713−719.

(2) Somers, T. C. The polymeric nature of wine pigments. *Phytochemistry* **1971**, *10*, 2175−2186.

(3) Timberlake, C. F.; Bridle, P. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. *Am. J. Enol. Vitic.* **1976**, *27*, 97−105.

(4) 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.

(5) Es-Safi, N.-E.; Fulcrand, H.; Cheynier, V.; Moutounet, M. Studies on the acetaldehyde-induced condensation of (−)-epicatechin and malvidin-3-*O*-glucoside in a model solution system. *J. Agric. Food Chem.* **1999**, *47*, 2096−2102.

(6) Jurd, L. Review of polyphenol condensation reactions and their possible occurrence in the aging of wines. *Am. J. Enol. Vitic.* **1969**, *20*, 191−195.

(7) Bishop, P. D.; Nagel, C. W. Characterization of the condensation product of malvidin 3,5-diglucoside and catechin. *J. Agric. Food Chem.* **1984**, *32*, 1022−1026.

(8) 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.

(9) Remy-Tanneau, S.; Guerneve, C. L.; Meudec, E.; Cheynier, V. Characterization of a colorless anthocyanin−flavan-3-ol dimer containing both carbon−carbon and ether interflavanoid linkages by NMR and mass spectrometries. *J. Agric. Food Chem.* **2003**, *51*, 3592−3597.

(10) Salas, E.; Fulcrand, H.; Meudec, E.; Cheynier, V. Reactions of anthocyanins and tannins in model solution. *J. Agric. Food Chem.* **2003**, *51*, 7951−7961.

(11) Salas, E.; Atanasova, V.; Poncet-Legrand, C.; Meudec, E.; Mazauric, J. P.; Cheynier, V. Demonstration of the occurrence of flavanol−anthocyanin adducts in wine and in model solutions. *Anal. Chim. Acta* **2004**, *513*, 325−332.

(12) Hayasaka, Y.; Kennedy, J. A. Mass spectrometric evidence for the formation of pigmented polymers in red wine. *Aust. J. Grape Wine Res.* **2003**, *9*, 210−220.

(13) Somers, T. C. Pigmented profile of grapes and wines. *Vitis* **1968**, *7*, 303−320.

(14) Somers, T. C. Pigment development during ripening of the grape. *Vitis* **1976**, *14*, 269−277.

(15) 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.

(16) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Effect of maturity and vine water status on grape skin and wine flavonoids. *Am. J. Enol. Vitic.* **2002**, *53*, 268−274.

(17) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

(18) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet. M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

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

(20) Sarni-Manchado, P.; Fulcrand, H.; Souquet, J. M.; Cheynier, V.; Moutounet, M. Stability and color of unreported wine anthocyanin-derived pigments. *J. Food Sci.* **1996**, *61*, 938−941.

(21) Håkansson, A. E.; Pardon, K.; Hayasaka, Y.; de Sa, M.; Herderich, M. Structure and colour properties of new red wine pigments. *Tetrahedron Lett.* **2003**, *44*, 4887−4891.

(22) Escribano-Bailon, T.; Alvarez-Garcia, M.; Rivas-Gonzalo, J. C.; Heredia, F. J.; Santos-Buelga, C. Color and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin-3-*O*-glucoside and (+)-catechin. *J. Agric. Food Chem.* **2001**, *49*, 1213−1217.

(23) Ferreira, D.; Slade, D. Oligomeric proanthocyanidins: naturally occurring O-heterocycles. *Nat. Prod. Rep.* **2002**, *19*, 517−541.
(24) Friedrich, W.; Eberhardt, A.; Galensa, R. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* **2000**, *211*, 56−64.
(25) Hayasaka, Y.; Waters, E. J.; Cheynier, V.; Herderich, M. J.; Vidal, S. Characterization of proanthocyanidins in grape seeds using electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 9−16.
(26) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Zang, Z.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *J. Mass Spectrom.* **2003**, *38*, 1272−1280.
(27) Karchesy, J. J.; Hemingway, R. W.; Foo, Y. L.; Barofsky, E.; Barofsky, D. F. Sequencing procyanidin oligomers by fast atom bombardment mass spectrometry. *Anal. Chem.* **1986**, *58*, 2563−2567.
(28) Foo, L. Y.; Lu, Y.; Howell, A. B.; Vorsa, N. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. *J. Nat. Prod.* **2000**, *63*, 1225−1228.

Received for review June 29, 2004. Revised manuscript received August 20, 2004. Accepted September 5, 2004. This work was supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Australian government, and by the Commonwealth Cooperative Research Centre Program. The work was conducted by the Australian Wine Research Institute and Unité Mixte de Recherche Sciences pour l'Oenologie, INRA, as part of the research program of the Cooperative Research Centre for Viticulture in Australia.

JF048939H