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Screening for Potential Pigments Derived from Anthocyanins in Red Wine Using Nanoelectrospray Tandem Mass Spectrometry

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Red wine extracts were screened for potential wine pigments derived from anthocyanins, using a combination of nanoelectrospray tandem mass spectrometry techniques. Fourteen aglycons were considered to be of anthocyanidin origin on the basis of their MS/MS spectra. The proposed structures of the aglycons were anthocyanidin C-4 substituted with vinyl linkage between C-4 and the hydroxy group at C-5. The anthocyanidin derivatives identified in the wine extracts were vinyl, vinylmethyl, vinylformic acid, 4-vinylphenol, 4-vinylguaiacol, and vinylcatechin adducts of malvidin as well as vinylformic acid and 4-vinylphenol adducts of peonidin and petunidin. The presence of vinyl alcohol, 4-vinylcatechol, and 4-vinylsyringol adducts of malvidin was also proposed.

KEYWORDS: Wine pigments; anthocyanins; electrospray; nanoelectrospray; tandem mass spectrometry (MS/MS)

INTRODUCTION

Anthocyanins are largely responsible for the attractive color of flowers. Red wine contains anthocyanins extracted from red or black grapes during vinification. The anthocyanins present in *Vitis vinifera* sp. grapes and the wines made from these grapes are structurally based upon five aglycons, namely, malvidin (**1a**), petunidin (**1b**), peonidin (**1c**), delphinidin (**1d**), and cyanidin (**1e**), as shown in **Figure 1**. The glycosylated anthocyanidins (anthocyanins) exist as 3-*O*-glucosides and 3-*O*-acylated glucosides. The major acylated glucosides are *p*-coumaroyl (*1*) or acetyl glucosides (*2*) and to a lesser extent caffeoyl glucosides (*1*), resulting from esterification of *p*-coumaric acid, acetic acid, or caffeic acid with the hydroxy group on carbon 6 of a glucose molecule (*3*).

Despite a progressive decline in anthocyanins during maturation (*4*), wine color is maintained, suggesting that new and more stable pigments (wine pigments) are formed by the reaction of anthocyanins with other wine constituents. Several wine pigments recently identified include the vinyl adduct of malvidin 3-*O*-glucoside (**2**; **Figure 1**) resulting from the reaction of malvidin 3-*O*-glucoside with acetaldehyde (*5*, *6*), the vinylformic acid adduct of malvidin 3-*O*-glucoside (**3a**) resulting from the reaction of malvidin 3-*O*-glucoside with pyruvic acid (*7*), and the 4-vinylphenol adduct of malvidin 3-*O*-glucoside (**7a**) (*8*). The presence of the vinylcatechin or vinylepicatechin adducts of malvidin 3-*O*-glucoside (**4**) was also proposed (*9*−*11*). All of these wine pigments are derived from anthocyanins present in the grape skin and are more resistant to attack by nucleophiles, especially sulfur dioxide, than their precursors due to the substitution at the C-4 position of malvidin (*12*).

Although the unambiguous identification of wine pigments via their isolation is highly desirable, the structural diversity and comparatively low concentration of these pigments make their isolation from the red wine matrix impractical. Therefore, a sensitive and reliable screening method for potential wine pigments (anthocyanin derivatives) in red wine is desirable prior to a comprehensive investigation, including the synthesis of reference compounds for structural confirmation and the influence of these wine pigments on red wine color.

The nanoelectrospray (nano-ESI) technique was theoretically and practically established by Wilm and Mann (*13*) and has been used widely for protein identification (*14*). The main advantages of the nano-ESI technique over conventional electrospray (ESI) are greater sensitivity and smaller sample size requirement (typically 1 μL) while still permitting the application of various tandem mass spectrometry (MS/MS) techniques (*15*). The present study describes the development and application for the screening of potential pigments in red wine using ESI and nano-ESI-MS/MS.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and solvents were of AR grade used and supplied by BDH Chemicals Ltd. (Poole, U.K.) unless otherwise indicated.

Malvidin 3-*O*-glucoside was extracted from *Vitis vinifera* cv. Shiraz grape skin using isoamyl alcohol (Ajax Chemicals) and was then purified by crystallization from acidified isoamyl alcohol using diethyl

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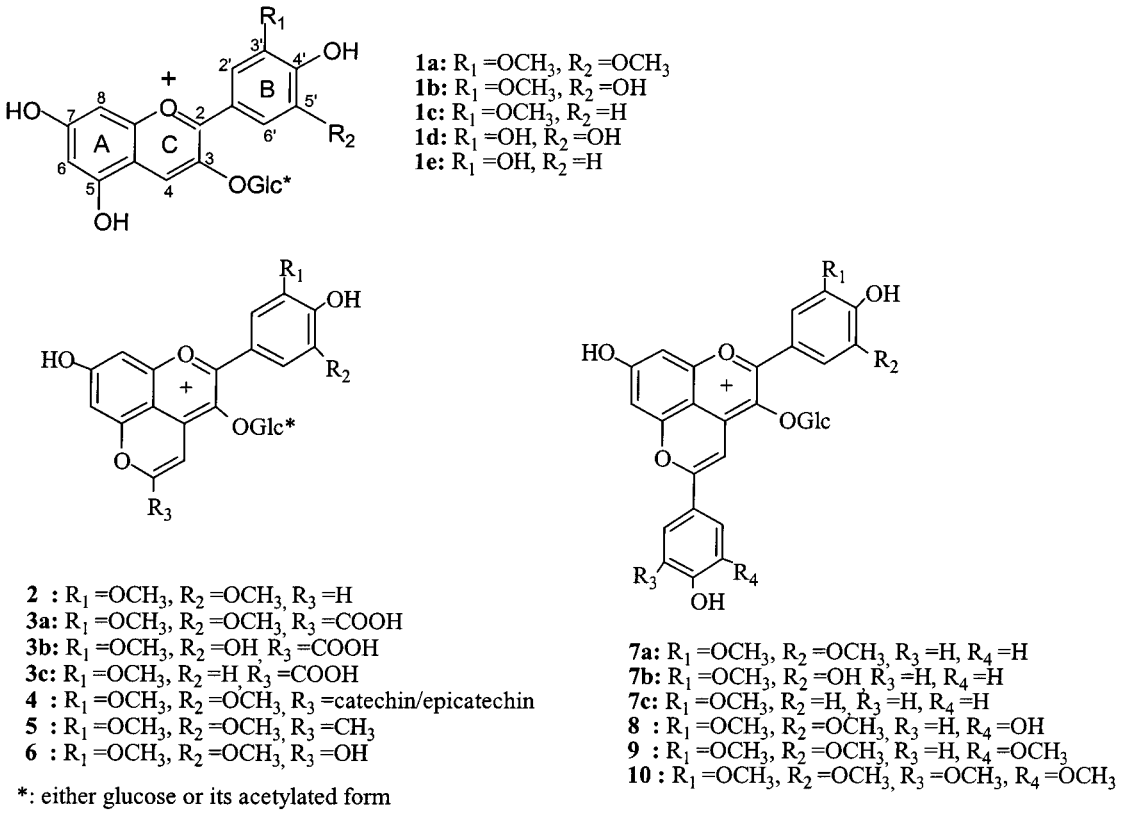

Figure 1. Structures of anthocyanins and proposed anthocyanin vinyl derivatives.

ether (*16*) and subsequently from an acidified methanol solution (*17*) until free of most impurities. The isolated malvidin 3-*O*-glucoside was used for the synthesis of the vinylformic acid adduct and 4-vinylphenol adduct according to the methods outlined by Fulcrand et al. (*7*, *8*) using pyruvic acid and 4-vinylphenol, respectively. These compounds were used as reference compounds.

Grape skin extract was prepared from grape skin extract powder supplied from Robert Bryce and Co. Ltd. (Glandore, SA, Australia). The powder (10 mg) was dissolved in a small volume of 50% aqueous ethanol and then diluted with distilled water (100 mL). The solution was loaded onto a C_{18} Sep-Pak cartridge (Waters, Milford, MA) and was extracted with methanol (2 mL).

A 3-year-old wine made from *V. vinifera* cv. Shiraz was fractionated according to the methods described by Asenstorfer et al. (*11*). Fractions **i** and **iv** were used in this study.

ESI-MS/MS Analysis. The sample solution was directly infused at a flow rate of 5 μL/min using a syringe pump (Cole-Parmer, Vernon, IL), into the ESI source of an API-300 triple-quadrupole mass spectrometer (PE Sciex, Thornhill, ON, Canada). The ESI needle potential was set at 5300 V, and the ring potentials varied from 120 to 250 V. The curtain gas (nitrogen) and nebulizer gases (air) were set at 8 and 10 units, respectively. Nitrogen gas was used as collision gas setting at 2 units. The collision energy and orifice potential varied between the experiments and therefore were optimized as appropriate. The instrument was operated in positive ion scan mode with a step size of 0.2 Da and a dwell time 1.0 ms. For the neutral loss experiment, the second mass analyzer was scanned from *m*/*z* 250 to 1000. The ion signals were consecutively accumulated using multichannel acquisition (MCA) mode within Sample Control software version 1.3 (PE Sciex).

Nano-ESI-MS/MS Analysis. The nano-ESI ion source (Protana, Odense M, Denmark) was interfaced with the mass spectrometer. One microliter of a sample was loaded into the glass capillary coated with gold (Protana) used as a nano-ESI needle. The needle voltage was set at 700 V, and other conditions were the same as those described above except that no nebulizer gas was required for nano-ESI. For the neutral loss experiment, the second mass analyzer was scanned from *m*/*z* 250 to 2000.

RESULTS AND DISCUSSION

Characterization of Anthocyanins and Vinylformic Acid and 4-Vinylphenol Adducts of Malvidin 3-*O*-Glucoside by ESI-MS/MS. Anthocyanins in the grape skin extract were examined by ESI-MS/MS. The molecular cations of malvidin 3-*O*-glucoside (*m*/*z* 493) and its 3-*O*-acetyl (*m*/*z* 535) and 3-*O*-*p*-coumaroylglucosides (*m*/*z* 629) all exhibited the same product ion alone at *m*/*z* 331 resulting from the elimination of 162, 204, and 308 u from the respective molecular cations (data not shown). This product ion can be identified as the malvidin (aglycon) cation produced by a loss of dehydrated sugar moieties, which is the common fragment pathway of glycosyl compounds (*18*−*20*). The synthetic vinylformic acid (*m*/*z* 561; **3a**) and 4-vinylphenol (*m*/*z* 609; **7a**) adducts of malvidin 3-*O*-glucoside also yielded solo product ions representing the respective aglycone cations at *m*/*z* 399 and 447 (data not shown). This indicated that the substitutions at the C-4 of malvidin did not change the primary fragmentation pathway under the low-energy CID MS/MS.

The anthocyanins in a skin extract were monitored by ESI-MS/MS in neutral loss scans for precursor ions with the elimination masses of 162, 204, and 308 u. As may be observed in the three different neutral scans (**Figure 2**), the five precursor ions corresponded to masses of the respective anthocyanins. Thus, neutral loss scanning may be used for the screening of anthocyanin derivatives that have the same glucosyl forms as those of genuine anthocyanins.

Further Characterization of Aglycon by ESI Source CID MS/MS. Because wine is a complex matrix and contains a broad range of the plant natural products that often exist in the form of glycosides, the precursor ions detected by the neutral loss scans can be derived from various sources. It was therefore necessary to further investigate the aglycon cation for its identity as the aglycon of an anthocyanin derivative. The mass spec-

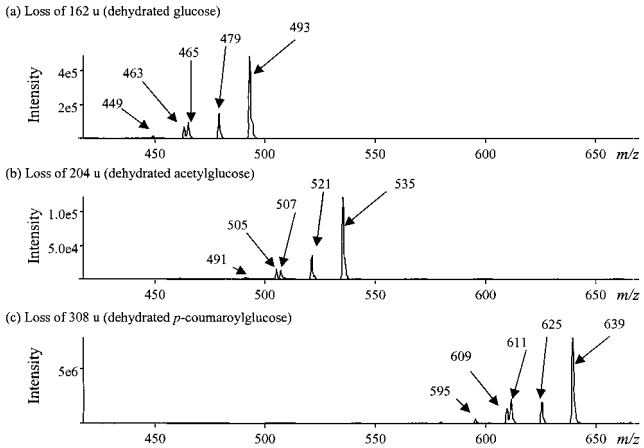

Figure 2. Neutral loss spectra obtained by analyzing the grape skin extract using ESI-MS/MS with monitoring of losses of (a) 162 u, (b) 204 u, and (c) 308 u representing the elimination of dehydrated glucose and acetylglucose and *p*-coumaroylglucose from anthocyanins, respectively.

Table 1. Elimination Masses from Aglycon Cations of Anthocyanins Resulting from ESI Source CID MS/MS in Product Ion Scan Mode

aglycon cation	*m*/*z*	elimination masses (u) from the aglycon cation					
malvidin (**1a**)	331	16	32	44	61	89	
petunidin (**1b**)	317	15	32	43	60	72	89
peonidin (**1c**)	301	15	32	43	58	72	88
delphinidin (**1d**)	303	18	46	74			
cyanidin (**1e**)	287	18	46	74			

trometric characterization of the aglycon cation was carried out by ESI source CID MS/MS, in which the aglycon cation was generated and enriched by source CID with the help of a higher orifice potential and subsequently isolated by the first mass analyzer followed by MS/MS in product ion scan mode. The product ion spectra of the aglycon cations of five anthocyanidin (**1a**−**e**) 3-*O*-glucosides were obtained by ESI source CID MS/MS. The elimination masses of <100 u from the aglycon cations are shown in **Table 1**. The elimination masses from malvidin, petunidin, and peonidin were very similar to each other, but all three were different from those of delphinidin and cyanidin. Malvidin had the characteristic elimination mass of 16 u, whereas petunidin and peonidin had elimination masses of 15 u.

Using the same method as above, the product ion spectra of the aglycon cations derived from the malvidin (**1a**) 3-*O*-glucoside and the vinylformic acid (**3a**) and 4-vinylphenol (**7a**) adducts of malvidin 3-*O*-glucoside were compared. The major elimination masses from the aglycon cation (malvidin; *m*/*z* 331) of malvidin 3-*O*-glucoside were 16, 32, 44, 61, and 89 u (**Table 1**). Notably these elimination masses were almost identical to those from the aglycon cations of the vinylformic acid (*m*/*z* 399) and 4-vinylphenol (*m*/*z* 447) adducts of malvidin 3-*O*-glucoside (data not shown). This indicated that these aglycon cations were fragmented by the same pathway regardless of the modification of the malvidin moiety. Furthermore, the elimination masses from the five different anthocyanidins (**Table 1**) may be used as a fingerprint of the precursor anthocyanidin for an anthocyanin derivative. Although it is possible that under the conditions used an anthocyanin aglycon cation may show a different fingerprint pattern from that reported in this study, the consistency of the fingerprint pattern for the various anthocyanin derivatives helps substantiate the method. Therefore, the screening of the aglycon cation derived from anthocyanin derivatives

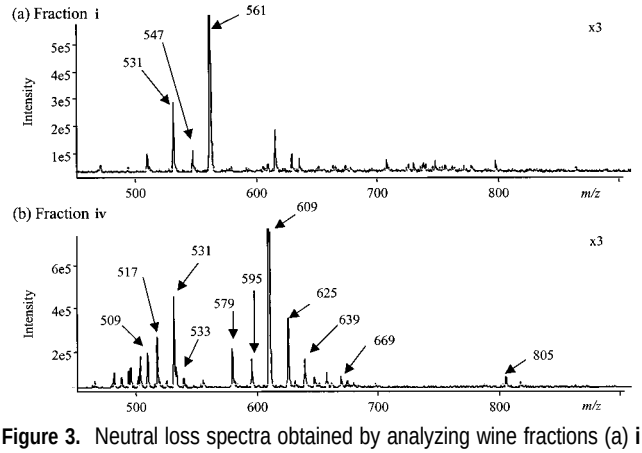

Figure 3. Neutral loss spectra obtained by analyzing wine fractions (a) **i** and (b) **iv** using nano-ESI-MS/MS with monitoring a loss of 162 u.

followed by the characterization of the aglycon cation was a potential method for the identification of novel wine pigments in red wine.

Characterization of Anthocyanin Derivatives in the Wine Fractions by Nano-ESI-MS/MS. The two wine fractions, **i** and **iv**, known to be rich in C4-substituted anthocyanins (*10*, *11*) were analyzed. The neutral loss spectra of 162 u obtained from fractions **i** and **iv** showed intense precursor ions at *m*/*z* 561 and 609 corresponding to the molecular cations of vinylformic acid (**3a**) and 4-vinylphenol adducts (**7a**) of malvidin 3-*O*-glucoside, respectively (**Figure 3a,b**). In fraction **i** there were 58 precursor ions detected (>1% of base peak intensity) showing a neutral loss of 162 u, 12 with a loss of 204 u, and 40 with a loss of 308 u. Similarly, in fraction **iv**, 33 ions were detected with a neutral loss of 162 u, 28 with a loss of 204 u, and 64 with a loss of 308 u. Eleven and 27 product ions (aglycon cation) in fractions **i** and **iv**, respectively, were derived from at least two precursor ions (162 u and either 204 or 308 u), thus indicating that they possibly originated from anthocyanin derivatives (**Table 2**).

Proposed Structural Determination of Anthocyanin Derivatives. The product ions (**Table 2**) were then further examined by nano-ESI source CID MS/MS to confirm their identities. As a result, 3 of the 11 product ions in fraction **i** and 11 of the 27 in fraction **iv** were postulated as aglycon cations of anthocyanin derivatives (**Table 3**). The aglycon with *m*/*z* 331 was malvidin itself. The other product ions (7 ions in fraction **i** and 15 ions in fraction **iv**) did not show the characteristic fragmentation pattern similar to that of the anthocyanidins.

In fraction **i**, the presence of vinylformic acid adducts of malvidin (**3a**), petunidin (**3b**), and peonidin (**3c**) 3-*O*-glucosides was confirmed according to the masses of the aglycon cations and their fragmentation pattern (**Table 3**). The characteristic elimination masses of these aglycon cations were similar; however, the ion of *m*/*z* 399 had a characteristic loss of 16 u identical to malvidin, whereas ions with masses of *m*/*z* 369 and 385 showed a loss of 15, similar to peonidin and petunidin. Consistent with previous work (*10*, *11*), the vinylformic acid adduct of malvidin glucoside (**3a**) was the most abundant species in fraction **i**.

Fraction **iv** contained a greater diversity of compounds that according to the mass spectra were thought to be of anthocyanin origin. The most abundant precursor ions monitored by neutral loss of 162, 204, and 308 u were *m*/*z* 609, 651, and 755, which had the same product ion with *m*/*z* 447. According to the molecular weight and product ion spectra (**Table 3**) and in agreement with data presented by Fulcrand et al. (*8*), this was

Table 2. Aglycon Cations of Potential Anthocyanin Derivatives in (a) Fraction **i** and (b) Fraction **iv**, Screened by Nano-ESI-MS/MS in Neutral Loss Scan Mode Monitoring Losses of 162, 204, and 308 u

aglycon	precursor ions detected			aglycon	precursor ions detected		
(*m*/*z*)	162 u	204 u	308 u	(*m*/*z*)	162 u	204 u	308 u
	(a) Fraction **i**				(b) Fraction **iv**		
308	470		616	303	465	507	611
331	493		639	319	481	523	
369	531	573	677	325	487	529	
371	533	575	679	331	493	535	639
385	547	589		333	495		641
399	561	603	707	339	501		647
401	563	605	709	341	503	545	649
443	605		751	347	509	551	655
467	629		775	355	517		663
489	651		797	357	519	561	665
589	751		897	369	531	573	677
				371	533	575	679
				377	539		685
				393	555		701
				417	579	621	725
				419	581		727
				433	595	637	741
				447	609	651	755
				463	625	667	771
				469	631	673	777
				477	639	681	785
				485	647	689	
				495	657	699	803
				507	669	711	
				517	679		825
				643	805	847	951
				655	817		963

identified as the 4-vinylphenol adduct of malvidin 3-*O*-glucoside (**7a**). Two aglycon cations with masses of *m*/*z* 417 and 433, which were 30 and 14 u smaller than the malvidin 4-vinylphenol adduct, exhibited product ion spectra with an elimination mass of 15 u, suggesting that these masses were peonidin and petunidin derivatives, respectively. It was therefore proposed that these aglycon cations represent petunidin 4-vinylphenol (*m*/*z* 433; **7b**) and peonidin 4-vinylphenol (*m*/*z* 417; **7c**) adducts.

A series of the aglycon cations with masses of *m*/*z* 463, 477, and 507 (**Table 3**) were 16, 30, and 60 u greater than malvidin 4-vinylphenol adduct (*m*/*z* 447; **7a**). These aglycons had elimination masses similar to those from malvidin, suggesting they were malvidin derivatives. Furthermore, the masses of 16, 30, and 60 u were consistent with additions of hydroxy, methoxy, and dimethoxy groups on the 4-vinylphenol, respectively. Therefore, these aglycon cations with masses of *m*/*z* 463, 477, and 507 were tentatively identified as malvidin 4-vinylcatechol (**8**), malvidin 4-vinylguaiacol (**9**), and malvidin 4-vinylsyringol (**10**) adducts. The malvidin 3-*O*-glucoside 4-vinylphenol adduct (**7a**) is the result of the enzymatic decarboxylation of *p*-coumaric acid into 4-vinylphenol (*21*), followed by the subsequent reaction of 4-vinylphenol with malvidin 3-*O*-glucoside (*8*). Caffeic, ferulic, and sinapic acids are also among the principal cinnamic acids found in grapes (*22*), and by analogy these cinnamic acids may be decarboxylated to form 4-vinylcatechol, 4-vinylguaiacol, and 4-vinylsyringol. The subsequent reaction of these 4-vinylphenols with anthocyanins was therefore likely to yield C-4-substituted wine pigments. The presence of malvidin 4-vinylcatechol and 4-vinylsyringol adducts in red wine has not been described previously.

One of the simplest of the vinyl malvidin derivatives, formed by the reaction of malvidin 3-*O*-glucoside with acetaldehyde (*5*), was observed in fraction **iv**. The aglycon cation of *m*/*z* 355 was characterized as the malvidin vinyl adduct (**2**) on the basis of the size of the molecule and its product ion spectrum (**Table 3**). A related aglycon cation was observed in fraction **iv** with a mass of *m*/*z* 369, a molecular weight 14 u greater than that of the malvidin vinyl adduct, and an elimination mass (16 u), which was consistent with its being malvidin derived (**Table 3**). This was consistent with a pigment described by Benabdeljalil et al. (*6*) with an aglycon mass of *m*/*z* 369 and formed by the reaction of malvidin with acetone (vinylmethyl adduct; **5**).

Another group of potential pigments in fraction **iv** had an aglycon cation mass of *m*/*z* 371. The aglycon cation mass agreed with the addition of a hydroxy group on the malvidin vinyl adduct; however, the vinylformic acid adduct of delphinidin has an identical mass. On the basis of the characteristic elimination masses from the aglycon cation using product ion scan, this aglycon was tentatively characterized as a malvidin vinyl alcohol adduct (**6**) and has not been previously proposed to be present in wine. The product ion spectrum of the aglycon cation observed in fraction **iv** with a mass of *m*/*z* 643 had a pattern different from those of the other aglycon cations (**Figure 4**).

Table 3. Mass Spectrometric Characterization of Potential Wine Pigments (Anthocyanin Derivatives) Screened by a Combination of Nano-ESI-MS/MS Techniques

precursor ions[a]			aglycon								proposed structure
162 u	204 u	308 u	(*m*/*z*)	elimination masses from aglycon cations[b] (u)							(Figure 1)
				Fraction **i**							
531	573	677	369	15	32	43	57	72	88		**3c**
547	589		385	15	32	44	72	89			**3b**
561	603	707	399	16	32	44	58	61	89		**3a**
				Fraction **iv**							
509	551	655	347	16	32	45	58	72	89		unknown
517		663	355	16	32	44	61	72	75	89	**2**
531	573	677	369	16	32	44	58	61	73	89	**5**
533	575	679	371	16	30	32	46	60	61	89	**6**
579	621	725	417	15	33	44	60	72	86	88	**7c**
595	637	741	433	15	32	44	60	72	88		**7b**
609	651	755	447	16	32	44	58	61	89		**7a**
625	667	771	463	16	30	32	44	58	61	89	**8**
639	681	785	477	16	32	44	58	61	89		**9**
669	711		507	16	32	44	58	61	86	89	**10**
805	847	952	643	152	168	180	196	213	241		**4**

[a] Detected by nano-ESI-MS/MS in neutral loss scan mode monitoring 162, 204, and 308 u. [b] Detected by nano-ESI source CID MS/MS in product ion scan mode.

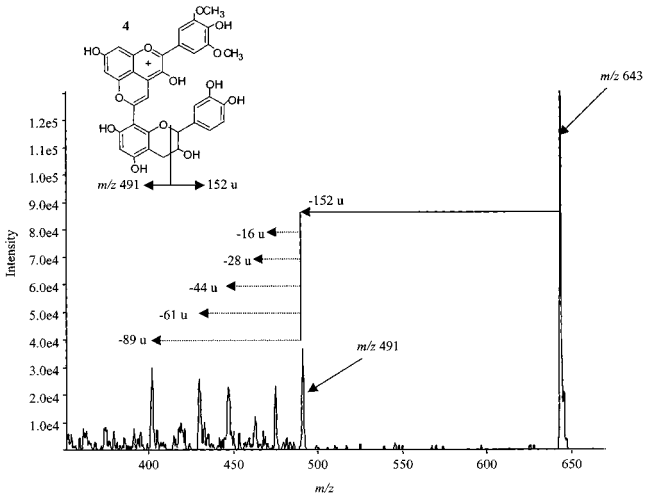

Figure 4. Product ion spectrum of aglycon cation *m*/*z* 643 obtained by nano-ESI source CID MS/MS.

The fragment ion of *m*/*z* 491 was further fragmented to *m*/*z* 475, 447, 430, and 402, which corresponded to the elimination masses of 16, 44, 61, and 89 u, respectively. This indicated that the aglycon cation was derived from malvidin. Furthermore, the loss of a fragment of 152 u (**Figure 4**), a common fragment of catechin/epicatechin resulting from retro-Diels−Alder cleavage (*23*, *24*), from the aglycon of *m*/*z* 643 suggested that either catechin or epicatechin was present in this compound. It was therefore proposed that this compound was either the malvidin 3-*O*-glucoside vinylcatechin or vinylepicatechin adduct (**4**) and belongs to the series of pigments first described by Francia-Aricha et al. (*9*).

A final series of glycosides that could be identified in fraction **iv** had an aglycon cation mass of *m*/*z* 347 and a product ion spectrum indicating that the aglycon cation was malvidin derived (**Table 3**). The aglycon cation mass was consistent with the addition of a hydroxyl group to malvidin but was 2 u smaller than that (*m*/*z* 349) of the hydrated malvidin, for example, carbinol pseudo-base or chalcone. Although mass spectrometry cannot give decisive identification, a simple interpretation would possibly be a hydroxy group substitution at the C-4 position of malvidin. Further structural study is needed to confirm whether this compound is a new wine pigment or one of the equilibrated forms of malvidin 3-*O*-glucoside.

Using a combination of the ESI-MS/MS techniques provided an effective method for the screening of ions of potential wine pigments. With the identification of putative anthocyanin derivatives, it is now possible to synthesize these compounds and confirm their presence in wine. Furthermore, it is possible using the techniques developed here to survey wines of different origins for new pigments.

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LITERATURE CITED

(1) Somers, T. Grape phenolics: The anthocyanins of *Vitis vinifera*, variety Shiraz. *J. Sci. Food Agric.* **1966**, *17*, 215−219.

(2) Fong, R. A.; Kepner, R. E.; Webb, A. D. Acetic-acid-acylated anthocyanin pigments in the grape skins of a number of varieties of *Vitis vinifera. Am. J. Enol. Vitic.* **1971**, *3*, 150−155.

(3) Hrazdina, G.; Franzese, A. Structure and properties of the acylated anthocyanins from *Vitis* species. *Phytochemistry* **1974**, *13*, 225−229.

(4) Nagel, C. W.; Wulf, L. W. Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and Cabernet Sauvignon. *Am. J. Enol. Vitic.* **1979**, *30*, 111−116.

(5) 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.

(6) Benabdeljalil, C.; Cheynier, V.; Fulcrand, H.; Hakiki, A.; Mosaddak, M.; Moutounet, M. Evidence of new pigments resulting from reaction between anthocyanins and yeast metabolites. *Sci. Aliments* **2000**, *20*, 203−220.

(7) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401−1407.

(8) Fulcrand, H.; dos Santos, P.; Sarni-Manchado, P.; Cheynier, V.; Favre-Bonvin, J. Structure of new anthocyanin-derived wine pigments. *J. Chem. Soc., Perkin Trans. 1* **1996**, 735−739.

(9) Francia-Aricha, E. M.; Guerra, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. New anthocyanin pigments formed after condensation with flavanols. *J. Agric. Food Chem.* **1997**, *45*, 2262−2266.

(10) Asenstorfer, R. E.; Hayasaka, Y.; Iland, P. G.; Lambert, S. G.; Jones, G. P. Wine phenolics: the development of pigments in red wine. *Proceedings of the 1999 Conference of the New Zealand Society for Viticulture and Oenology*; Steans, G., Ed.; Auckland, New Zealand, 1999; pp 83−87.

(11) Asenstorfer, R. E.; Hayasaka, Y.; Jones, G. P. Isolation and structures of oligomeric wine pigments by bisulfite-mediated ion-exchange chromatography. *J. Agric. Food Chem.* **2001**, *49*, 5957−5963.

(12) Timberlake, C. F.; Bridle, P. Flavylium salts resistant to sulphur dioxide. *Chem. Ind. (London)* **1968**, 1489.

(13) Wilm, M. S.; Mann, M. Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *Int. J. Mass Spectrom. Ion Processes* **1994**, *136*, 167−180.

(14) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **1996**, *379*, 466−469.

(15) Wilm, M.; Mann, M. Analytical properties of the nanoelectrospray ion source. *Anal. Chem.* **1996**, *68*, 1−8.

(16) Willstätter, R.; Zollinger, E. H. XVI. Über die Farbstoffe der Weintraube und der Heidelbeere, II. *Justus Liebigs Ann. Chem.* **1916**, *412*, 195−216.

(17) Levy, L.; Robinson, R. Experiments on the synthesis of anthocyanin. Part IX. Synthesis of oxycoccicyanin chloride. Observations on the distribution numbers of the anthocyanins. *J. Chem. Soc.* **1931**, 2715−2722.

(18) Wolfender, J. L.; Maillard, M.; Marston, A.; Hostetmann, K. Mass spectrometry of underivatized naturally occurring glycosides. *Phytochem. Anal.* **1992**, *3*, 193−214.

(19) Stobiecki, M. Application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry* **2000**, *54*, 237−256.

(20) Favretto, D.; Flamini, R. Application of electrospray ionization mass spectrometry to the study of grape anthocyanins. *Am. J. Enol. Vitic.* **2000**, *51*, 55−64.

(21) Steinke, R. D.; Paulson, M. C. The production of steam-volatile phenols during the cooking and alcoholic fermentation of grain. *J. Agric. Food Chem.* **1964**, *12*, 381−387.

(22) Ribéreau-Gayon, P.; Dubourdieu, D.; Donèche, B.; Lonvaud, A. *Handbook of Enology. Vol. 1. The Microbiology of Wine and Vinifications*; Wiley: Chichester, U.K., 2000.

(23) 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.
(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.

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