Isolation and Structures of Oligomeric Wine Pigments by Bisulfite-Mediated Ion-Exchange Chromatography

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Methods have been developed that are based on cation exchange chromatography in the absence and presence of excess bisulfite for the isolation of wine pigments from Australian red wine and grape marc extract. The pigments were identified using HPLC and electrospray ionization mass spectrometry. The mass spectral data indicate that these pigments are C4-substituted anthocyanins with a tetracyclic structure. The pigments form a series of closely related oligomeric pigments which include those previously described in the literature, such as pigment A and vitisin A, as well as some newly identified pigments.

Keywords: *Wine pigments; oligomeric pigments; anthocyanin polymers; bisulfite; vitisin; acetaldehyde*

INTRODUCTION

Anthocyanins and their derived pigments are important constituents of red wine. The relative instability of anthocyanins isolated from grapes during the winemaking process suggests that they participate in reactions during the fermentation and maturation of wine to form more complex pigments. Although theories have been put forward regarding their identities, the nature of these pigments has remained elusive. For example, Somers (*1*) reported that the concentration of anthocyanins steadily declines during wine aging and that these anthocyanins are replaced by more stable "polymeric pigments". Evidence suggests that these polymeric pigments include a range of pigments, the smallest of which is vitisin B (**1**, Figure 1) (*2*) that has a molecular ion at *m*/*z* 517 on mass spectrometric analysis.

Isolation of stable pigments from wine has proven difficult, and until recently there has been little evidence as to the actual identity of the pigments formed during wine maturation. However, with the use of higher performance chromatographic methods and the advent of electrospray mass spectrometry there is increasing evidence for the existence of a series of oligomeric pigments (Figure 1) in wine (*2*−*4*), grape marc (*5*), and model wine solutions (*6*, *7*) with an anthocyanin-based tetracyclic structure. The overall structure of these pigments is based principally on malvidin-3-glucose with a fourth ring that results from the cyclization between a substituent group on carbon 4 and the hydroxyl group at carbon 5 (see Figure 1 for numbering). Anthocyanins with a carbon atom covalently bonded to the 4-carbon are resistant not only to bisulfite addition (*8*), but also to the formation of the hemiketal through pH changes (*9*, *10*). These types of compounds are also resistant to oxidation (*11*). Thus, as the wine matures, the monomeric grape-derived anthocyanins become less important and these C4-substituted anthocyanins become more important.

Since the discovery and identification of vitisin A (**2**, Figure 1), or the pyruvate adduct to malvidin-3-glucose **(3)** which has identical spectral properties (*2*, *3*, *5*), research has been conducted to determine the importance of this pigment in Australian red wines. The pigment was found in higher concentrations than other oligomeric pigments, and from the HPLC data it was estimated that it, together with its acetyl and *p*-coumaryl derivatives, contributes typically between 1 and 4%, and as high as 10%, of total red wine pigments (*12*). However, other non-anthocyanin pigments are also observed in addition to vitisin A and its derivatives.

Methods were developed to isolate these oligomeric pigments from wine based upon the reactivities of the pigments to bisulfite anion. Most anthocyanins, in the presence of the bisulfite ion, form an anionic addition product (*6*). However, the resistance of the 4-substituted anthocyanins to bisulfite addition prevents the formation of this anionic species. The method developed uses ion-exchange chromatography to separate those anthocyanins and anthocyanin-derivatives which form anionic bisulfite addition products from the 4-substituted anthocyanins. This method has allowed the identification of a number of oligomeric pigments in both wine and grape marc extracts.

MATERIAL AND METHODS

Isolation of Oligomeric Pigments. Approximately 11 L of four-year-old Shiraz wine from the Riverland, South Australia (made by fermenting to dryness using small-lot winemaking procedures followed immediately by storage in glass bottles) was concentrated by using both rotary evaporation and reversed-phase C18 chromatography, as follows. The C18 column consisted of 50 g of material loaded in a 100-mm Büchner funnel with diatomaceous earth as supporting material. The material retained on this column was eluted with 1

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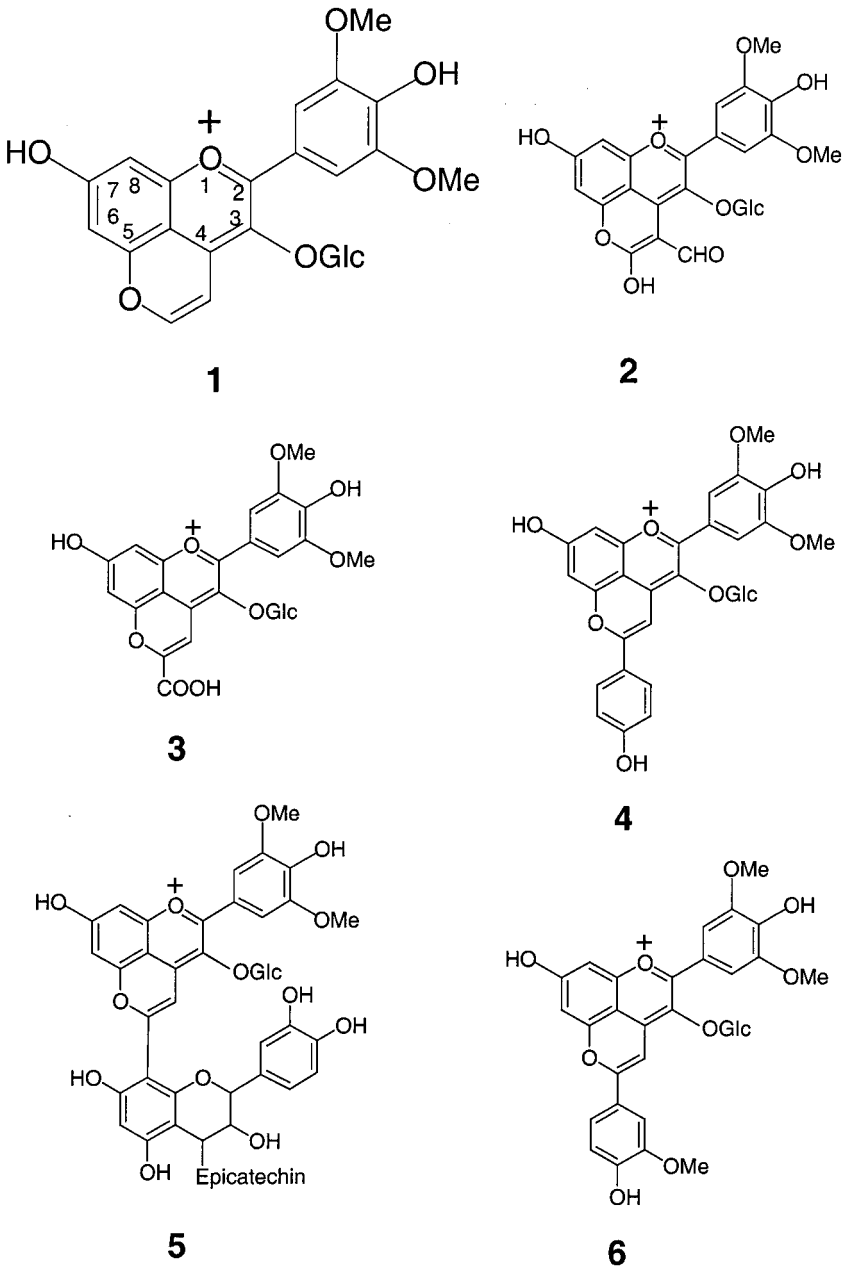

Figure 1. Structures of chemically similar oligomeric pigments identified in wine, grape marc, and model wine solutions: **(1)** vitisin B (*2*); **(2)** vitisin A (*3*); **(3)** pyruvate adduct of malvidin-3-glucose (*5*); **(4)** pigment A (*4*); **(5)** pigment B2-III (*6*); and **(6)** 3″-*O*-methyl-pigment A.

L of methanol. The methanol eluate (wine concentrate) was further concentrated using rotary evaporation until almost dry, and then water was added to give a volume of 200 mL. Approximately 50 mL of this aqueous wine concentrate was loaded onto a sulfoxyethyl cellulose column (40 × 200 mm) prepared according to the method outlined by Spagna and Pifferi (*13*). The neutral/anionic fraction (fraction **i**) was eluted from the column using 2 L of 10% methanol. The wine pigments retained on the sulfoxyethyl cellulose column were then removed using a solution of 2 M NaCl in 50% (v/v) aqueous methanol (fraction **ii**). A solution of NaCl in aqueous methanol was used in preference to acidified methanol to avoid acid hydrolysis of the retained pigments.

Fraction **ii** was cleaned up by removing methanol on a rotary evaporater, and any NaCl present was removed using a C18 column (C18 material in a Büchner funnel) washed with water. The pigment retained on the C18 material was eluted with methanol and fractionated further as follows. The methanol was rotary evaporated almost to dryness, and the pigment was dissolved in 500 mL of 0.1 M potassium metabisulfite solution. A sulfoxyethyl cellulose cation exchange was prepared as before and a portion of the pigment solution (approximately 100 mL) was loaded onto this column. Pigments, for the most part grape anthocyanins existing primarily as their anionic bisulfite addition products, were eluted from the column using a 0.1 M potassium metabisulfite solution (fraction **iii**). The pigments that were retained on the column were eluted using a solution of 2 M NaCl in 50% methanol (fraction **iv**). The methanol was evaporated from this eluate, and any residual salt was removed using the C18 material as described previously. Pigments were eluted from the C18 column with methanol and were concentrated by rotary evaporation.

Fraction **iv** was further purified by preparative TLC using a silica gel plate without binder (Merck, Darmstadt, Germany) and developed using 70% (v/v) aqueous propanol. The red band (R_f 0.8) was extracted with a 10% (v/v) aqueous methanol solution. The solution was centrifuged, and any silica remaining was removed using a C18 (Sep-Pak Classic) cartridge. Finally, the pigmented eluate was concentrated by rotary evaporation for analysis by HPLC and mass spectrometry.

This general procedure was repeated using a commercial sample of concentrated grape marc extract supplied by Tarac

Table 1. Mass Spectral Data of Pigments Isolated in Fraction iv from Grape Marc and Wine Accompanied by the Proposed Pigment that Each Mass Represents[a]

peak	marc (M^+ *m/z*)	wine (M^+ *m/z*)	proposed compound
A	609.4	609.4	pigment A
	nd	639.4	3″-*O*-methyl- pigment A
B	651.4	651.4	(acetyl)pigment A
C	707.2	707.2	(*p*-coumaryl)vitisin A
	nd	755.6	(*p*-coumaryl)pigment A
D	805.4	805.4	malvidin-3-glucose-4-vinyl-catechin
E	847.4	nd	malvidin-3-(acetyl)glucose-4-vinyl-catechin
F	951.4	951.4	malvidin-3-(*p*-coumaryl)glucose-4-vinyl-catechin
G	1093.4	1093.4	malvidin-3-glucose-4-vinyl-dicatechin
H	1135.4	nd	malvidin-3-(acetyl)glucose-4-vinyl-dicatechin
I	1239.6	nd	malvidin-3-(*p*-coumaryl)glucose-4-vinyl-dicatechin
J	1381.6	nd	malvidin-3-glucose-4-vinyl-tricatechin
K	1423.4	nd	malvidin-3-(acetyl)glucose-4-vinyl-tricatechin
L	1527.6	nd	malvidin-3-(*p*-coumaryl)glucose-4-vinyl-tricatechin
M	1669.4	nd	malvidin-3-glucose-4-vinyl-tetracatechin

[a] Reference to catechin represents either catechin or epicatechin. nd: Not detected.

(Nuriootpa, South Australia). The aqueous grape marc extract was applied directly to the sulfoxyethyl cellulose column without any prior preparation.

HPLC Analysis. The HPLC apparatus used was a Waters system (Waters 501 pump, Wisp auto-sampler 710B; Waters Corporation, Milford, MA) equipped with a diode array detector (Waters diode array 996). The column was a reversed-phase C18 column (250 × 4 mm Licrosphere 100; Merck, Darmstadt, Germany) and was protected by a C18 (NovaPak, Waters) guard column. The elution conditions consisted of a binary solvent system. Solvent A was dilute HCl (pH 2.4) and solvent B was 80% acetonitrile solution acidified using concentrated HCl to pH 2.1. A flow rate of 0.6 mL/min and a column temperature of 30 °C were used. The linear gradient consisted of 0% to 100% solvent B over 50 min. The elution was monitored at 254 nm and UV−visible spectra were recorded from 200 to 600 nm. Wines were diluted 1:10 using distilled water and then filtered using a 0.45-μm syringe filter (Schleicher und Schuell GmbH, Dassel, Germany). The data were analyzed with Waters Millennium software (version 3.05).

Electrospray Mass Spectrometry by Direct Injection. Electrospray mass spectra of the compounds were obtained using an API-300 mass spectrometer with an electrospray interface (PE Sciex, Thornhill, ON). The ion source and orifice potentials were 5.5 kV and 30 V, respectively, for the positive ion mode and −4.5 kV and −30 V, respectively, for the negative ion mode. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 10 units, respectively. The sample was introduced into the mass spectrometer using a flow injector (8125, Rheodyne, Cotati, CA) with a 5 μL sample loop connected to the ion source. The injected solution was delivered in 50% acetonitrile acidified with 2.5% acetic acid at a rate of 5 μL/min, using a syringe pump (Cole-Parmer, Niles, IL).

Liquid Chromatography−Mass Spectrometry (LC−MS). The sample was injected using the flow injector with a 5 μL loop coupled to a C18 reversed-phase HPLC column (2 × 150 mm, Nova-Pak, Waters) at a flow rate of 100 μL/min using the syringe pump. The column was equilibrated in a mixture of 90% solvent A (2.5% (v/v) aqueous acetic acid) and 10% solvent B (2.5% acetic acid in 90% aqueous acetonitrile (v/v)). The pigments were eluted from the column with a gradient of solvent B from 10% to 70% in the first 60 min and from 70% to 100% from 60 to 70 min, with a flow rate of 10 μL/min. All mass spectral data were processed using Bio-Multiview software 1.2β3 (PE Sciex).

Electrospray Mass Spectrometry via Desalting Trap. Electrospray mass spectrometry was also performed by direct injection via a desalting trap. The sample (20 μL) was loaded onto the desalting trap cartridge (Michrom BioResources, Auburn, CA) with a dual-syringe pump (140B solvent delivery system, Applied Biosystems, Foster City, CA) at a rate of 100 μL/min through the flow injector. After the cartridge had been washed with 2.5% acetic acid at a rate of 100 μL/min for 5 min, pigments were eluted using a binary solvent system consisting of solvent A (2.5% acetic acid) and solvent B (2.5% acetic acid in 90% acetonitrile) at a rate of 10 μL/min. A linear gradient of 0 to 100% of solvent B over 30 min was used. The eluent from the cartridge was delivered directly to the ionspray mass spectrometer.

RESULTS AND DISCUSSION

The fractionation and isolation of pigments from wine and grape marc extracts was achieved using cation-exchange chromatography in the absence and presence of a bisulfite buffer. The pigments were separated into two fractions initially, fractions **i** and **ii**. Pigments which are neutral or anionic at wine pH such as vitisin A and its acetyl derivative (*12*) are present in fraction **i**. Indeed, this method was developed to purify vitisin A in sufficient quantities to permit electrophoretic and spectroscopic studies (*12*). Fraction **ii** was fractionated further into fraction **iii**, which contains bisulfite-bleached compounds including anthocyanins, and fraction **iv**. Preparative TLC was applied to fraction **iv** to yield an extract containing mixtures of bisulfit-bleach-resistant pigments. The individual compounds in this extract were not purified further but were investigated directly using HPLC and mass spectrometry. A HPLC chromatogram of the sample measured at both 520 and 280 nm indicated that it was free of non-pigmented phenolic contaminants.

A number of oligomeric pigments in fraction **iv** (the fraction eluted from the second sulfoxyethyl cellulose column with aqueous NaCl/methanol) were tentatively identified in both the grape marc and wine samples using mass spectrometry (Table 1). The mass spectrum of a grape marc sample, injected into the mass spectrometer via a desalting column, is shown in Figure 2. Peaks in the mass spectrum show that the sample contains pigment A (**4**) as well as *p*-coumaryl-vitisin A. Another pigment with *m/z* 30 greater than that of pigment A was also observed which represents an *O*-methyl group. *p*-Vinylguaiacol has been identified in wine (*14*) and originates from the degradation of ferulic acid, a naturally occurring compound in grapes, by a process identical to the degradation of *p*-coumaric acid which yields *p*-vinylphenol (*15, 16*). It is therefore proposed that the new compound is 3″-*O*-methyl-pigment A (**6**, Figure 1) resulting from the reaction of *p*-vinylguaiacol and malvidin-3-glucoside by a mechanism similar to that leading to the formation of pigment A.

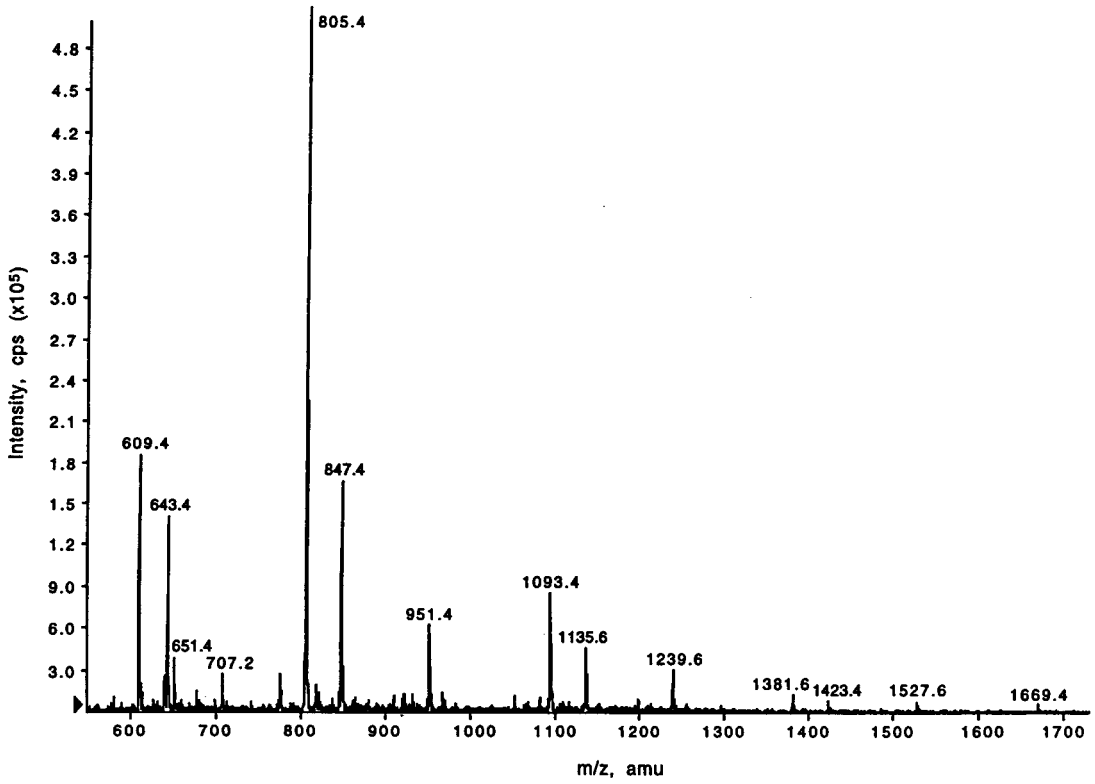

Figure 2. Direct injection electrospray mass spectrum of fraction **iv** isolated from grape marc (see Table 1).

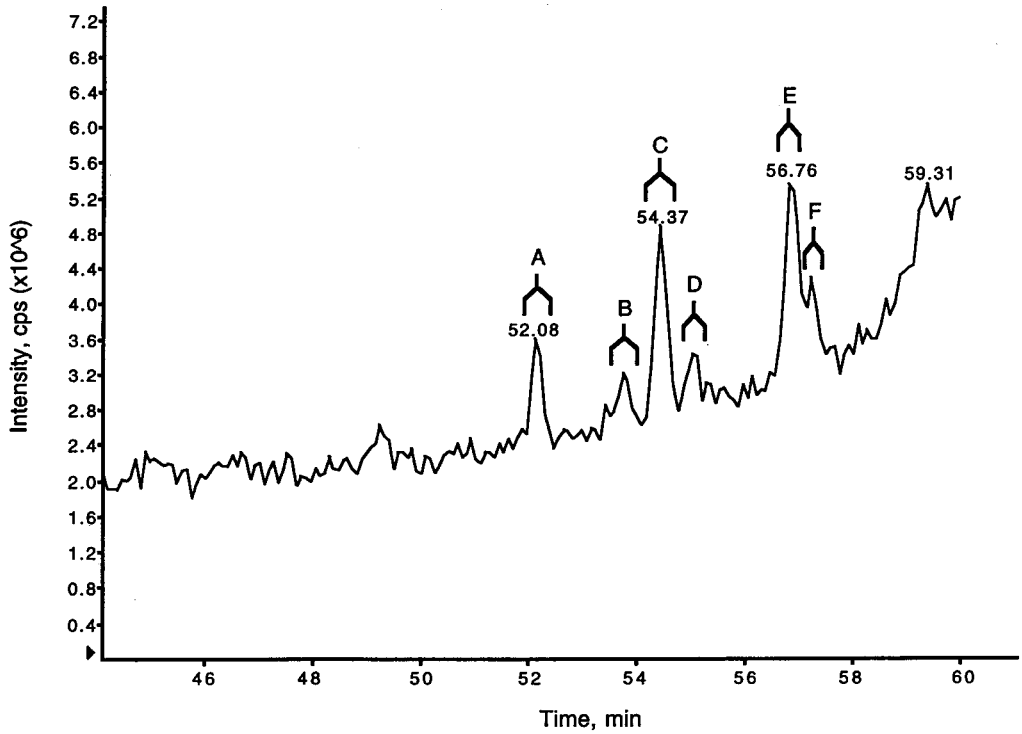

Figure 3. A portion of the LC/MS analysis of fraction **iv** of a grape marc sample indicating the peaks of interest. The mass spectra of each of the marked peaks are shown in Figure 4.

The grape marc sample was also analyzed using LC–MS. The chromatogram shows two peaks, A and C (Figure 3) with different retention times but have identical parent ions with a mass of *m*/*z* 805 (Figure 4). Similarly, peaks B and D gave a parent ion with a *m*/*z* of 847. It is suggested that these peaks represent the catechin and epicatechin isomers of malvidin-3-glucose-4-vinyl-catechin and malvidin-3-(acetyl)glucose-4-vinyl-catechin, respectively. The identity of the compound associated with peak E has not been ascertained, whereas the molecular ion with mass *m*/*z* 609 and major fragment of *m*/*z* 447 indicates that the compound associated with peak F is pigment A (**4**, Figure 1) (*4*).

A number of complex oligomeric pigments were also identified in fraction **iv** in both the wine and marc samples. It is proposed that the pigments with the masses of *m*/*z* 805.4, 847.4, 951.4, 1093.4, 1135.4, 1239.6, 1381.6, 1423.4, 1527.6, and 1669.4 belong to the

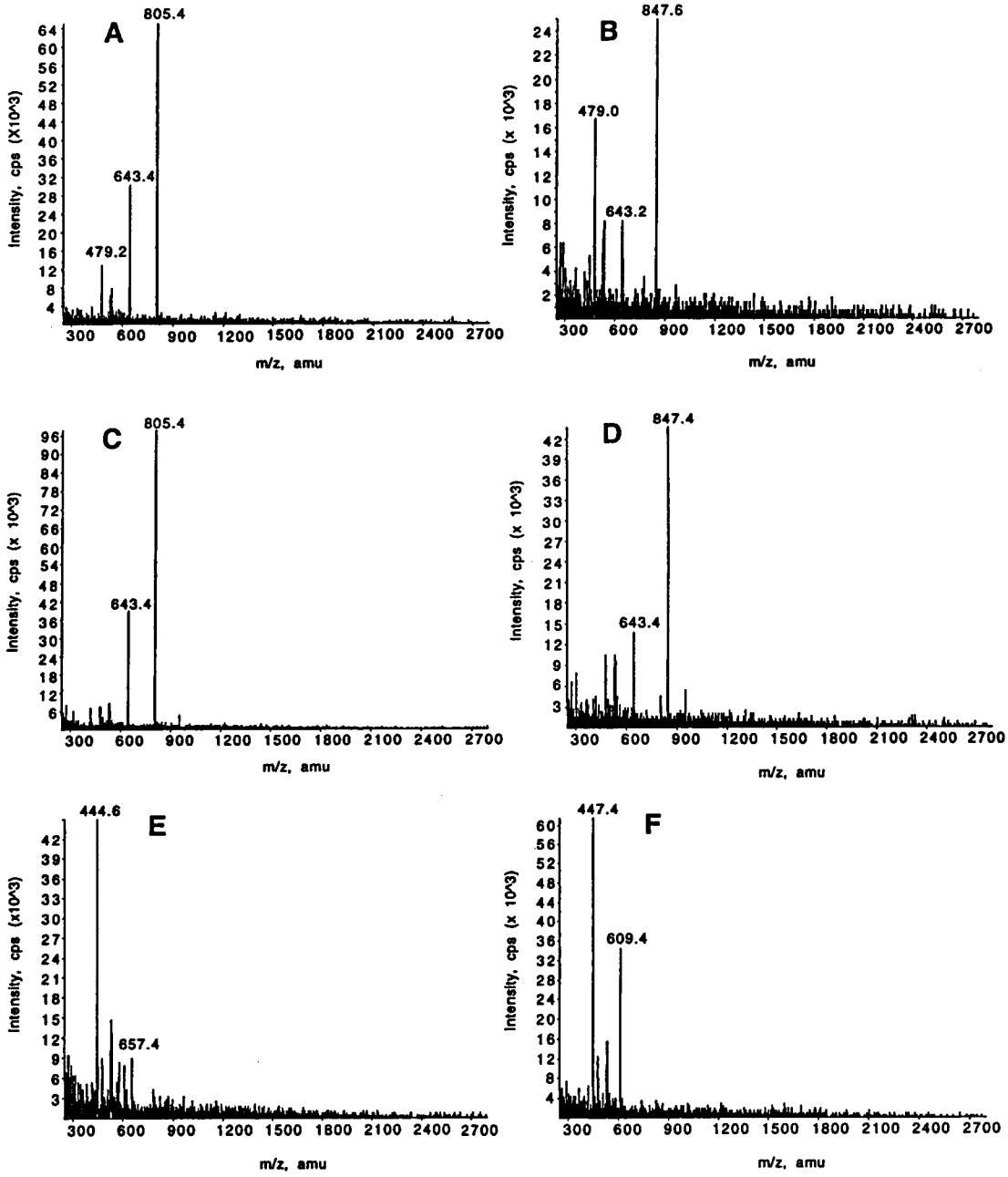

Figure 4. Mass spectra obtained for the peaks identified in Figure 3. Peaks A and C have identical molecular ions of *m*/*z* 805. Similarly, peaks B and D have molecular ions of *m*/*z* 847.

group of pigments described by Francia-Aricha et al. (*6*), whereby malvidin-3-glucose is linked at the C4 position, via a vinyl linkage, to either catechin/epicatechin or procyanidin (**5**, Figure 1). Compared with the grape marc extract, the wine sample lacked a number of the larger oligomeric pigments (Table 1). In contrast, the wine sample contained some smaller oligomeric pigments not evident in the grape marc sample. Many additional minor peaks observed in the mass spectral data can be assigned to similar compounds with anthocyanin entities other than malvidin. The complex oligomeric pigments may arise from the reaction of vinyl-catechins and/or vinyl-procyanidins with malvidin-3-glucose by a mechanism similar to the formation of pigment A from *p*-vinyl phenol and malvidin-3-glucose (*4*). In model solutions Francia-Aricha et al. (*6*) were able to synthesize pigment B2−III (**5**, Figure 1) from acetaldehyde, malvidin-3-glucose, and procyanidin B2. It is proposed that the mechanism of this synthesis reaction involves the addition of acetaldehyde to catechin, epicatechin, or procyanidins, to yield vinyl derivatives. Acetaldehyde reacts with flavan-3-ols to yield unstable ethanol adducts (*17*). Two proposed mechanisms for the decomposition of the ethanol adducts to give chemically reactive intermediates are indicated in Figure 5. In the presence of acid, protonation and then loss of water from the ethanol adducts results in the formation of a carbonium ion (**7**) (*17*). The carbonium ion, acting as an electrophile, then reacts with malvidin-3-*O*-glucose (or other anthocyanins) to form ethyl-linked type pigments. These ethyl-linked pigments have been characterized by HPLC, UV−visible spectroscopy, and mass spectroscopy (*9*, *18*). A second pathway for the decomposition of the ethanol adduct is by elimination of a water molecule prior to protonation to produce vinyl catechin (**8**). The A ring of the vinyl catechin or vinyl

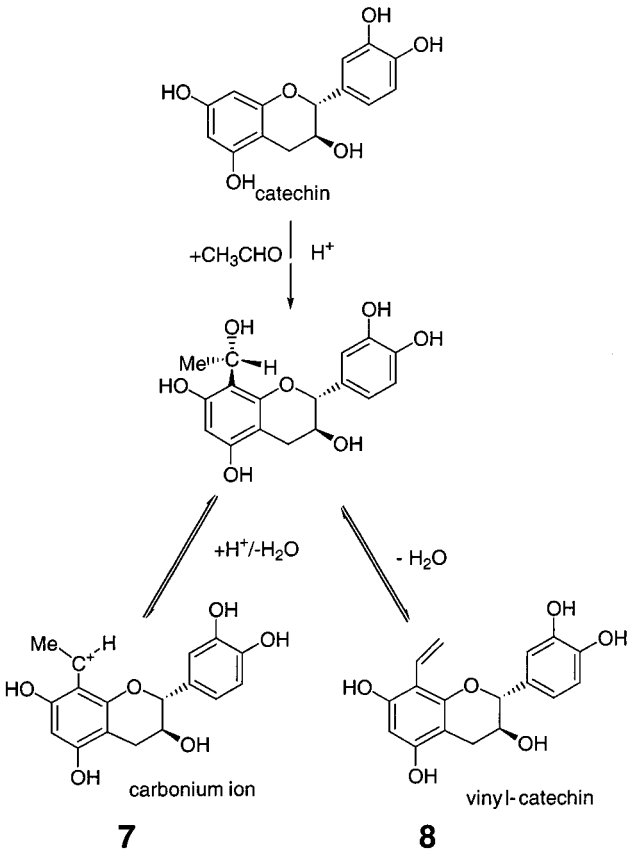

Figure 5. Proposed pathways for the reaction of acetaldehyde with catechin and malvidin-3-glucose. The ethanol−catechin intermediate can form either the carbonium ion **7** as proposed by Fulcrand et al. (*5*) or a vinyl-catechin **8**; the latter then reacts with malvidin-3-glucose to form malvidin-3-glucoside-4-vinyl-catechin.

procyanidin is chemically similar to *p*-vinyl phenol and these vinyl flavan-3-ols may then react with malvidin-3-*O*-glucose or other anthocyanins to form the complex oligomeric pigments (**5**) with the vinyl moeity acting as a nucleophile. It is also noted that the formation of a vinyl flavan-3-ol entity from an ethyl flavan-3-ol is a reversible reaction in aqueous acidic solution. The particular pathway by which the ethanol flavan-3-ol is consumed is expected to be influenced by a range of winemaking parameters including pH, temperature, and substrate concentrations. Because both flavan-3-ols and anthocyanins are substrates for the formation of ethyl-bridged polymers from carbonium ion intermediates, whereas only anthocyanins are substrates for the reaction to form vinyl-bridged polymers, the formation of the latter will be favored by higher anthocyanin concentrations in the must. The relative rates for the two reaction pathways with anthocyanins as substrates, which are unknown at the present time, will be an important factor in determining the concentrations of the vinyl-linked pigments in red wine.

The method for pigment extraction presented here is designed for the separation of C4-substituted pigments from those pigments that can form bisulfite-addition products. For example, polymeric pigments with an acetaldehyde bridge (**10**, Figure 6) such as catechin-ethyl-malvidin-3-glucoside (M^+ 809) (*19*) have an unsubstituted C4 carbon. Because they do not appear in fraction **iv** it is proposed that these compounds can form bisulfite addition products. As such, it is proposed that they will appear in fraction **iii** along with grape anthocyanins. Additional techniques need to be developed for the separation and isolation of these types of compounds; the techniques may involve removal of the bisulfite ion under mild conditions.

As a result of the mass spectral data and their resistance to bisulfite bleaching, it is suggested that all of the pigments identified in fraction **iv** are C4-substituted with a core structure similar to that of vitisin A. It is interesting to note that none of the interflavan-linked C4-substituted polymeric pigments (**9**) as proposed by Somers (*1*) were identified in either the grape marc extract or wine in this study. This does not prove their nonexistence but it suggests that their concentrations, in the samples examined, are very low. Recently, the existence of a Somers-type polymer in wine, determined by electrospray MS and thiolysis degradation studies, has been reported but its concentration is unknown (*20*). Because the interflavan bond in this compound is between C4 of the flavanol and C8 of the anthocyanin, leaving C4 unsubstituted, this type of compound is expected to be bisulfite-bleachable and to appear in fraction **iii**.

The mechanism for separation of the various pigments using the sulfoxyethyl cellulose column is not fully understood. Addition of the bisulfite ion to malvidin-3-glucoside not only alters the charge characteristic of this compound but also changes its solubility and the ability of the anthocyanin to engage in hydrophobic interactions. Any cationic or hydrophobic materials retained on the column were removed using sodium chloride, as the use of acid was deliberately avoided. However, the material retained on the column generally

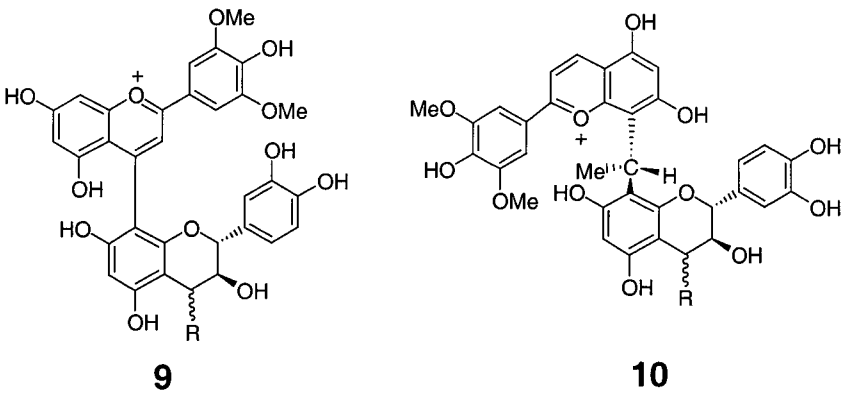

Figure 6. Structures of potential pigments resulting from the reaction of malvidin-3-glucoside and catechin or procyanidin not observed in fraction **iv**: **(9)** interflavan-linked compound as proposed by Somers (*1*); **(10)** ethyl-linked compound as proposed by Timberlake and Bridle (*9*).

exhibited high hydrophobicity in aqueous sodium chloride solution and the inclusion of 50% methanol in the solvent was required. It is therefore proposed that the major differences in retention between parent pigments and their bisulfite adducts are due to differences in hydrophobicity as well as differences in charge properties. This could explain why vitisin A, which is neutral at wine pH is present in fraction **i**, whereas the *p*-coumaryl vitisin A, which is also expected to be neutral, elutes in fraction **iv**.

There are compositional differences between the pigments extracted from grape marc and those extracted from wine (Table 1). The data suggest that while the smaller pigments remain in the wine, larger pigments are lost into the marc portion. It is known that the physicochemical adsorption of anthocyanins to yeast cell walls is an important mechanism for the loss of anthocyanins during fermentation (*21*). Furthermore, the cell wall protein and polysaccharide matrix of the grape skins may preferentially adsorb the larger pigments, which are thereby lost from the wine during pressing.

Many of the observed oligomeric pigments are present in wine in low concentrations. Although these wine pigments provide a pool of color-stable compounds in wine, their importance remains in question. In aged wine, where the concentration of grape-derived anthocyanins is negligible, the stable C4-substituted wine pigments may contribute significantly toward the color of wine. The color of vitisin A at wine pH is brick red ($\lambda_{Max} = 501$ nm) (*12*), and therefore the tawny color of aged wine may be due to the presence of these stable wine pigments. However, in younger wines these C4-substituted pigments will still contribute to the total pool of pigments and therefore may be considered as important pigments in the overall expression of wine color. Currently, it is not possible to estimate the relative contribution of the oligomeric pigments to wine color because molar absorbances for the individual compounds are not available and methods need to be developed for the routine quantification of these compounds.

Whereas pigments are primarily of interest for their visual sensory properties, the taste properties of these wine pigments is also of interest in wine research. Sensory evaluation of taste requires substantial amounts of material for evaluation. Synthesis, followed by separation and purification (*5*, *7*, *17*), may prove to be effective in obtaining sufficient amounts of the compounds for such sensory studies. On the other hand, while the concentrations of many of the individual oligomeric pigments are low in wine, grape marc extract may serve as an alternative source of many of these compounds. The separation method using bisulfite addition and ion-exchange chromatography developed here may prove useful in these studies.

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