Isolation and Structural Characterization of Anthocyanin-furfuryl Pigments

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Condensation reactions of malvidin-3-glucoside with two representative oak wood furanic aldehydes (furfural and methylfurfural) were conducted in wine-like model solutions. Methylfurfural led to the formation of malvidin-3-glucoside-methylfurfural (603 *m/z*), whereas furfural led to the formation of malvidin-3-glucoside-furfural (589 *m/z*). The latter was structurally characterized by 1D and 2D NMR, allowing an elucidation of the formation mechanism of these anthocyanin-furanic aldehyde adducts in the absence of flavanols.

INTRODUCTION

The aging of wine is a complex process in which organoleptic properties such as color and flavor are likely to change. In fact, young red wines evolve from a purple red color, arising from anthocyanins that are extracted from red grape skins, to a reddish brown hue, resulting from structural changes in these pigments (*1*, *2*). Such changes may occur through diverse mechanisms, giving rise to new pigments that present different chromatic features. One of these mechanisms is the aldehyde-mediated association of anthocyanins and flavanols, which is now well characterized: aldehydes react with flavanols and anthocyanins through a Bayer acid catalyzed condensation, giving rise to an adduct composed of two flavanols or one flavanol and one anthocyanin linked by an interflavonoid bridge, resulting from the aldehyde initially present in the reaction (*3−9*). Some of these aldehydes are extracted from the wood of the oak barrels. The maturation of wine in oak barrels is a common procedure in the wine industry, and the extraction of volatile and nonvolatile compounds from the wood influences important characteristics in matured wine, namely, color, aroma, and taste (*10*).

The extraction of compounds depends on the period of contact between wine and wood and on the chemical composition of the wood, which is affected by the species and origin of the trees, the seasoning of the staves, the age of the barrel, and most importantly by the heat treatment or toasting of barrels (*11−14*). In this procedure, macromolecular components such as lignins and polysaccharides of the wood are degraded into smaller compounds, including several aldehydes (*15*).

Furanic aldehydes (furfural, methylfurfural, and hydroxymethylfurfural), which are minor components in oak wood, are produced in large quantities during heat treatment of the barrels (*16*). Although these aldehydes are at subthreshold concentrations in matured wines, they may interact with other wine compounds during aging in oak barrels and subsequently contribute to color changes.

The present work reports our studies on the formation of new pigments resulting from a direct reaction of malvidin-3-glucoside, the main anthocyanin found in *Vitis vinifera* red wines, with two furanic aldehydes (furfural and methylfurfural). The newly formed pigment resulting from the reaction of malvidin-3-glucoside with furfural was structurally characterized, confirming for the first time the formation of anthocyanin-furanic aldehyde adducts in the absence of flavanols.

MATERIALS AND METHODS

Standards. Furfural and methylfurfural were purchased from Sigma-Aldrich (Spain).

Isolation and Purification of Malvidin-3-glucoside. Malvidin-3-glucoside (mv3glc) was obtained from a young red wine extract rich in anthocyanins. Wine was concentrated by nanofiltration membrane system, the aqueous residue was then spray dried on a Büchi Mini Spray Drier B-290, and the powder obtained was extracted three times with ethyl acetate. Malvidin-3-glucoside was then obtained and purified by semipreparative HPLC. The extract was applied onto a Hibar Purospher Star reversed-phase C18 (Merck) column (25 cm length and 10 *μ*m particle size), at room temperature. Solvents were (A) water/formic acid (90:10) and (B) methanol/water/formic acid (50:40:10). The elution gradient was performed using a k-1001 Merck pump from 35% to 85% B for 70 min at a flow rate of 10.0 mL min^{-1}. The purity of malvidin-3-glucoside (> 95%) was confirmed by HPLC and NMR.

Reaction between Malvidin-3-glucoside and Furanic Aldehydes. Malvidin-3-glucoside (4 mM, 2 mg) was incubated with furfural (200 mM) and methylfurfural (200 mM) separately in 1 mL of 12% (v/v) hydroalcoholic solutions at two different pH values (1.5 and 3.2). These model solutions were kept at a temperature of 35 °C and protected from light.

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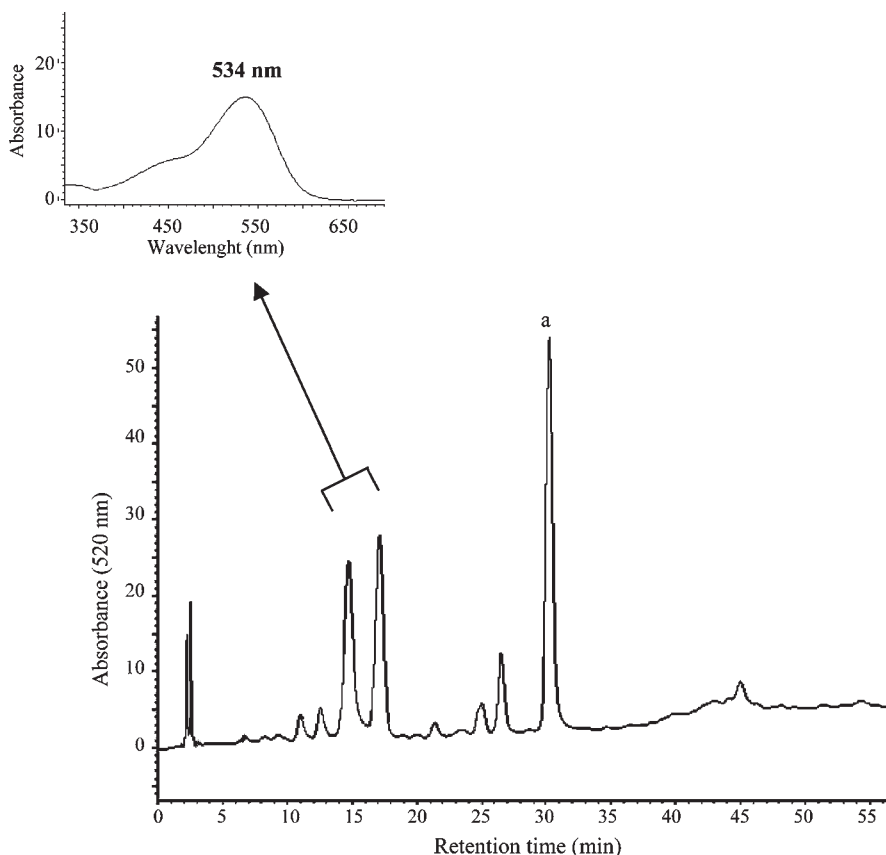
Figure 1. HPLC chromatogram recorded at 520 nm of the model solution containing malvidin-3-glucoside and furfural, after 4 days of reaction at pH 1.5 and 35 °C. UV/vis spectrum of the two isomers. Peak a: Malvidin-3-glucoside.

The formation of new compounds was analyzed by HPLC-DAD using a reversed-phase C18 (Merck) column (250 mm × 4.6 mm i.d., particle size 5 μm), at 25 °C. Solvents were (A) water/formic acid (90:10) and (B) acetonitrile/water/formic acid (30:60:10). The elution gradient was performed using a L-2130 Merck pump from 20% to 85% B for 70 min at a flow rate of 1.0 mL min^{-1}.

Analysis of Deuterated Pigments by Mass Spectrometry. The synthesized pigment malvidin-3-glucoside-furfural (2 mg) was dissolved in a D_2O/CD_3OD solution for 5 min and then directly analyzed by mass spectrometry at positive ion mode ESI, in order to observe the protons of hydroxyl groups that exchange with deuterium.

LC−MS Conditions. Mass spectrometry analysis was performed using a Finnigan SurVeyor series liquid chromatograph, equipped with an API source, using an electrospray ionization (ESI) probe. Solvents were (A) aqueous 0.1% acetic acid and (B) acetonitrile. The elution conditions were as follows: 0.5 mL min^{-1} flow rate; oven temperature, 35 °C; elution began with linear gradient from 5% to 30% B in 40 min, from 30% to 40% in 10 min, and from 40% to 100% in 5 min, followed by washing and re-equilibration of the column. The capillary voltage was 11 V, and the capillary temperature was 200 °C. Spectra were recorded in positive ion mode between m/z 100 and 1200. The mass spectrometer was programmed to do a series of three scans: a full mass spectrum, a MS^2 spectrum of the most intense ion, and a MS^3 spectrum of the most intense ion in the second scan, using a relative collision energy of 45 V.

Synthesis and Purification of the Malvidin-3-glucoside-furfural Adduct. Malvidin-3-glucoside (4 mM, 345 mg) was incubated with furfural (300 mM) in 175 mL of a 12% (v/v) hydroalcoholic solution at pH 1.5. The model solution was kept at a temperature of 35 °C and protected from light, and the formation of new compounds was followed by HPLC-DAD. When the reaction was completed, the sample was applied on a silica gel C-18 reversed-phase SPE cartridge in order to remove inorganic salts and other impurities, and the pigments were eluted with methanol acidulated with 2% HCl. Methanol was evaporated in a rotary evaporator at 38 °C, and the sample was freeze-dried and stored at −18 °C until use.

The sample was further applied on a 200 mm × 40 mm polyamide resin > 80−100 mesh (Balin Petrol Chemical Co. Ltd., Human, China) column and was eluted with 2% aqueous HCl with increasing percentages of methanol in water to obtain different fractions: F1, 30%; F2, 50%; and F3, 100%. The criterion used for changing the percentages of methanol was the decrease in color intensity of the solution eluted from the column. The solvent of each fraction was partially evaporated in a rotary evaporator at 38 °C, and the samples were freeze-dried and stored at −18 °C until use.

Semipreparative HPLC. Semipreparative HPLC was performed in order to isolate and purify the two isomers of mv3glc-furfural adducts eluted in fraction F2 from polyamide resin. This fraction was injected into a reversed-phase C18 (Merck) column (250 mm × 4.6 mm i.d., particle size 5 μm) at room temperature (volume injected was 2 mL). Solvents were (A) water/formic acid (90:10) and (B) acetonitrile/water/formic acid (30:60:10). The elution gradient was performed using a k-2501 Knauer pump from 10% to 85% B for 80 min at a flow rate of 2.0 mL min^{-1}, and detection was carried out at 520 nm using a Knauer k-2501 detector.

NMR Measurements. In the NMR characterization of mv3glc-furfural, ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in DMSO/TFA (95:5) on a Bruker Avance 500 spectrometer at 25 °C with TMS as internal standard. ^{1}H assignments were made with the aid of 2D gCOSY (^{1}H−^{1}H) and gNOESY spectra (mixing time of 800 ms), whereas ^{13}C assignments were made on the basis of 2D gHSQC (^{1}H−^{13}C) and gHMBC experiments (delay for long-range $J_{C−H}$ couplings were optimized for 4 and 20 Hz).

RESULTS AND DISCUSSION

The nucleophilic character of the phloroglucinol ring of anthocyanins (ring A) and catechins is well documented in reactions of anthocyanins with catechins mediated by aldehydes in acidic medium to form the catechin-alkyl/aryl-anthocyanin linked dimers (*4*, *5*, *8*, *17*). It is also important to note that these reactions first start by a nucleophilic attack of the catechin to the aldehyde
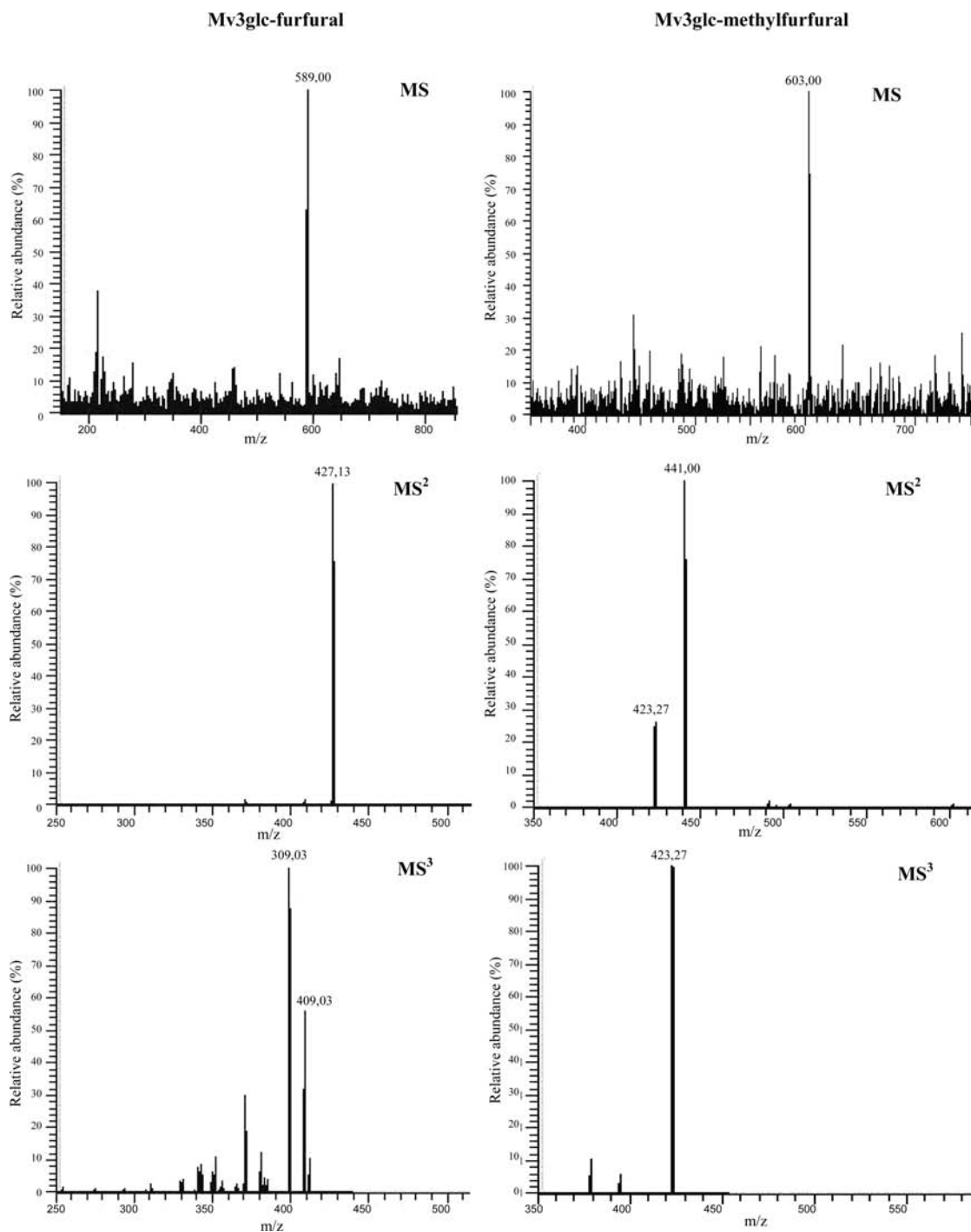
Figure 2. Mass spectra and respective MS[2] and MS[3] fragmentations for malvidin-3-glucoside-furfural (both isomers) and malvidin-3-glucoside-methylfurfural.

carbocation form, and then the anthocyanin can react with the aldehyde moiety, forming the adduct. However, attending to the nucleophilic character of the ring A of anthocyanins in its hemi-ketal form, anthocyanins may react directly with some aldehydes in their protonated form, as it is well established for methylmethine-linked anthocyanin dimers and polymers formed by the reaction with acetaldehyde (*18*). It is also expected that anthocyanins could react directly with some aldehydes such as furanic aldehydes (furfural and methylfurfural) in the absence of flavanols.

Synthesis of Anthocyanin−Furanic Aldehyde Adducts. The reaction of furfural or methylfurfural with malvidin-3-glucoside in acidic medium (pH 1.5 and 3.2) led to the formation of new compounds (**Figure 1**). In the reaction of malvidin-3-glucoside with furfural the formation of two chromatographic peaks was

detected, whereas only one chromatographic peak was detected in the reaction with methylfurfural. These new compounds showed maximum absorption in the visible range at λ_{max} 534 nm (both isomers) and at λ_{max} 537 nm from the reaction with furfural and methylfurfural, respectively, indicating a red/purple color. The λ_{max} of these pigments are bathochromically shifted from that of their precursor malvidin-3-glucoside (λ_{max} = 528 nm).

The molecular weight (mass spectra) of these pigments was determined by LC−ESI-MS. The mass spectra and the respective MS[2] and MS[3] fragmentations of the new compounds formed in the reaction with furfural and methylfurfural are presented in **Figure 2**.

Both compounds resulting from the reaction of malvidin-3-glucoside with furfural have the same molecular ion [M]⁺ at
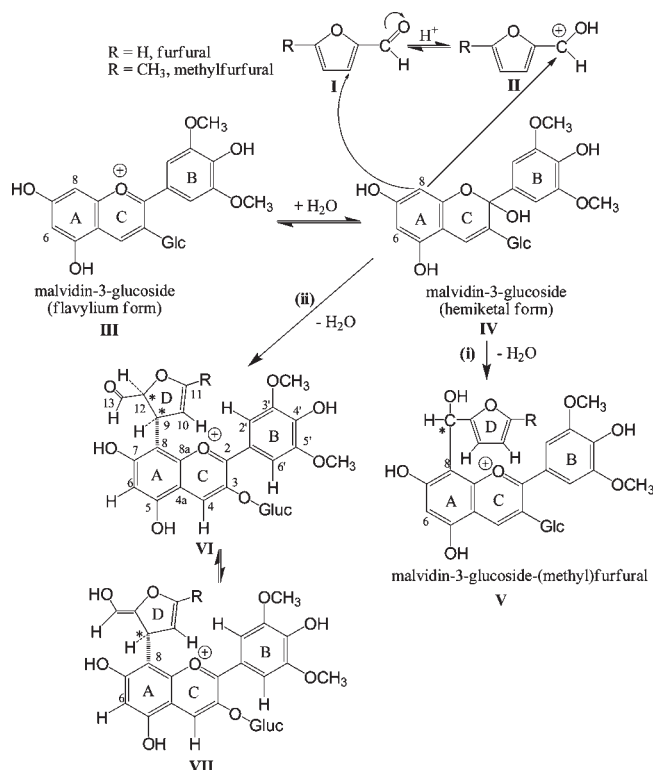
Figure 3. Mechanisms proposed for the reaction between malvidin-3-glucoside and furanic aldehydes.

Table 1. ^{1}H and ^{13}C NMR Data and HMBC and HSQC Correlations of Isomer **1** and **2** of Malvidin-3-glucoside-furfural, Determined in DMSO/TFA (95:5)

position	δ ^{1}H (ppm); J (Hz)^a	δ ^{13}C (ppm)	HMBC^b	HSQC
		Anthocyanin Moiety		
2C		162.3	H-2′/6′B^{a,b}, H-4C^{a,b}	
3C		144.6	H-2′/6′B^{a,b}, H-4C^{a,b}	
4C	8.98; s	136.3		H-4C
4aA		112.1	H-6A^{a,b}, H-4C^{a,b}	
5A		157.3	H-6A^{a,b}, H-4C^{a,b}	
6A	6.80; s	102.7		H-6A
7A		166.0		
8A		109.6	H-6A^b, H-9D^b	
8aA		154.7	H-4C^{a,b}	
1′B		109.7	H-2′/6′B^{a,b}	
2′/6′B	7.90; s	109.6		H-2′/6′B
3′/5′B		148.7	H-2′/6′B^{a,b}	
4′B		144.6	H-2′/6′B^{a,b}	
OMe	3.87; s	57.2		OCH$_3$
		Furfural Moiety		
9D	4.00; brs	53.1		H-9D
10D	6.35; brs	133.2		H-10D
11D	7.69; brs	163.5	H-9D^b	H-11D
12D	4.90; brs	77.1	H-10D^b, H-9D^b	H-12D
13D	na	203.5	H-9D^b, H-10D^a, H-11D^b	
		Sugar Moiety		
G1-1	5.46; d, 7,4	102.8		H-G1−1
G1-2	3.46^c	73.8		H-G1−2
G1-3	3.37^c	76.6		H-G1−3
G1-4	3.21^c	69.8		H-G1−4
G1-5	3.44^c	48.0		H-G1−5
G1-6a	3.70^c	61.2		H-G1−6a
G1-6b	3.50^c	61.2		H-G1−6b

^{a} na not attributed; s singlet; d doublet; brs broad singlet. ^{b} a,b: only detected in the HMBC spectrum running with a long range C/H coupling constant of 4 Hz (a) or 20 Hz (b). ^{c} Unresolved.

m/z 589, indicating they are isomers. The MS^2 spectrum shows a fragment at m/z 427 ([M − 162]$^+$), corresponding to the loss of a glucose unit. The ion at m/z 427 was also fragmented and yielded two other fragments at m/z 409 [loss of a water molecule, (M − 162 − 18)$^+$] and at m/z 399 [loss of CO, (M − 162 − 28)$^+$], which were detected in the MS^3 spectrum. The other pigment formed in the reaction of malvidin-3-glucoside with methylfurfural with a molecular ion [M]$^+$ at m/z 603 yields two fragments at m/z 441 ([M − 162]$^+$), corresponding to the loss of a glucose unit, and at m/z 423 [loss of a glucose unit and a water molecule, (M − 162 − 18)$^+$] detected in the MS^2 spectrum. The MS^3 spectrum shows also the fragment at m/z 423, corresponding to the losses of a glucose unit and a water molecule [(M − 162 − 18)$^+$].

The molecular ions of these new pigments correspond to the sum of the molecular weight of malvidin-3-glucoside (493 g·mol^{-1}) and the molecular weight of the furanic aldehyde (furfural and methylfurfural, 96 and 110 g mol^{-1}, respectively). The formation of new pigments in reactions of anthocyanins with furanic aldehydes may follow two different pathways, which are represented in **Figure 3**. The mechanisms proposed may start with the protonation of the furanic aldehyde **I** in acidic medium, forming a carbocation in the carbonyl carbon **II**, followed by a nucleophilic attack of the A ring of malvidin-3-glucoside in its hemiketal form **IV**, and leading to the malvidin-3-glucoside-(methyl)furfural **V**. The other probable mechanism involves a Michael-type addition of the A ring of malvidin-3-glucoside in its hemiketal form **IV** to the furanic aldehydes, leading to the malvidin-3-glucoside-(methyl)furfural **VI** in equilibrium with their enolic form **VII** (**Figure 3**). The A ring malvidin-3-glucoside attack may occur from position C6 or preferentially from position C8. The negative formal charge in ring A is expected to be higher at carbon 8, as is well documented for flavylium compounds (*19*) in the reaction leading to the formation of catechin-alkyl/aryl-anthocyanin adducts (*17*). The aldehyde form of malvidin-3-glucoside-(methyl)furfural **VI** has two asymmetric carbons

(C-9D and C-12D), which means that this compound may have four isomers (2^n, n = 2). However only two were observed.

Structural Characterization of Malvidin-3-glucoside-furfural by NMR. The structure of the two isomers of malvidin-3-glucoside-furfural was characterized by NMR. These results point that the reaction follows the second pathway (**ii**) originating the aldehyde form of adduct **VI** (**Figure 3**).

The ^{1}H and ^{13}C NMR chemical shifts of both isomers are exactly the same and are reported in **Table 1**. This indicates that both compounds, despite being diastereoisomers due to the sugar moiety, present chemically and magnetically equivalent H-9D and H-12D because they are far enough from the sugar moiety and thus behave as enantiomers. The ^{1}H chemical shifts were assigned using 1D and 2D NMR techniques (*g*COSY), and the assignment of the carbon resonances was made using 2D techniques (*g*HSQC and *g*HMBC techniques). The HMBC spectra were optimized for two different long-range coupling constants (4 and 20 Hz), in order to visualize long distance coupling ^{1}H−^{13}C ($^2J_{C,H}$, and $^3J_{C,H}$) important to assign most of the carbons, especially those from the furfural moiety.

Anthocyanidin Moiety. All protons of the anthocyanidin moiety were easily assigned by comparison with those already reported on anthocyanin-ethyl-catechin adducts (*4*). Protons H-4C and H-6A were assigned to the characteristic singlets at 8.98 and 6.80 ppm, respectively. The equivalent protons H-2′,6′B and the two methoxyl groups of ring B were attributed to the singlets at 7.90 and 3.87 ppm, respectively.

The assignments of carbon C-4C at 136.3 ppm, carbon C-6A at 102.7 ppm, carbon C-8A at 109.6 ppm, carbons C-2′,6′B at 109.6 ppm, and the methoxyl carbons at 57.2 ppm were obtained from the HSQC spectrum. The remaining carbons were assigned through long-range $^1H-^{13}C$ correlations obtained in the HMBC spectrum.

The anomeric carbon of the glucose moiety at 102.8 ppm and its correspondent proton doublet at 5.46 ppm ($J = 7.4$ Hz) were easily assigned. All other glucose protons and carbons were attributed through correlations found in the COSY and HSQC spectra.

Furfural Moiety. The assignment of the proton H-4C to the anthocyanidin moiety proves that the furfural molecule is not linked to that position. The presence of a singlet corresponding to only one proton of ring A (H-6A or H-8A) proves that the furfural is linked to that ring. The only possible way to elucidate the linked position at C-6 or C-8 in this kind of compound is by NOESY experiment throughout NOE cross-peaks between furanic protons and those of the B ring methoxyl groups (5). In fact, a weak NOE cross-peak was observed between H-2′,6′B and H-9D, which indicates a spatial proximity between ring B of the anthocyanidin and furfural, possible only in the case of a C-8 linkage rather than a C-6 linkage. Moreover, spatial correlations in NOESY spectrum were observed between H-11D and H-12D, H-9D and H-10D (weak), and H-10D and H-11D. A parallel analyses by mass spectrometry of the malvidin-3-glucoside-furfural dissolved in a D_2O/CD_3OD solution showed $[M]^+$ at m/z 597, which means that the 5 sugar hydroxyl protons and the 3 protons from those of the flavylium moiety were exchanged by the deuterium from the solvent. This result is important since it proves that the two OH groups of the A ring of the anthocyanidin do not participate in any process of cyclization with the furfural moiety.

Carbon C-9D was attributed to the signal at 53.1 ppm (HSQC), which is characteristic of a methyne carbon. H-9D at 4.00 ppm correlates in the HMBC spectrum (20 Hz) with the carbon resonances of C-10D, C-11D, and C-13D (203.5 ppm). The chemical shift of C-13D is characteristic of an aldehyde carbon. The proton H-12D was attributed to a signal at 4.9 ppm and the corresponding carbon at 77.1 ppm (HSQC). Through the COSY spectrum analysis this proton correlates with the protons H-9D, H-11D, and a small signal with H-10D. The proton H-10D was attributed to a signal at 6.33 ppm and the corresponding carbon at 133.2 ppm (HSQC). Through HMBC observation, using the small long-range C−H coupling constant (4 Hz), it was possible to verify a correlation between proton H-10D and carbon C-13D. On the other hand, HMBC analysis using the high long-range C−H coupling constant (20 Hz) could not show the previous correlation, although it was possible to observe a C−H long correlation between proton H-10D and carbon C-12D. The proton H-11D was attributed to a signal at 7.7 ppm and the corresponding carbon at 163.5 ppm (HSQC). The HMBC spectrum (20 Hz) shows correlation of this proton with the carbonyl group C-13D.

The aldehydic proton H-13D could not been attributed, since it must be very broad as a result of the fast equilibrium with their enolic form and also the CHO fast rotation around the C12−C13 bond.

The signals of the protons H-9D and H-12D linked to the asymmetric carbons are broad singlets, indicating that they have a small coupling constant (< 2 Hz), which is characteristic of a *cis* configuration. Thus, the two isomers obtained must have (9*S*,12*S*) and (9*R*,12*R*) configurations.

Malvidin-3-glucoside showed the ability to react directly with furanic aldehydes, namely, furfural and methylfurfural, in the absence of flavanols. This is the first report of anthocyanin-furfuryl adducts and their characterization by mass spectrometry and NMR. Other studies of condensation reactions in the presence of flavanols, namely, catechins, had shown the formation of catechin-furfuryl, catechin-furfuryl-catechin, and catechin-furfuryl-malvidin-3-glucoside adducts, but the formation of a pigment containing only the anthocyanin and the furanic aldehyde was never observed(9). In other work (20) it was showed that furfural could react with cyanidin but not with its 3-glucoside form, following different condensation pathways. Other aldehydes such as acetaldehyde, vanillin, coniferaldehyde, and hydroxymethylfurfural were studied, but they did not yield new pigments. The data suggest that the isomers formed in the reaction between malvidin-3-glucoside and furfural are present in the aldehyde form, with configurations (9*S*,12*S*) and (9*R*,12*R*). The formation mechanism of these pigments occurs via a Michael-type addition of the malvidin-3-glucoside C-8 to the furanic aldehyde, unlike the proposed mechanism of formation for catechin-ethyl-anthocyanin adducts, in which the first step in the reaction occurs by nucleophilic attack on the carbocation of the carbonyl group of the aldehyde (17, 19). The furanic aldehydes that are extracted to wine during aging in oak barrels and that contribute to a sweet and pleasant aroma may also be involved in reactions with polyphenols present in wine, such as anthocyanins, giving rise to new compounds with different chromatic characteristics.

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