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Anthocyanone A: A Quinone Methide Derivative Resulting from Malvidin 3-*O*-Glucoside Degradation

PAULO LOPES,† TRISTAN RICHARD,‡ CÉDRIC SAUCIER,*,† PIERRE-LOUIS TEISSEDRE,† JEAN-PIERRE MONTI,‡ AND YVES GLORIES†

Faculté d'Oenologie de Bordeaux, Université Victor Segalen Bordeaux2 UMR 1219 INRA, 351 Cours de la Libération, 33405 Talence Cedex, France, and GESVAB, EA 3675, Université Victor Segalen Bordeaux2, 146 rue Léo Saignat, Bordeaux, 33076, France

A new compound resulting from the oxidative degradation of maldivin 3-*O*-glucoside under acid conditions was detected in a wine model solution stored under 90 and 25 °C. It was isolated by semipreparative HPLC, and its structure was elucidated by UV−vis spectra, mass spectrometry (LC/MS), and NMR spectroscopy (1-D and 2-D). The compound was identified as 8-β-D-glucopyranosyl-2,4-dihydroxy-6-oxo-cyclohexa-2,4-dienyl acetic acid (anthocyanone A), which results from nucleophilic attack of hydrogen peroxide to maldivin 3-*O*-glucoside through a Baeyer−Villiger oxidation followed by other oxidations steps.

KEYWORDS: Maldivin 3-*O*-glucoside; Anthocyanone A; syringic acid; mass spectrometry; NMR

INTRODUCTION

Anthocyanins (glycosylated polyhydroxy derivatives of 2-phenylbenzopyrylium salts) are unstable plant pigments responsible for a wide range of colors in vegetables, fruits, and derived products, such as juices or red wines (*1*). They occur in nature as glycosides with an anthocyanidin C6-C3-C6 skeleton and may have aliphatic or aromatic acids attached to their glycosidic residues. The acylating agents include cinnamic acids (caffeic, *p*-coumaric, ferulic, and sinapic) and aliphatic acids (acetate, malonate, oxalate, and succinate) (*2*).

The anthocyanins present in *Vitis vinifera* grape and red wines are cyanidin, delphinidin, petunidin, peonidin, and maldivin under their glycoside and acylated forms. Among all anthocyanins, maldivin 3-*O*-glucoside is always the most important one (*3*). These compounds occur in four molecular species, which are extremely dependent on the pH value of the solution. At low pH, the red flavylium cation is the predominant form. An increase in pH will favor displacement of the equilibrium toward the other three secondary structures: the carbinol colorless base, the chalcone pseudobase, and the quinoidal base (*4*, *5*).

A decrease in anthocyanin content is observed from the alcoholic fermentation, throughout maturation and bottle aging (*6*). The color evolution of red wine over this period is partially attributed to copigmentation phenomena and to a progressive formation of new stable pigments involving anthocyanins. Studies conducted on model solutions and wines propose different mechanisms that could participate in the formation of these new pigments: direct condensation between anthocyanins and flavanols, which can form orange xanthylium salts by dehydration and protonation (*7*−*10*); direct condensation between anthocyanins and quinones (*11*); aldehyde bridging of anthocyanins with flavanols (*12*−*15*); and reactions described as cycloaddition of acetaldehyde, vinlyphenols, pyruvic acid, and vinylflavanols at C4 and OH in position 5 of the anthocyanins (*16*−*19*).

Anthocyanins are unstable, particularly once removed from their native environment and the protection conferred by copigmention. Several authors have studied various factors that affect the stability of anthocyanins during processing, such as temperature, pH, oxygen, enzymes, metallic ions, ascorbic acid, and sulfur dioxide (*20*−*26*). The storage temperature was found to be the main factor responsible for anthocyanin loss by breaking the *O*-glycosidic bond. Thermal and enzymatic treatment under anaerobic conditions moves the equilibria toward the chalcone forms and then yields 2,4,6-trihydroxybenzaldehyde and syringic acid from maldivin 3-*O*-glucoside, and other benzoic acids would be produced from other wine anthocyanins (*27*). When a close analogue to maldivin 3-*O*-glucoside, maldivin 3,5-diglucoside, was oxidized in the presence of hydrogen peroxide under acid conditions, *ortho*-benzoyloxyphenylacetic acid esters were formed through a Baeyer−Villiger-type oxidation (*28*−*30*). However, little is known about the influence of oxygen on maldivin 3-*O*-glucoside stability.

The purpose of this work was to study the oxidative degradation of malvidin 3-*O*-glucoside and the structural characterization of the newly formed compounds under acidic conditions.

MATERIALS AND METHODS

Chemicals. The water used was deionized water purified with a Milli-Q water system (Millipore, Bedford, MA). Ethanol (HPLC grade)

* Corresponding author. E-mail: saucier@oenologie.u-bordeaux2.fr. Fax: 33-5-40-00-646.
† Faculté d'Oenologie de Bordeaux.
‡ GESVAB, EA 3675.

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was obtained from Carlo Erba (Val de Reuil, France), and methanol (HPLC grade), formic acid (RP), iron-ferrous sulfate monohydrate rectapur, and L-tartaric acid were from Prolabo-VWR (Fontenay s/Bois, France). Toyopearl HW-50F chromatography media was obtained from Supelco (St. Louis, MO).

Isolation and Purification of Malvidin 3-*O*-Glucoside. *V. vinifera* L. cv. Merlot berries grown during the 2001 harvest season were used as the source material for anthocyanins. Grape berries were then stored at −20 °C until extracted.

For extraction, 2.5 kg of grape berries was placed in a 5 L Erlenmeyer flask with 2 L of methanol containing 0.1% v/v hydrochloric acid under agitation at room temperature for 16 h. Afterward, the extract was filtered through no. 1 Whatman filter paper to remove seed and skin tissue. The methanol extract was then concentrated under reduced pressure at 40 °C to a volume of approximately 300 mL. The crude anthocyanin extract was then stored at −20 °C, prior to purification.

Volumes of 30 mL of crude anthocyanin were poured onto a glass column (180 mm × 45 mm) filled with Toyopearl HW-50F size exclusion media, which was previously equilibrated with water. The column was then rinsed with 500 mL of water containing 0.1% v/v hydrochloric acid to remove sugars, organic acids, proteins, and salts. The monoglucoside anthocyanins were recovered by eluting the column with 1000 mL of water/methanol (60:40, v/v) containing 0.1% v/v hydrochloric acid. The eluent was then concentrated under reduced pressure at 40 °C to remove methanol and water and then lyophilized to a dry powder.

Malvidin 3-*O*-glucoside was purified from the lyophilizate extract using a semipreparative HPLC Varian (Walnut Creek, CA) on a 250 mm × 25 mm, 5 μm LiChrospher 100 RP-18 column. A total of 100 mg of lyophilizate extract was dissolved in 10 mL of mobile phase A, prior to injection. Elution conditions were as follows: flow rate 1 mL min^{-1}; room temperature; 500 μL sample loop; solvent A, water/formic acid (90:10, v/v); and solvent B, methanol/formic acid (90:10, v/v). The elution gradient was as follows: 30% B in 2 min; 30−40% B in 10 min; 40−100% B in 2 min; 100% B in 10 min; and 100−0% B in 5 min. Finally, malvidin 3-*O*-glucoside was obtained with a purity of 92 ± 2% determined by HPLC.

Reactions. A model wine solution was prepared with 12% (v/v) ethanol, 5 g L^{-1} tartaric acid, and adjusted to pH 3.4 with 1 N sodium hydroxide. The reagents used were malvidin 3-*O*-glucoside (250 mg L^{-1}, 0.5 mmol L^{-1}) and iron (18 mg L^{-1}, 0.32 mmol L^{-1}) under a ferrous sulfate monohydrate form. A total of 2 mL of each mixture was separated into 2 mL sealed vials under oxygen-poor conditions (nitrogen flush). The other 2 mL of mixture solution was put into 4 mL sealed vials under oxygenated conditions favoring the oxidative reactions. Each reaction was replicated 2 times and incubated at 90 °C in total darkness prior to injection. An identical experiment was conducted at 25 °C over 6 months of storage to evaluate the temperature effect on the previous reactions.

Samples were analyzed at 2, 4, 6, 12, and 24 h by puncturing the vial septum. Reactions were monitored by high performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) and a mass spectrometer (MS).

Analytical HPLC/UV Analysis. HPLC/UV analyses were performed by means of a Beckman System Gold (Beckman, Roissy Charles-de-Gaulle, France), which included a manual injector, a 126 pump module, and 168 diode array detector with all systems operating using 32 Karat 5.0 software. UV−vis absorption spectra were recorded from 200 to 900 nm. The column was a Beckman ODS ultraphere (5 μm packing, 250 mm × 4.6 mm i.d.) (Beckman Coulter, Fullerton, CA). Elution conditions were flow rate 1 mL min^{-1}; room temperature (20 °C); 20 μL sample loop; solvent A, water/formic acid (99:1, v/v); and solvent B, methanol/formic acid (99:1, v/v). The elution gradient was 0−2% B in 3 min, 2−10% B in 15 min, 10−20% B in 10 min, 20−30% B in 15 min, 30−70% B in 20 min, 70−100% B in 1 min, 100% B for 5 min, and 100−2% B in 5 min.

Malvidin 3-*O*-glucoside and its degradation compounds were quantified by comparing the compound area (280 nm) with that of malvidin and syringic acid standard, respectively. All reaction products were estimated as equivalent syringic acid. The compound identity was determined by mass spectrometry and NMR.

MS Apparatus and LC/MS Analysis. LC/MS analyses were performed on a Micromass Platform II simple quadrupole mass spectrometer (Micromass-Beckman, Roissy Charles-de-Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in the positive and negative ion modes. Source temperature was 120 °C, capillary voltage was set at ±3.5 kV, and there was a cone voltage of −30 and −90 V. Mass spectra were recorded from 100 to 1500 amu. HPLC separations were performed on a Hewlett-Packard 1100 series (Agilent, Massy, France) including a pump module and a UV detector. Both systems were operated using Masslynx 3.4 software. Column and separation conditions were identical to those used for analytical HPLC/UV analysis. The flow rate was 1 mL min^{-1} for the column and 0.1 mL min^{-1} for the MS source, and the injected volume was 50 μL. The absorbance was recorded at 280 nm.

Isolation of Breakdown Products Resulting from the Degradation of Malvidin 3-*O*-Glucoside. Malvidin 3-*O*-glucoside (100 mg) and iron (5 mg) were dissolved in 500 mL of model wine solution in a 1 L flask under oxygenated conditions and then incubated at 90 °C in total darkness over 24 h. Afterward, the extract was filtered through no. 1 Whatman filter paper and then concentrated under reduced pressure at 20 °C. The reaction medium of the residual malvidin 3-*O*-glucoside and the breakdown products were then dissolved in 10 mL of mobile phase A, prior to injection. The purification of these products was performed using a semipreparative Varian HPLC (Walnut Creek, CA) on a 250 mm × 25 mm, 5 μm LiChrospher 100 RP-18 column. Elution conditions were similar to those used in analytical HPLC/UV analysis with a flow rate of 12 mL min^{-1}; at room temperature; and 2000 μL sample loop.

NMR Analysis. NMR experiments were performed at 300 K, using a Bruker AVANCE 300 MHz spectrometer. The sample was dissolved in D_2O/TFA (99:1 v/v). Complete structure elucidation was further elucidated by ^1H and ^{13}C NMR spectroscopy using 2-D techniques (COSY, HSQC, HMBC).

RESULTS AND DISCUSSION

Malvidin 3-*O*-Glucoside Degradation and Formation of New Compounds. Malvidin 3-*O*-glucoside disappearance was investigated in a wine-like solution with or without oxygen. The solutions were maintained at 90 °C to accelerate reaction rates, and the disappearance of malvidin 3-*O*-glucoside and the appearance of new compounds were monitored as a function of time (**Figure 1**). After 24 h, 82 and 95% of malvidin 3-*O*-glucoside had disappeared with or without oxygen, respectively. The progressive formation of new compounds was observed in both model solutions; most compounds (**A**−**H**) were formed in larger amounts in the presence of oxygen, but **I** and **J** seemed to be in higher amounts in the sample incubated with low oxygen. The products **I** and **J** displayed a maximum UV−vis absorption at 330 nm. The mass spectrum of these compounds obtained by LC/MS in the negative ion mode showed a molecular ion at *m*/*z* 509 (**Table 1**). These characteristics were coherent with *cis*- and *trans*-chalcone forms, which had already been detected in wine model solutions (*31*).

Compounds **C** and **D** were preferably formed in wine model solutions rich in oxygen, displaying a λ_{max} at 293 nm and a shoulder at 340 nm. The mass spectrum of these compounds showed a molecular ion $[M]^-$ at *m*/*z* 343. In addition, a fragment ion was detected at *m*/*z* 181 in the negative ion mode, consistent with the loss of a glucose moiety $[M - 162]^-$ (**Table 1**). The fact that compounds **C** and **D** have the same λ_{max} and molecular mass with different retention times on the LC chromatogram suggests that they are isomers.

Compounds **E** and **F** displayed a UV λ_{max} at 293 and 275 nm, respectively. These compounds revealed a $[M]^-$ ion at *m*/*z* 153 and 197, which fits exactly with the mass of 2,4,6-

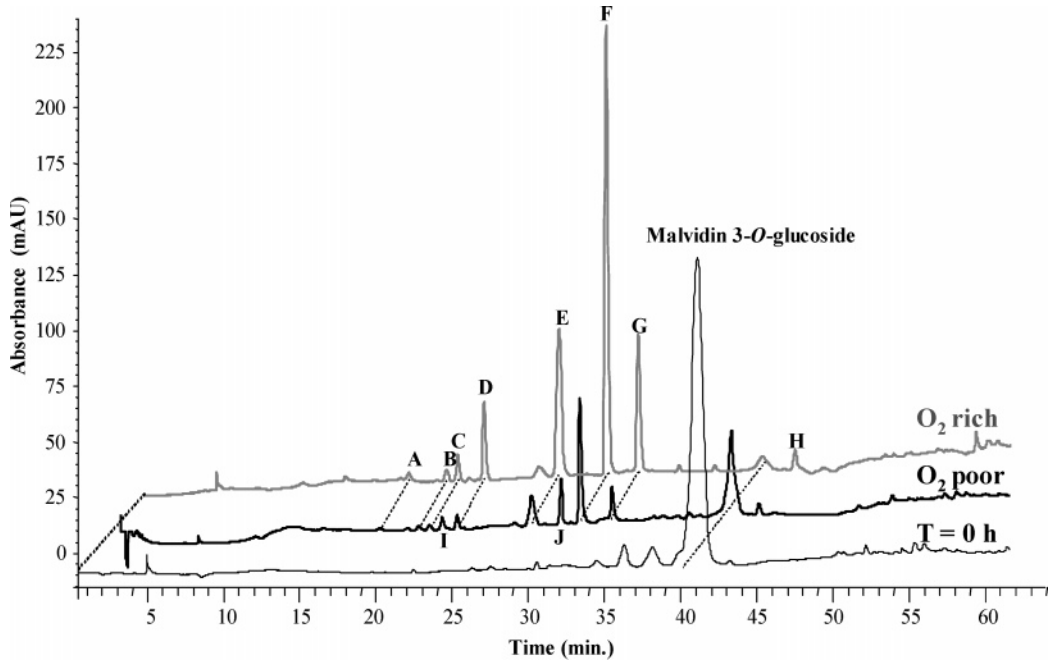

Figure 1. HPLC chromatogram recorded at 280 nm representing the malvidin 3-*O*-glucoside degradation over 24 h of reaction in oxygen-poor and -rich environments. Compounds **A**–**J** correspond to breakdown products.

Table 1. UV–Vis Spectra, Mass, and Identification of Compounds Representing the Breakdown Products of the Malvidin 3-*O*-Glucoside Degradation[a]

		mass/charge (*m*/*z*)		
compound	λ_{max}, shoulder	$[M-H]^-$	fragments	identification
A	310	225	153, 123	unknown
B	275	221		unknown
C	293, 340	343	181	unknown
D	293, 340	343	181	unknown
E	293	153		2,4,6-trihydroxybenzaldehyde
F	275	197		syringic acid
G	293, 330	333	179	unknown
H	280, 305	363	305, 147	unknown
I	330	509	347, 221	chalcone glucoside
J	330	509	347, 221	chalcone glucoside

[a] Fragment ions were detected in negative ion mode (60 eV).

trihydroxybenzaldehyde (THBA) and syringic acid, respectively (**Table 1**). Their identification was fully confirmed by HPLC injection of respective standard compounds. These compounds had already been detected in model solutions as the major breakdown products of malvidin and malvidin 3,5-*O*-diglucoside (*23*, *27*).

Compound **G** had a UV λ_{max} at 293 nm and a shoulder at 330 nm. The mass spectrum had a molecular ion $[M]^-$ at *m*/*z* 333, yielding one fragment at *m*/*z* 179, which probably results from the loss of a 2,4,6-THBA structure (**Table 1**).

In spite of the UV−vis and mass characterization, the chemical structure of minor compounds **A**, **B**, and **H** were unknown.

The malvidin 3-*O*-glucoside disappearance was also investigated in wine-like solutions maintained at 25 °C (**Figure 2**). After 6 months of storage, 74 and 82% of malvidin 3-*O*-glucoside had disappeared in rich and poor oxygen solutions, respectively. The gradual formation of four major breakdown compounds (compounds **D** and **G**, syringic acid, and 2,4,6-THBA) was observed in both model solutions. However, the amounts of compound **D** and syringic acid were significantly higher when the solution was rich in oxygen (see **Table 3**). More important amounts of compound **H** were produced in the assays carried out at 25 °C than at 90 °C. However, because compounds **D** and **G** represented the major unknown compounds of malvidin 3-*O*-glucoside degradation at 90 °C (easier to isolate), these compounds were isolated, and their structure was studied by NMR.

NMR Analysis of Compound D. The compound **D** complete structure was then elucidated by ^1H and ^{13}C NMR spectroscopy using 2-D techniques (COSY, HSQC, HMBC) (**Table 2**). The ^1H and ^{13}C NMR spectra of **D** exhibited signals for a set of two meta-coupled protons on a 1,2,4,6-tetrasubstituted benzene ring at δ_H 6.03 [(1H, d, J = 1.4 Hz, H-3), δ_H 6.12 (1H, d, J = 1.4 Hz, H-5)], an olefinic proton at δ_H 5.83 (1H, s, H-7), and a glucopyranosyl moiety, for which β-anomeric ^1H and ^{13}C NMR resonances appeared at δ_H 4.88 (1H, d, J = 7.4 Hz, H-1′) and δ_C 103.6, four oxymethines were seen between δ_H 3.40 and 3.54 and δ_C 72.0 and 79.0, with one oxymethylene resonating at δ_H 3.78 (1H, dd, H-6′a) and δ_H 3.90 (1H, dd, H-6′b) in the ^1H NMR spectrum and δ_C 63.2 in ^{13}C NMR spectrum.

HSQC and HMBC NMR experiments confirmed the substitution pattern of the molecule together with the position of the glucoside moiety. In the HMBC spectrum, the signal at δ_H 5.83 related to the olefinic proton showed correlations through ^3J with the signals for C-2 and C-6; this allowed its position to be established on the aromatic ring. Correlations were observed between C-1/H-5 and C-6/H-5, indicating the position of aromatic proton H-5. The relatively unusual downfield chemical shift of the carbon at 172.2 ppm could be explained by a fast equilibrium between isomeric forms (**Figure 4**). The lack of HMBC correlation with the second aromatic proton at δ 6.03 ppm could be in agreement with NMR silent, which was previously reported in quinones (*32*). Finally, in the HMBC spectrum, the long-range correlations from carbonyl carbon C-8 at δ_C 175.7 to the olefinic proton H-7 and to the anomeric proton H-1′ indicated its position between C-7 and C-1′.

Thus, negative and positive mass experiments together with NMR data led to the structure of the proposed molecule (**Figure**

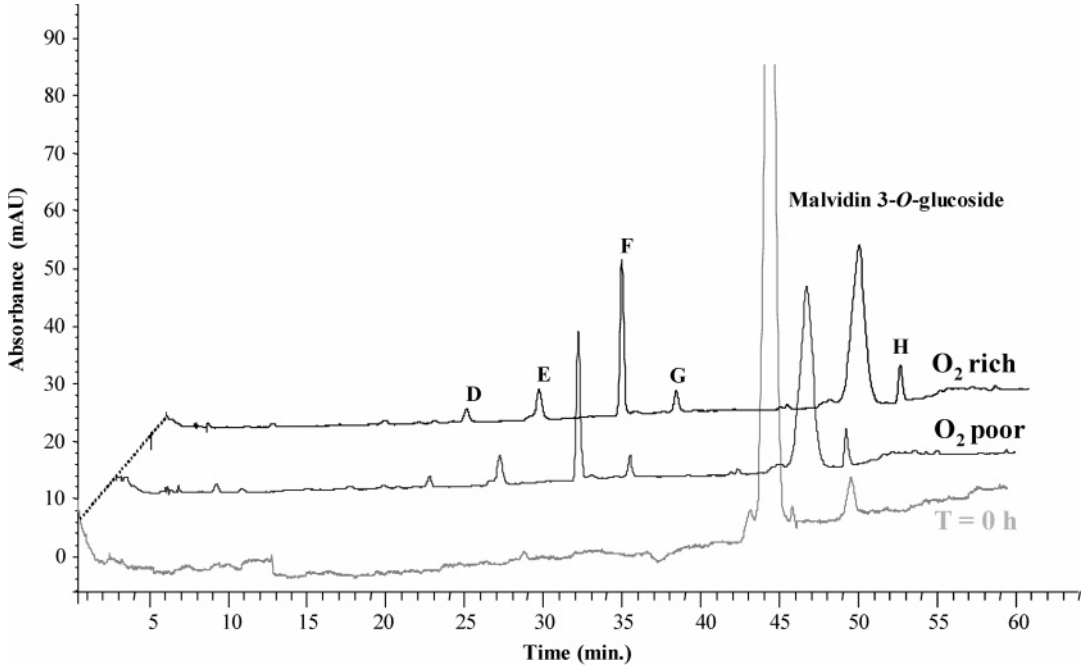

Figure 2. HPLC chromatogram recorded at 280 nm representing the malvidin 3-*O*-glucoside degradation over 6 months of reaction in oxygen-poor and -rich environments. Compounds **D**–**H** correspond to breakdown products.

Table 2. ^{1}H (300 MHz) and ^{13}C (75 MHz) Spectroscopic Assignments for Anthocyanone A, in D_2O/TFA (99:1 v/v)

position	δ^{1}H (ppm); m, *J* (Hz)	δ^{13}C
1		104.1
2		172.2
3	6.03; d, 1.4	99.8
4		160.7
5	6.12; d, 1.4	94.3
6		175.9
7	5.83; s	102.2
8		175.7
1′	4.88; d, 7.9	103.6
2′	3.40; dd, 7.9, 9.2	75.2
3′	3.54; m	78.0
4′	3.48; m	72.0
5′	3.51; m	79.0
6′	3.90; dd, 1.5, 12.5 3.78; dd, 4.8, 12.5	63.2

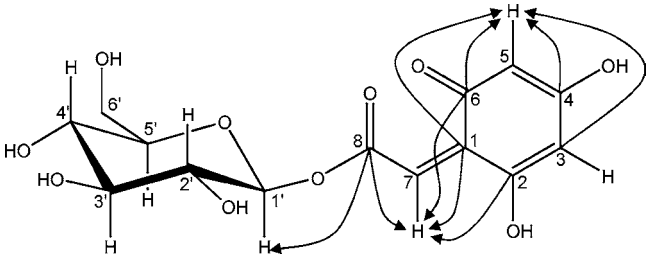

Figure 3. Structure and long-range correlations observed by HMBC (arrows) for compound **D**.

3). Compound **D** was identified as 8-β-D-glucopyranosyl-2,4-dihydroxy-6-oxo-cyclohexa-2,4-dienyl acetic acid and given the name anthocyanone A.

The ^{1}H NMR spectrum of compound **G** showed that it structure contains one aromatic ring B of the malvidin 3-*O*-glucoside without the glucose moiety (data not shown). However, this compound was very unstable in solution, hindering it complete structure elucidation by 2-D techniques (COSY, HSQC, HMBC). Probably, this compound contains the basic

Table 3. Average Concentration of Breakdown Compounds of Malvidin 3-*O*-Glucoside (250 mg L^{-1}, 0.5 mmol L^{-1}) Degradation under Different Temperatures (25 and 90 °C) and Oxygenation Conditions over Time[a]

wine model solutions				compounds			
T (°C)	time	O_2	iron (18 mg L^{-1})	anthocyanone A	THBA	syringic acid	**G**
90	2 h	–	0	0.9	0.9	2.1	1.4
	2 h	+	0	1.1	1.5	3.9	1.5
	24 h	–	0	1.2	2.1	8.6	2.6
	24 h	+	0	5.5	8.2	29.1	8.9
90	2 h	–	+	0.3	0.5	1.1	1.4
	2 h	+	+	2.2	2.0	5.0	1.4
	24 h	–	+	0.8	1.8	8.2	2.0
	24 h	+	+	10.5	7.2	35.0	5.1
25	1 month	–	0	0.4	0.8	1.9	0.5
	1 month	+	0	0.4	0.8	2.5	0.6
	6 months	–	0	0.7	3.5	11.8	2.2
	6 months	+	0	2.1	3.6	15.4	2.4

[a] Compound concentrations are expressed as mg/L of syringic acid.

malvidin structure with the two aromatic rings without a glucose moiety, once its molecular weight is 334. However, further research efforts are still needed to complete elucidation of the structure of this compound and its formation mechanism.

Kinetics of Formation of the Four Major Breakdown Compounds. In **Table 3** are represented the average concentration of the four major breakdown compounds of malvidin 3-*O*-glucoside degradation under different temperatures (25 and 90 °C) and oxygenation conditions over the time.

The progressive formation of the four major compounds of malvidin 3-*O*-glucoside degradation was observed in both model solutions under 90 °C, but in much more larger amounts when it was oxygenated. However, after 6 months of storage under less drastic temperature (25 °C), only anthocyanone A and syringic acid were formed in significantly larger amounts when the solution was rich in oxygen. Moreover, the addition of iron to solutions resulted in the formation of more than 48% of anthocyanone A and more than 17% of syringic acid (**Table**

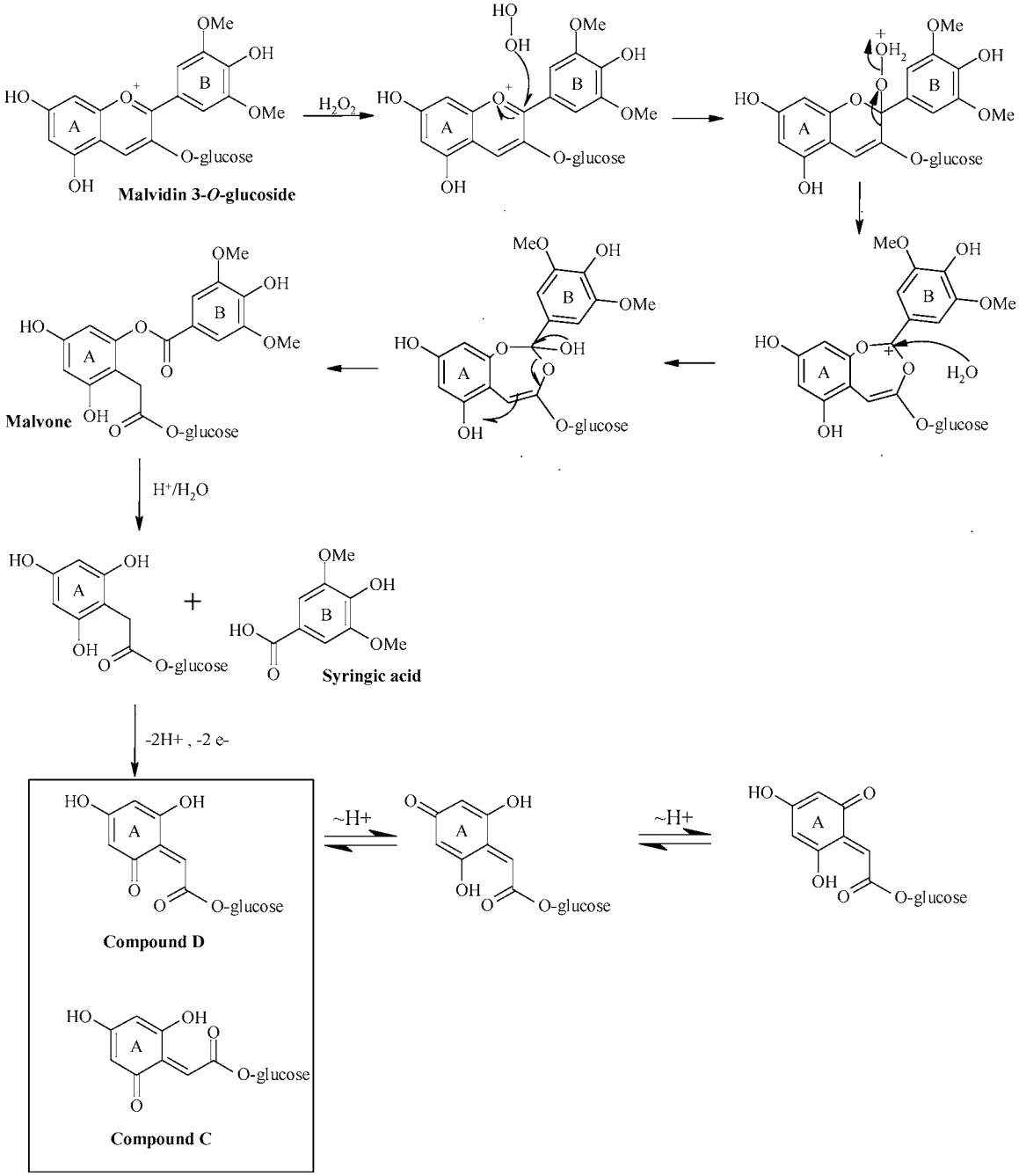

Figure 4. Hypothetical scheme for the formation of compounds **C** and **D** (anthocyanone A) from malvidin *O*-glucoside through a Baeyer–Villiger-type oxidation.

3). These findings suggest that both compounds were formed through oxidative degradation of malvidin 3-*O*-glucoside.

On other hand, the gradual formation of 2,4,6-trihydroxybenzaldehyde and compound **G** was also favored by the presence of oxygen at 90 °C. Conversely, the addition of iron led to a decrease of their concentrations in both model solutions. However, under a less drastic temperature (25 °C), 2,4,6-trihydroxybenzaldehyde as well as the compound **G** were formed in both solutions at similar concentrations (**Table 3**).

Probably, the formation of 2,4,6-trihydroxybenzaldehyde resulted from the degradation of malvidin 3-*O*-glucoside by a mechanism similar to the hypothetical reaction scheme described by Piffaut et al., which also forms syringic acid (*27*). These authors have suggested that an unknown intermediary should exist, to explain the loss of C3 in the anthocyanin ring C that implies the formation of 2,4,6-trihydroxybenzaldehyde and syringic acid. The compound **G** could probably be this unknown intermediary. However, only the complete elucidation of its structure will highlight the reaction mechanisms involved.

Mechanism of Formation of Anthocyanone A. The hypothetical mechanism of formation of anthocyanone A under the reaction conditions is supposed to be similar to that described by Hrazdina (*29*). Upon nucleophilic attack of hydrogen peroxide at carbon 2 of the malvidin 3-*O*-glucoside, the heterocyclic ring was cleaved between C_2 and C_3 to form *ortho*-benzoyloxyphenylacetic acid esters through a Baeyer−Villiger-type oxidation. These esters were then hydrolyzed under acid conditions to the syringic acid and 2,4,6-trihydroxyphenylacetic acid with a glucose moiety. This compound is further oxidized to compound **D** (anthocyanone A), which is in fast equilibrium

with its tautomeric quinone forms (**Figure 4**). These three should be present as a mixture in fast equilibrium so that the NMR spectrum can correspond to an average spectrum of the three forms. The fact that compounds **C** and **D** have identical UV spectra and molecular mass suggests the possibility that they are isomers.

The formation of important amounts of anthocyanone A and syringic acid under more acid conditions (pH 2.5), in the presence of iron and oxygen, confirms that these compounds result from an oxidation degradation pathway of the malvidin 3-*O*-glucoside under its flavylium form (data not shown).

In conclusion, under wine pH and anthocyanin concentrations, malvidine 3-*O*-glucoside was degraded to 2,4,6-trihydroxybenzaldehyde, syringic acid, and the 8-*β*-D-glucopyranosyl-2,4-dihydroxy-6-oxo-cyclohexa-2,4-dienyl acetic acid (anthocyanone A). This new molecule results from the degradation of malvidin 3-*O*-glucoside, formed by Baeyer−Villiger-type oxidation. Other compounds of unknown structure were also formed. However, further research is still required, and the complete identification of other compounds from malvidin 3-*O*-glucoside degradation detected in this study should provide more information about the actual mechanism(s) involved in anthocyanin disappearance in model solutions.

The structural characterization of these compounds provides important information regarding the chemical transformations involved in the changes of color during red wine's aging. Probably, electrophilic compounds such as anthocyanone A and 2,4,6-trihydroxybenzaldehyde will not be present in the wine solution as free forms due to their strong reactivity with nucleophilic species. However, it will be interesting to understand the mechanism of reaction of these compounds with others wine compounds (anthocyanins, flavanols, flavonols, etc.).

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