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Research Article

Identification by HPLC-MS of Anthocyanin Derivatives in Raisins

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The anthocyanin composition in red grapes dried under controlled conditions has been studied. Pyranoanthocyanins and condensed anthocyanins with flavanols by a methylmethine bridge have been identified. Typically, these compounds appear in wine after the fermentation process, as they require compounds such as pyruvic acid, acetoacetic acid, and acetaldehyde for their formation. During the chamber-drying process a stress situation is originated, inducing significant changes in the grape metabolism from aerobic to anaerobic, and as a result it produces the activation of the alcohol dehydrogenase enzyme (ADH) and others that would cause the formation of these compounds. These derivatives are very interesting because they give greater stability to the color of red wine.

1. Introduction

The pyranoanthocyanins are compounds formed in wine during aging. They are responsible for the red-purple color of red wines and evolve progressively toward brown colors, since these adducts have a more reddish-orange color than their anthocyanins counterparts.

The pyranoanthocyanins are formed from condensation reactions on anthocyanins, which are modified to result in stable oligomers from substitutions at position C4. Their general structure (Figure 1) includes an additional D-ring formed between the OH group at C5 and the C4 of the anthocyanin pyran ring [1]. This new pyran D-ring formed, which is responsible for the name of these compounds, may have different substituents directly linked to C10. This structural characteristic is the source of the greatest intensity of color and stability of pyranoanthocyanins in a broader pH range than the original anthocyanins [2, 3]. These compounds have an absorption peak between 495 and 520 nm, that is, a hypsochromic effect is observed compared to the starting anthocyanins [4–6]. In addition, they have an absorption peak in the 420 nm region, explaining the orange hues of these compounds [7].

The vitisins are one of the most studied pyranoanthocyanin families and are originated by the reaction of anthocyanins with some metabolites released during the fermentation of yeast, such as pyruvic acid, acetoacetic acid, and acetaldehyde [2, 7, 8], the latter could be in wine as a result of the ethanol oxidation.

Since a long time ago, the condensation of the anthocyans with flavanols by a methylmethine bridge or not has been studied by different authors [9–11]. Specifically, in the anthocyanin-methyilmethine-flavanol adducts the absorption peak presents a bathochromic shift compared to the corresponding monomer, finding the λ_{max} between 530 and 540 nm, which gives the molecules blue-red or purple hues. This color shift has been observed both in model solutions [12, 13] and in experimental red wines [14].

The inclusion of anthocyanin C4 into the pyran ring causes the steric hindrance which makes the pyranoanthocyan molecule more stable to bleaching by the SO_2 [2, 5, 15], increase of pH [5, 11, 16], to oxidative degradation [17], and even to temperature [18]. Therefore, the aim of this work has been to identify, by HPLC-MS, the presence of condensation adducts of anthocyanins with flavanols via methylmethine bridges and pyranoanthocyanins in red grapes dried under controlled humidity and temperature.

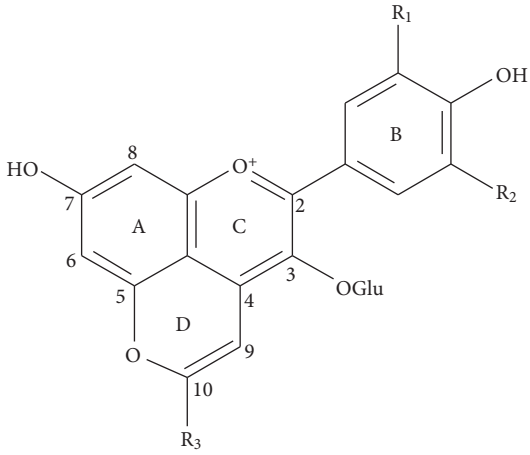

Figure 1: General structure of pyranoanthocyanins.

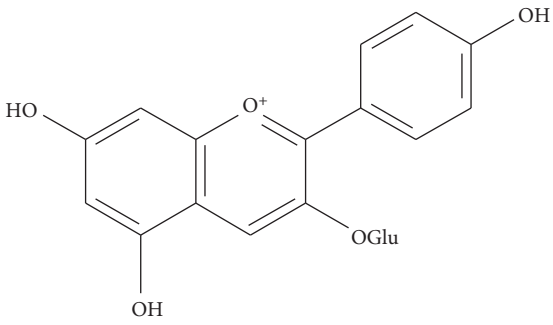

Figure 2: Structure of pelargonidin-3-glucoside.

2. Experimental

2.1. Reagents. All the anthocyanins standards were purchased from Extrasynthèse (Genay, France) and a calibration curve was obtained by injection of different concentration. Methanol, formic acid, ethyl acetate, acetonitrile and purified water were purchase from Merck (Madrid, Spain).

2.2. Grape Drying. The material used in this study consisted of Syrah grapes from the Montilla-Moriles region (southern Spain). This was uniformly distributed in several trays and allowed to dry in a Frisol Climatronic chamber at an air temperature of 40°C and a constant relative humidity of ca. 20% [19].

2.3. Analytical Techniques. The measurement of reducing sugars was performed by refractometry, using a refractometer model Atago Master (Master Baume 2594, Atago, Japan).

Pyruvic acid was determined enzymatically, using a K-PYRUV 03/07 kit from Megazyme (Wicklow, Ireland).

The ethanol content was determined according to Crowell and Ough [20], to this end, ethanol in the sample was collected by steam and then reacted with acid potassium dichromate. The reaction was spectrophotometrically monitored via the absorbance at 600 nm against a blank on a Perking Elmer Lambda 25 spectrophotometer.

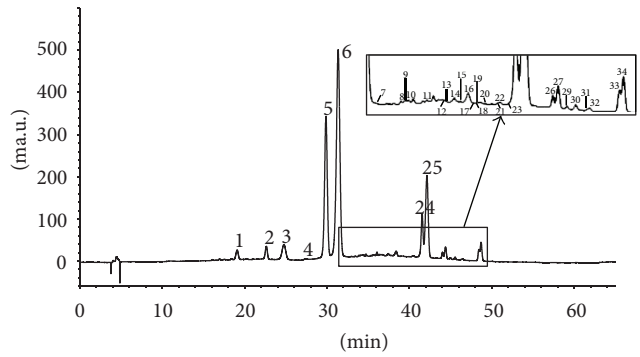

Figure 3: HPLC-DAD chromatogram at 520 nm of anthocyanins of the must from raisins.

The extraction of anthocyanins was performed by using Sep-Pak C18 cartridge (Long Body Sep-Pak Plus; Waters Associates, Milford, MA). A volume of 2 mL of must was passed through the cartridge, with 900 mg of filling that was previously activated with 5 mL of methanol and washed with aqueous HCl 0.01% (v/v). The cartridge was washed successively with 10 mL of 0.01% aqueous HCl and 5 mL of ethyl acetate and anthocyanins were recovered with 5 mL of acidified methanol at pH 2. The extracts were analysed using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) [19]. Detection was carried out in a diode array detector (DAD), using 520 nm as the preferred wavelength, and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet. MS detection was performed in a API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controled by the Analyst 5.1 software [21].

For acetaldehyde quantification, an Agilent 6890 series plus gas chromatograph (Agilent Technologies, Waldbronn. Germany) with electronic pressure control was used [22]. The column, a CPWAX-57 CB model from Chrompack (Middelburg, The Netherlands), was fused silica 60 m × 0.25 mm and 0.40-μm film thickness. The chemstation software package (Agilent Technologies, Waldbronn. Germany) was used. 1 ml of a solution containing 1 g/L of 4-methyl-2 pentanol as internal standard was added to 10 mL of sample, and an aliquot of 0.5 μL was injected.

3. Results and Discussion

Table 1 shows the identified compounds in the musts from red grapes by HPLC-DAD-MS. As can be seen, firstly, monoglucosides derivatives were identified, five of which are present in most red varieties: delphinidin-3-glucoside (peak 1), cyanidin-3-glucoside (peak 2), petunidin-3-glucoside (peak 3), peonidin-3-glucoside (peak 5), and

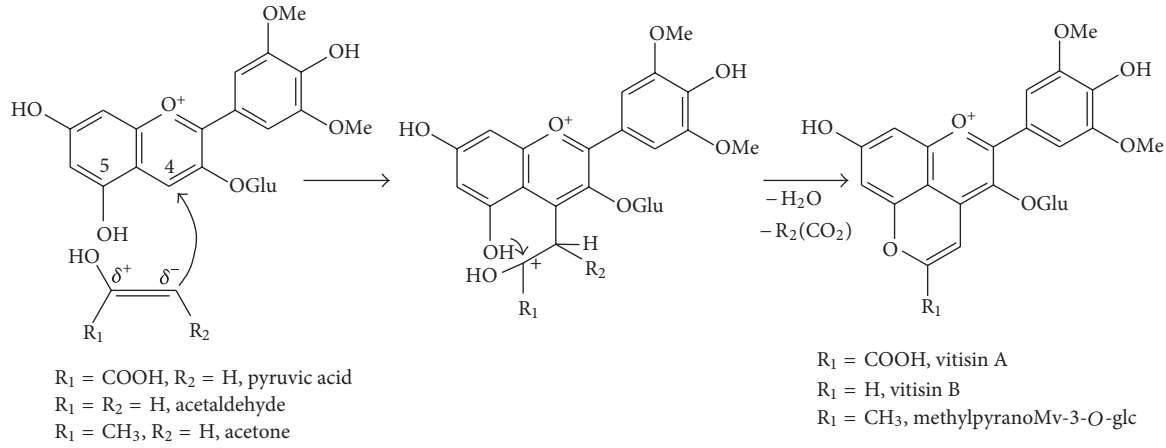

Figure 4: Formation of pyranoanthocyanin compounds by reaction between malvidin-3-glucoside and carbonyl compounds (enolic forms).

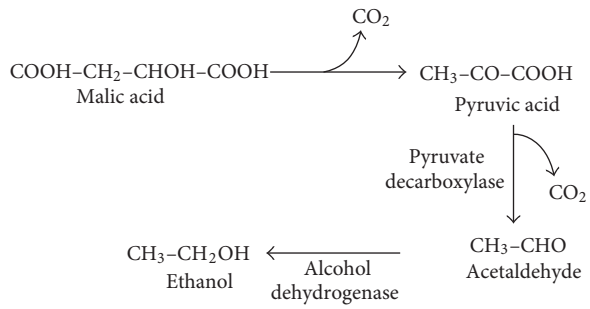

Figure 5: Enzymatic reactions of carbonic maceration.

malvidin-3-glucoside (peak 6), peak 4 was identified as pelargonidin-3-glucoside, whose structure is shown in Figure 2. The MS analysis of pelargonidin-3-glucoside produced a $[M^+]$ peak at *m/z* 433 and a fragment at *m/z* 271. This anthocyanin was identified in grapes of nonvinifera varieties Concord, Rubired, and Salvador [23, 24] and recently in *Vitis vinifera* grapes of Garnacha Tintorera [25]. These authors suggested that the presence of this compound could be used as a chemical marker to identify red wines of this variety, however, after finding this compound in new grape varieties the hypothesis could be revised.

Other monomeric anthocyanins identified were the acetic esters of delphinidin (peak 8), cyanidin (peak 14), petunidin (peak 16), peonidin (peak 24), and malvidin (peak 25). Also, the caffeoylglucosides of petunidin, peonidin, and malvidin were identified as peaks 23, 26, and 27 and finally the peaks 29-34 were assigned to the *p*-coumarylglucosides derivatives of cyanidin, petunidin, peonidin, and malvidin. In relation to these latter compounds, it was possible to differentiate the *cis*- and *trans*-isomers of coumarylglucosides of peonidin (peaks 31 and 33), both with a $[M^+]$ ion at *m/z* 301 and malvidin (peaks 32 and 34), with $[M^+]$ at *m/z* 331.

All of these anthocyanin derivatives have been identified in the musts from fresh grapes. However, in musts from raisins new compounds were also identified, which were synthesized during the drying process of the grapes, pyranoanthocyanins, and condensation compounds anthocyanin-methylmethine-flavanol, whose chromatograms are shown in Figure 3.

Some authors insure that the way of formation of the pyranoanthocyanins, called vitisins (Figure 4), begins with the addition of small metabolites of the positions 4 (carbon) and 5 (hydroxyl group) of anthocyanins, followed by a dehydration and an oxidation, obtaining the D-ring [1]. Specifically, the formation of type A vitisins, also called carboxipyranoanthocyans, under acidic conditions such as exist in red wines are caused by the interaction between the enol form of pyruvic acid with an anthocyanin [7]. The chromatographic characteristic of peak 7 showed a $[M^+]$ ion at *m/z* 561 and a fragmentation with *m/z* 399 and the peak 10 a $[M^+]$ ion at *m/z* 603 and a fragmentation with *m/z* 399. These peaks agreed with those of vitisin A and acetylvitisin A, respectively, first identified by Bakker and Timberlake [2] and whose starting anthocyanins are malvidin-3-glucoside and malvidin-3-acetilglucoside. To confirm the formation of these derivatives, the pyruvic acid content in musts was determined, finding concentrations of 20.5 ± 0.437 mg/L in raisins and below the detection limit in musts from fresh grapes. Considering that the analyzed musts were not fermented, the presence of pyruvic acid would be due to another way of formation during the drying process of the grapes.

So, the fruit dehydration involves a stress situation inducing significant changes in their metabolism [26]. In the particular case of grapes, Bellicontro et al. [27] showed that the stress situation took place when the weight loss was 10–15% if they were dried in tunnels at 21°C. Furthermore, according to Chkaiban et al. [28] the loss of water in the grape berries during the drying process leads to changes in the membrane permeability through the activation of lipooxygenase enzyme

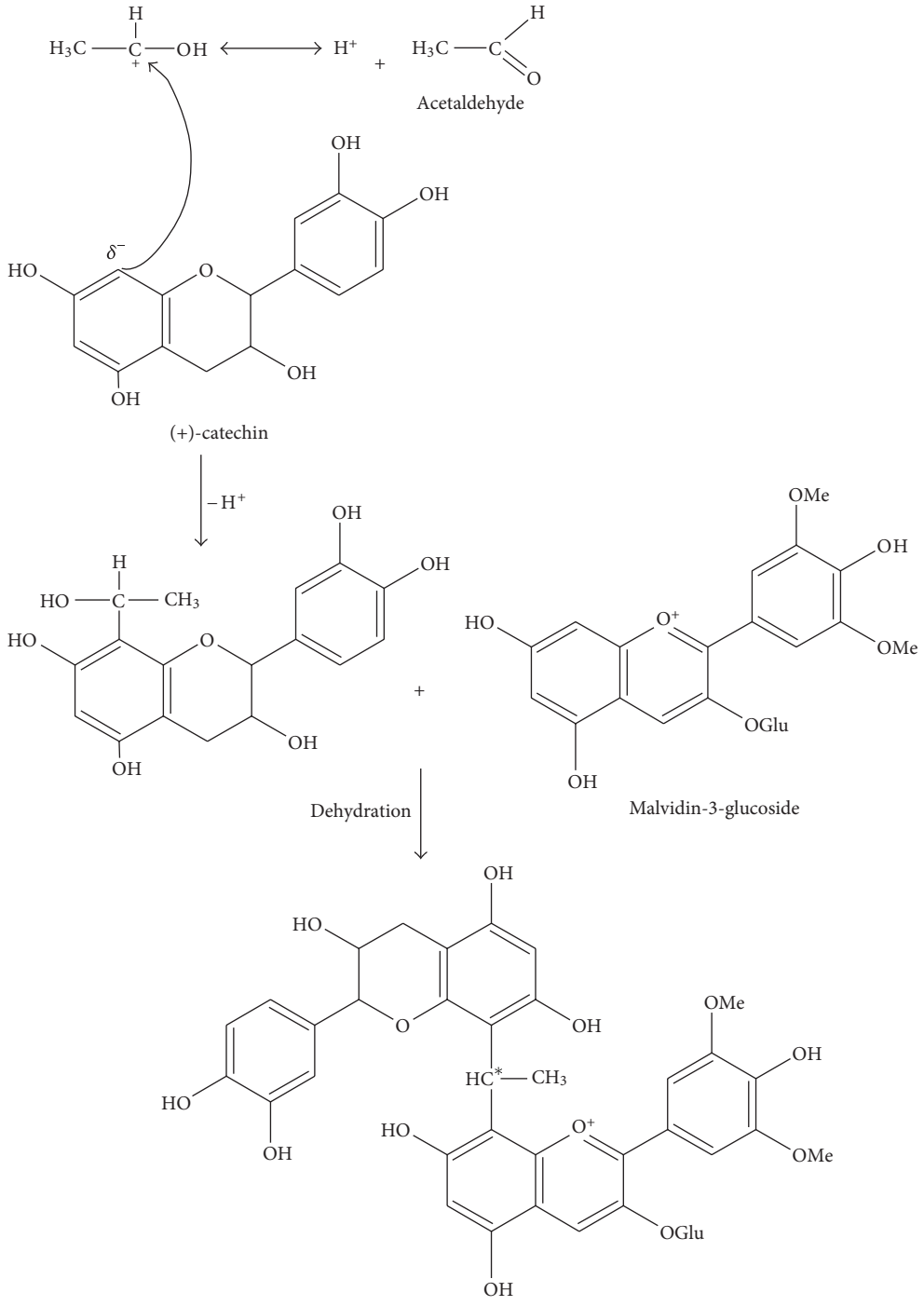

Figure 6: Formation of anthocyanin-methylmethine-flavanol compounds by reaction between (+)-catechin and malvidin-3-glucoside.

(LOX). This fact produces a change in the berry's metabolism from aerobic to anaerobic, resulting in the activation of the alcohol dehydrogenase enzyme (ADH). Under these anaerobic conditions during drying other enzymes could be activated, capable of degrading sugars and/or malic acid from grapes to pyruvic acid, which would justify the contents of this acid found in raisin musts.

In addition, in the musts from raisins, type B vitisins were determined, which differ from carboxipyranoanthocyans because the carboxyl group is missing at the C10 position of the D-ring. These vitisins are formed by the addition of one acetaldehyde molecule on an anthocyanin molecule (Figure 4). Peak 11 showed the chromatographic characteristics of the so-called vitisin B. This piranoanthocyan is

Table 1: Retention times, mass spectral, and UV-Vis data of anthocyanins identified in musts from raisins after chamber drying.

Peak	R_t	$M^+(m/z)$	MS^2 frag.	λ_{max} (nm)	Compound
1	19.10	465	303	524	Dp-3-glc
2	22.65	449	287	516	Cy-3-glc
3	24.81	479	317	526	Pt-3-glc
4	27.46	433	271	506	Pg-3-glc
5	29.91	463	301	518	Pn-3-glc
6	31.37	493	331	528	Mv-3-glc
7	32.25	561	399	490	Vitisin A
8	33.90	507	303	528	Dp-3-acetylglc
9	34.23	487	325	486	B-type vitisin Pn-3-glc
10	34.43	603	399	490	A-type vitisin Mv-3-acetylglc
11	35.66	517	355	490	Vitisin B
12	36.90	779	—	530	Pn-3-glc-methylmethine(epi)catechin
13	37.25	809	357	530	Mv-3-glc-methylmethine(epi)catechin
14	37.46	491	287	518	Cy-3-acetylglc
15	37.86	809	357	540	Mv-3-glc-methylmethine(epi)catechin
16	38.42	521	317	528	Pt-3-acetylglc
17	38.60	529	325	494	B-type vitisin Pn-3-acetylglc
18	39.07	779	—	528	Pn-3-glc-methylmethine(epi)catechin
19	39.07	559	355	494	B-type vitisin Mv-3-acetylglc
20	39.28	809	357	542	Mv-3-glc-methylmethine(epi)catechin
21	40.47	809	357	530	Mv-3-glc-methylmethine(epi)catechin
22	40.50	779	—	528	Pn-3-glc-methylmethine(epi)catechin
23	41.10	641	317	522	Pt-3-caffeoylgluc
24	41.58	505	301	520	Pn-3-acetylglc
25	42.16	535	331	532	Mv-3-acetylglc
26	44.05	625	301	522	Pn-3-caffeoylglc
27	44.41	655	331	534	Mv-3-caffeoylglc
28	44.46	851	—	—	Mv-3-acetylglc-methylmethine(epi)catechin
29	44.99	595	287	520	Cy-3-coumaroylglc
30	45.55	625	317	534	Pt-3-coumaroylglc
31	46.20	609	301	526	Pn-3-coumaroylglc *cis*
32	46.50	639	331	538	Mv-3-coumaroylglc *cis*
33	48.44	609	301	524	Pn-3-coumaroylglc *trans*
34	48.71	639	331	538	Mv-3-coumaroylglc *trans*

originated when the cycloaddition is carried out on malvidin-3-glucoside [4, 15]. Spectroscopic characteristics of this compound showed a $[M^+]$ at m/z 517, fragmentation ion at m/z 355 and λ_{max} 490 nm. Likewise, the peaks 9, 17, and 19 were identified as type B vitisins of peonidin-3-glucoside, peonidin-3-acetilglucoside, and malvidin-3-acetilglucoside, respectively.

The presence of acetaldehyde in the musts from raisins, in the formation of these compounds, would be essential. As a result of the metabolism change discussed above, the pyruvic acid could have been converted into acetaldehyde by decarboxylation. To prove this hypothesis, the musts were analyzed by gas chromatography (GC-FID) for quantification of acetaldehyde. The results showed the absence of this compound in fresh grapes and a content of 128 ± 7.9 mg/L in the musts from raisins, indicating that during chamber-drying the acetaldehyde synthesis should take place.

According to the hypothesis about the synthesis of pyruvic acid and acetaldehyde during grape drying, the latter could be reduced to ethanol by the action of the alcohol dehydrogenase enzyme. As a result, ethanol would be the major metabolite produced from sugars and/or malic acid in grapes subjected to anaerobic stress, similar to the reaction that occurs during carbonic maceration (Figure 5). To confirm this hypothesis, the concentration of ethanol in fresh grapes was measured, finding a content of zero, while in raisin musts the concentration was 0.789 ± 0.003 (v/v), indicating that the alcohol dehydrogenase enzyme could convert acetaldehyde into ethanol.

Besides the pyranoanthocyanins, the HPLC-DAD-MS analysis also showed condensation products between anthocyanins and (epi)catechin via a methylmethine bridge, whose compounds need the presence of acetaldehyde. According to the mechanism proposed by Pissarra et al. [29], the

acetaldehyde in acid medium is subjected to an initial protonation forming the corresponding carbocation, inducing an electrophilic attack on the flavanol phloroglucinol ring, preferably in the C8 position. The formed adduct reacts with the anthocyanin to yield the new colored compound. The presence of an asymmetric carbon (C^*) on the created link leads to the formation of two diastereoisomeric structures (R and S). This mechanism is shown in Figure 6. In the corresponding chromatograms, the following adducts were identified: three compounds of formula Pn-3-glc-methylmethine(epi)catechin (peaks 12, 18, and 22) with *m/z* 779 and four compounds of formula Mv-3-glc-methylmethine(epi)catechin (peaks 13, 15, 20, and 21) of ratio *m/z* 809 and fragmentation ions at *m/z* 357.

In summary, the presence of pyruvic acid, acetaldehyde, and ethanol would confirm the enzymatic transformations and, therefore, the formation of pyranoanthocyanins and condensation compounds of anthocyanin with flavanols via methylmethine bridge that occurred during the raisining process. These anthocyanin derivatives have a great oenological interest because they confer stability to the color of red wines and have always been identified in wine and not in unfermented musts.

Acknowledgments

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