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REACTIONS OF POLYPHENOLOXIDASE GENERATED CAFTARIC ACID *o*-QUINONE WITH MALVIDIN 3-*O*-GLUCOSIDE

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Key Word Index—anthocyanins; degradation; oxidation; phenolic compounds; polyphenol oxidase; caffeoyl tartaric acid *o*-quinone.

Abstract—Polyphenol oxidase (PPO)-catalysed oxidation of solutions containing malvidin 3-*O*-glucoside and caftaric acid was studied by HPLC and mass spectrometry. The reaction of the anthocyanin with *o*-quinones and the nature of the products formed were compared as two pH values. The oxidized solution contained, in addition to the precursors, coloured and colourless compounds which eluted between caftaric acid and malvidin 3-*O*-glucoside. Liquid chromotography with ion spray mass spectrometry (LC-ISP-MS) indicated that they were adducts of malvidin 3-*O*-glucoside and caftaric acid, either in the flavylium or in the hemiacetal form.

INTRODUCTION

Phenolic compounds are important grape constituents, responsible for major organoleptic properties of wine, in particular colour. The phenolic composition of wines depends on the initial level of phenolics in the grapes and also of other compounds which may interfere with them, as well as on the wine-making process used, which greatly influences the extraction of various components and their subsequent reactions. In the normal wine-making process, enzymatic oxidation catalysed by grape PPO (EC 1.10.3.1) starts when the grapes are crushed or pressed. The major substrates of PPO in grapes are thought to be caftaric acid (caffeoyl tartaric acid) and, to a lesser extent, coutaric acid (coumaroyl tartaric acid) which are both transformed to caftaric acid *o*-quinone. Grapes and wines contain other phenolic substrates, including flavan-3-ols, flavonols and, in the case of red grapes, anthocyanins. All of these compounds are poor PPO substrates but are rapidly altered by enzymically generated *o*-quinones, due either to coupled oxidation or to condensation reactions between phenolic compounds and quinone [1, 2]. Whereas coupled oxidation by *o*-quinones is well documented, few studies on the role of caftaric acid *o*-quinone in the process of anthocyanin degradation and subsequent discolouration in oxidizing grape must are available. Although it cannot be oxidized by coupled oxidation, malvidin 3-*O*-glucoside, the major grape anthocyanin, was degraded in the presence of enzymically generated caftaric acid quinones [2]. Complex mixtures including new red pigments and colourless molecules arose from oxidation of must-like model solutions. The purpose of the present work was to study the mechanism of malvidin 3-*O*-glucoside degradation in the presence of caftaric acid *o*-quinone and to characterize the new products formed.

RESULTS AND DISCUSSION

Electrospray ionization mass spectrometry which is a soft ionisation technique was used to determine the masses of the products. Preliminary mass analyses were performed in order to distinguish new products from the native compounds and from the buffer molecules.

It has been demonstrated that in acidic aqueous solutions, four anthocyanin species exist in equilibrium: the quinonoidal base, the flavylium cation, the hemiacetal pseudobase and the chalcone [3]. Interconversion between these four structures is a function of pH. For malvidin 3-*O*-glucoside, the pigment occurs under acidic conditions (pH 0–6) as an equilibrium mixture of the red flavylium cation and the colourless hemiacetal form. At low pH, flavylium structure dominates the hemiacetal form (at pH 1.7, 90 and 10%, respectively), whereas at pH 4, the opposite is true [3]. Because of the positive charge of the flavylium, anthocyanin flavylium mass spectrum

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response in positive-ion mode corresponds to its real mass $[M]^+$. Acidic malvidin 3-*O*-glucoside solution gave rise to an *m/z* peak at 493 in the positive mode as reported by Baldi *et al.* [4]. In the negative-ion mode, the $[M-2H]^-$ ion was detected at *m/z* 491. These peak signals correspond to the flavylium form of malvidin 3-*O*-glucoside. In contrast, other anthocyanin chemical forms give classical signals corresponding to $[M+H]^+$ for positive-ion experiments and $[M-H]^-$ for negative-ion analysis. The flavylium form was the major species of malvidin 3-*O*-glucoside present in acidic medium (pH 1.7), but the presence of the hydrated form at *m/z* 509 was noticed in negative-ion mode. The corresponding peak at 511 in the positive-ion mode was not detected in the acidic solution. The solution of malvidin 3-*O*-glucoside in potassium hydrogen tartrate buffer (pH 3.4) presented an intensity of the *m/z* 493 signal lower than that observed for the acidic solution; in contrast, the $[M-H]^-$ peak at 509 was larger than in the acidic solution and was also detected in the positive mode as $[M+H]^+$ peak at *m/z* 511. These results are in agreement with the well-known equilibrium of anthocyanins in aqueous solution [3]. The red flavylium compound (*m/z* 493) is unstable in weakly acidic solution (i.e. pH 3.4) due to the formation of the colourless hemiacetal form (*m/z* 511 in positive-ion mode and *m/z* 509 in negative-ion mode).

No degradation of malvidin 3-*O*-glucoside occurred in the presence of grape PPO without caftaric acid confirming that it was not oxidized directly by PPO. HPLC profiles and LC-ISP-MS analyses of PPO-free controls containing malvidin 3-*O*-glucoside and caftaric acid at pH 1.7 and pH 3.4 showed that no new product appeared in the media, confirming that the native caftaric acid and the malvidin 3-*O*-glucoside do not interact.

Malvidin 3-*O*-glucoside was degraded in the presence of a crude grape PPO extract and of caftaric acid at pH 3.4 with only 20% of each precursor remaining in the resulting solution after 90 min. The resulting solution was monitored by HPLC and LC-ISP-MS. Figure 1 shows typical HPLC chromatograms of the malvidin 3-*O*-glucoside/caftaric acid solution oxidized for 90 min with grape PPO. After 90 min, the solution showed numerous new compounds, some of which absorbed in the visible range. Most of the new products formed eluted between caftaric acid and malvidin 3-*O*-glucoside, showing intermediate polarity. UV-visible spectra of the reaction products recorded from 250 to 600 nm by means of a diode array detector, exhibited two absorption maxima around 290 and 328 nm in the UV-region and eventually a third absorption in the visible region at 540 nm indicating that the chromophore moiety of the anthocyanin was maintained (Fig. 2). The bathochromic shift in the visible range is often observed in the case of condensation of anthocyanins with other compounds [5]. The shoulder around 320–330 nm suggests the presence of a cinnamic acid moiety in the pigment structure. Thus,

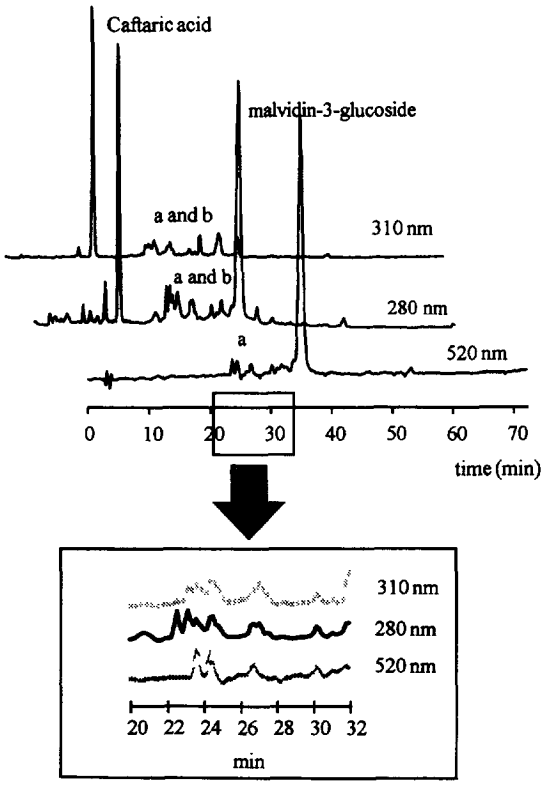

Fig. 1. HPLC pattern of enzymatic caftaric acid malvidin 3-*O*-glucoside oxidation mixture at pH 3.4.—Detection at 280, 310 and 520 nm; peaks a, coloured adducts; peaks b, colourless adducts.

examination of the UV-visible spectra of the produced pigments suggests that they contained both anthocyanin and caftaric acid moieties.

The oxidized solution was submitted to LC-ISP-MS analysis. Besides the malvidin 3-*O*-glucoside peak ($[M]^+$ at *m/z* 493 and $[M\text{-}2H]^-$ at *m/z* 491) and caftaric acid peak ($[M-H]^-$ at *m/z* 311 and $[M-H]^+$ at *m/z* 313), LC-IPS-MS spectra showed molecular ion peaks at 803 and 821 in the positive-ion mode and 801 and 819 in the negative-ion mode. These peaks (803 and 801) corresponding to $[M']^+$ values of 803 and $[M'-2H]^-$ of 801 confirmed that the products detected are malvidin 3-*O*-glucoside and caftaric acid adducts whose predicted mass was $(493+312)-2 = 803$. The detection of additional peaks at *m/z* 821 ($[M'+H]^+$) and at *m/z* 819 ($[M'-H]^-$ allowed us to postulate that they are malvidin 3-*O*-glucoside and caftaric acid adducts in which the malvidin 3-*O*-glucoside is not in the flavylium form but in the hydrated hemiacetal form. Moreover, detection of these compounds was easier in the negative-ion mode than in the positive-ion mode because of the addition of the caftaric moiety on a non-charged anthocyanin form. Thus, the results of earlier stoichiometric studies [2] indicating equimolar 1/1 condensation of malvidin 3-*O*-glucoside and caftaric acid quinone were confirmed by the mass values observed in these experiments.

Comparison of the mass response and *A* at 520 nm

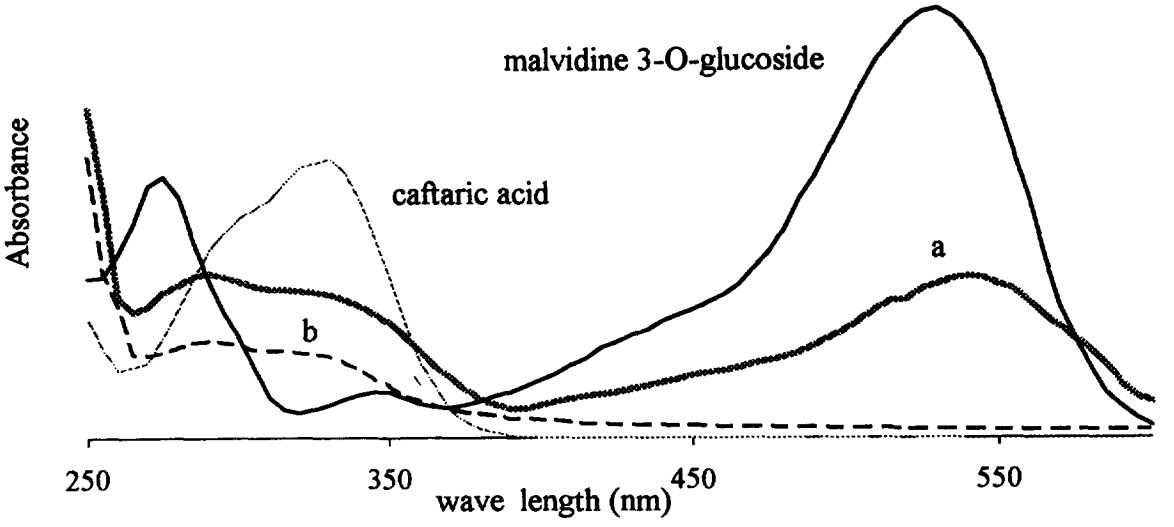

Fig. 2. UV-visible spectra registered by the diode array detector of coloured reaction product (a), colourless reaction product (b), malvidin 3-*O*-glucoside and caftaric acid.

(Fig. 3) showed that the coloured products formed during the reaction were related to 801 mass responses, whereas colourless compounds were detected at *m/z* 819 (negative-ion mode). The mass determination allowed us to demonstrate that the colourless products are malvidin 3-*O*-glucoside and caftaric acid condensation products too, but in which the anthocyanin moiety is in the hydrated state, whereas the coloured products are flavylium adducts. As shown on the three-dimensional map of the LC-ISP-MS (Fig. 3), six hydrated adducts and five flavylium adducts (at least) could be numbered.

Thus, both coloured flavylium/caftaric acid adducts and hemicetal-caftaric adducts were present in the reaction mixture. Since the flavylium and the hydrated forms of malvidin 3-*O*-glucoside coexist in the initial pH 3.4 solution, they may both have reacted with caftaric acid *o*-quinone. However, it is also possible that reaction occurs only with one of the anthocyanin forms and that the resulting products are themselves in equilibrium with their hydrated or flavylium form. In order to study separately the reactivity of each malvidin form towards the quinone, two sets of experiments were carried out respectively at pH 1.7 and pH 3.4. Solutions containing equimolecular amounts of malvidin 3-*O*-glucoside and caftaric acid *o*-quinone were prepared. To avoid eventual conversion between flavylium and hemiacetal forms in the acidic HPLC solvent, the reaction mixtures were analysed directly by ISP-MS.

Comparison of these solutions prepared at two pH values (Fig. 4, negative-ion mode A, positive-ion mode B) allows three groups of reaction products to be distinguished in addition to anthocyanin and caftaric acid. The first one, consisting of products

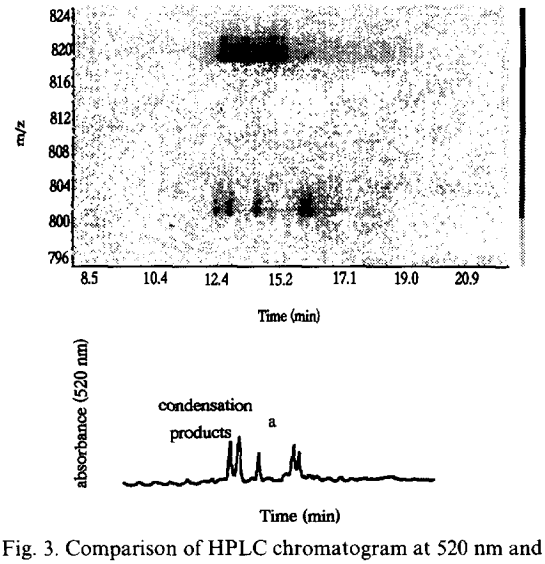

Fig. 3. Comparison of HPLC chromatogram at 520 nm and LC-ISP-MS three-dimensional map of adducts.

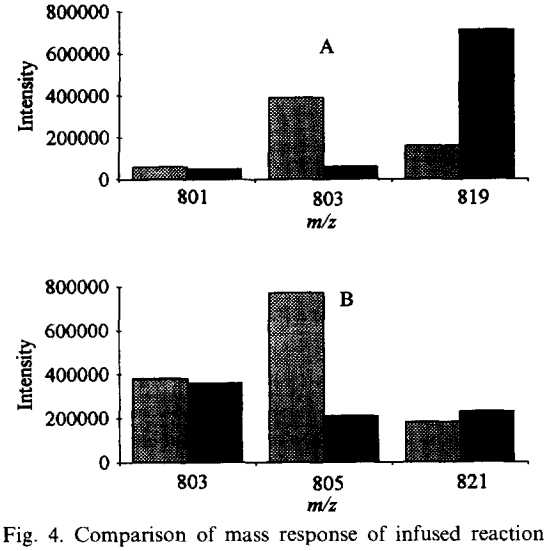

Fig. 4. Comparison of mass response of infused reaction mixtures. In: negative-ion mode (A) and in positive-ion mode (B) at pH 1.7 ▨ and at pH 3.4. ■

detected at m/z 803 in the positive-ion mode was formed in identical amounts with regards to their signal intensity at both pH values. The second one corresponds to products detected at m/z 819 in the negative-ion mode which are more abundant at pH 3.4 than at pH 1.7. Both types of adducts are formed at both pH values. In agreement with the anthocyanin equilibrium the malvidin 3-*O*-glucoside flavylium form was predominant in the pH 1.7 solution; therefore 803 adducts were expected as the major adducts and in a higher proportion than in the pH 3.4 solution. In contrast, the results showed that the amounts of 803 adducts are similar at both pH values whereas the 820 adducts are more abundant in the pH 3.4 solution, in agreement with the prediction based on anthocyanin equilibrium. These results suggest that the hydrated forms are more reactive than the flavylium form. Moreover, a third group was noticed at m/z 805 in the positive-ion mode and at m/z 803 in the negative-ion mode at pH 1.7. This form is more responsive in the positive-ion mode than in the negative-ion mode, supporting the hypothesis that it corresponds to stacking of malvidin 3-*O*-glucoside flavylium form and caftaric acid which are not detected under LC conditions.

Unfortunately, the small amounts of each adduct formed do not allow their isolation and structural characterization. However, their mass determination clearly demonstrates that they are formed by condensation involving covalent binding, and that the hydrated forms are predominant. Thus, with regard to these data and the number of products detected, some hypotheses may be formulated to explain their mechanisms of formation.

Previous reports have shown that PPO generated caftaric acid *o*-quinone reacts with glutathione and leads to grape reaction produced (GRP) [6] by nucleophilic addition of glutathione to the *o*-quinone. Moreover, Cheynier *et al.* [7] demonstrated that anthocyanin addition delays GRP formation and lowers its maximum level, suggesting some kind of competition between glutathione and anthocyanin for caftaric acid *o*-quinones. This may also mean that the anthocyanin-caftaric acid adducts can proceed from the same mechanism. Guyot *et al.* [8] demonstrated that enzymatic oxidation of catechin leads to numerous condensation products, by reaction of the catechin *o*-quinone (*) with non-oxidized catechin unit. Some of them, linked by C-6′B*—C-8A bond, result from nucleophilic Michael 1-4 addition of a flavanol A ring onto the *o*-quinone which is favoured by high pH [10]. In addition, owing to the partial negative charges on C-6 and C-8 of the anthocyanin and the electrophilic character of the caftaric acid *o*-quinones, condensation is likely to proceed via nucleophilic attack of the anthocyanin onto the caftaric acid *o*-quinone. The hemiacetal forms, whose C-8 and C-6 carbons should be more nucleophilic than those of the flavylium ion, are more reactive towards the quinone.

Considering the possibilities of competitive substitutions involving the 2, 5, or 6 positions of the caftaric acid *o*-quinones and the two nucleophilic positions C-6 and C-8 on the anthocyanin moiety, six adducts may be expected with C-6*-C-8A, C-6*-C-6A, C-2*-C-8A, C-2*-C-6A, C-5*-C-8A or C-5*-C-6A biphenyl linkages. The side chain double bond of the caftaric acid *o*-quinone may also be the site of nucleophilic attack, as described in the case of caffeic acid oxidation products [11]. However, some side chain conjugation still exists in the anthocyanin/caftaric acid adducts as demonstrated by the residue *A* at 328 nm, indicating that the double bond is not involved in the condensation reaction. Thus, this position seems protected, as for tartaric and quinic acid [12].

Finally, a radical mechanism involving the semiquinone cannot be ruled out, as Guyot *et al.* (1996) [8] found products showing O-3′B*-C-8A or O-4′-B-C-8A ether bonds directly related to this mechanism [13] which is favored by low pH values [10]. This mechanism should lead to the formation of O-3* caftaric acid-C-8A or O-4* caftaric acid C-8A malvidin 3-*O*-glucoside-caftaric acid adducts and the equivalent O-3* caftaric acid-C-6A or O-4* caftaric acid C-6A malvidin 3-*O*-glucoside/caftaric acid adducts.

The positions of substitution for the anthocyanins adducts described in this work have to be explored. Moreover, the different mechanisms could give rise to different adducts depending on the anthocyanin form involved.

EXPERIMENTAL

Materials. Malvidin 3-*O*-glucoside was prepared from skins of *Vitis vinifera* var. Grenache noir as described previously [2]. Purity was checked by HPLC DAD (Diode Array Detector) analysis and LC-ISP-MS (liquid Chromatography with Ion Spray Mass Spectrometry) analysis. Crude PPO extract and caftaric acid were prepd from grape (*Vitis vinifera* var. Grenache noir) according to ref [14].

Generation of o-*quinones and incubation conditions.* The degradation of malvidin 3-*O*-glucoside was studied in the presence of grape PPO and caftaric acid or of its enzymically generated *o*-quinone. All grape must-like model solns contained initially 0.5 mM of caftaric acid (or caftaric acid *o*-quinone), 0.5 mM of the malvidin 3-*O*-glucoside and grape PPO (2.5 nkatal ml^{-1}). As we demonstrated before [2], that maximum adduct yield was obtained for a reaction time of 90 min, enzymatic incubations were conducted at 30° using air agitation on a magnetic stirrer for 90 min. To study the reaction of malvidin 3-*O*-glucoside with the quinone at different pH values, the caftaric acid *o*-quinones were enzymatically generated. The latter components were generated by PPO oxidation of caftaric acid in 0.01 M KH tartrate buffer pH 3.4. Once the maximum quinone yield (80%) was obtained, the reaction was stopped by addition of HCO_2H (2% final) for pH 1.7 value. For pH 3.4 value, the enzyme

was eliminated and the quinones recovered by centrifugation on a Centricon system (Amicon, Epernon, France). Under these conditions, caftaric acid *o*-quinones were stable and PPO activity inhibited. The *o*-quinones recovered from the reaction mixts (0.5 mM) were added to malvidin 3-*O*-glucoside (0.5 mM) previously equilibrated at pH 1.7 or pH 3.4 and the resulting soln incubated at 30° under air.

HPLC analyses. The HPLC apparatus was a Waters-Millipore (Millipore Corp, Milford, MA, USA) system equipped with a diode array detector (DAD). The column reversed-phase Lichrospher 100-RP18 (5 μm packing) (250 × 4 mm i.d.) (Merck, Darmstadt, Germany) was protected with a guard column of the same material.

Elution conditions: 1 ml min^{-1} flow rate; oven temp. 30°; solvent A, 2% HCO_2H in H_2O; solvent B, CH_3CN–H_2O–HCO_2H (40:9:1); linear gradient from 5 to 30% B in 40 min, from 30 to 50% in 20 min and from 50 to 80% in 10 min, followed by washing and reconditioning of the column. The elution was monitored simultaneously at 280, 310 and 520 nm and UV-visible spectra were recorded from 250 to 600 nm.

Electrospray-ionization mass spectrometry. The formation of reaction products was monitored by liquid chromatography with ion spray mass spectrometry (LC-ISP-MS) analysis. LC-ISP-MS analyses were carried out using a Superspher 100 RP18 (125 × 2 mm, i.d. 3 μm packing) (Merck) column with a UV detector set at 280 nm and a Sciex API I Plus (Perkin–Elmer Sciex, Thornhill, Ontario, Canada) simple quadrupole mass spectrometer equipped with an ion spray source. The sepn was achieved by using a gradient adapted from that used for HPLC/DAD analyses. The mass spectrometer was operated with −4000 V applied to the electrospray needle and −60 V to the orifice in the negative-ion mode, and +5000 V and +60 V in the positive-ion mode. The flow was split so that 50 μl min^{-1} went to the electrospray source through a fused-silica capillary (length 100 cm, 100 μm i.d.)

The mass spectrometer scanned from *m/z* 300 to 1650, in steps of 0.1 amu and with a dwell time of 25 msec at each step, referred as scan-mode. Some experiments were performed by direct inlet mass spectrometery. For direct injection, the soln was introduced into the electrospray source at a constant flow rate of 10 μl min^{-1} with a medical syringe infusion pump (Harvard Apparatus, Model 22, South Natick, USA) in combination with a syringe (100 μl).

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