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Antiradical Properties of Red Wine Portisins

Katharina F. Pirker,[†] Joana Oliveira,[§] Victor de Freitas,[§] Bernard A. Goodman,[†] and Nuno Mateus*[,§]

[†]Health and Environment Department, Environmental Resources and Technologies, AIT Austrian Institute of Technology GmbH, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria

[§]Centro de Investigação em Química, Departamento de Química, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal

ABSTRACT: The free radical chemistry of two polyphenolic pigments from red wine, belonging to the family of portisins, has been investigated after reaction with $O_2^{\bullet-}$ radicals using electron paramagnetic resonance (EPR) spectroscopy. Two portisins derived from malvidin-3-glucoside and cyanidin-3-glucoside were used for this study. Stable free radicals were detected with both portisins and correspond to unpaired electrons localized on the B as well as F rings of the portisins. Interpretations were confirmed by comparison with the spectra of free radicals formed by oxidation of the model compounds cyanidin-3-glucoside, malvidin-3-glucoside, and catechin. These results concur with previous work reporting the higher antiradical properties of these pigments compared to their anthocyanin precursors.

KEYWORDS: portisins, red wine, EPR spectroscopy, superoxide anion radical, free radical

INTRODUCTION

Moderate consumption of red wine has often been assumed to have health-promoting effects, including protection against cardiovascular disease.[1] These important biological properties of red wine have been associated with their antioxidant and free radical scavenging abilities, which are attributed to the presence of various classes of flavonoids (flavanols, anthocyanins, flavonols, and phenolic acids).[2−5] During red wine aging, anthocyanins undergo several chemical transformations, leading to the production of new families of pigments. Pyranoanthocyanins, a major class of anthocyanin derivatives occurring in red wine, are structurally characterized by the presence of a fourth ring between C-4 and the C-5 hydroxyl group of an anthocyanin moiety, that is, portisins (Figure 1). Members of this family are distinguished by the chemical nature of substituents at C-10 of the new ring.[6−14] Portisins are a class of red wine pyranoanthocyanins that have a flavanol moiety linked through a vinyl bond to produce an extended conjugated system of π electrons (Figure 1). They display an unusual bluish color under acidic conditions[15] and have thus been suggested as having potential applications as food colorants.[16]

Although the antioxidant properties of anthocyanins have been studied extensively (mostly as natural extracts), there have been fewer studies of their free radical scavenging abilities, which are by definition possible antioxidant reactions.[17,18] These studies on free radical scavenging are even more limited in the case of anthocyanin derivatives.[19] Furthermore, there is currently little information on the chemical reactions that occur as a result of the free radical scavenging reactions of these compounds, especially portisins. On the other hand, a recent study has highlighted the higher antiradical properties of portisins compared to their anthocyanin precursors.[20]

The present study describes the free radical chemistry of two red wine portisins derived from malvidin-3-glucoside and cyanidin-3-glucoside after oxidation by superoxide anion ($O_2^{\bullet-}$) radicals using electron paramagnetic resonance (EPR) spectroscopy. The $O_2^{\bullet-}$ radical is a biologically relevant reactive oxygen species (ROS); to avoid any side reactions with other ROS, the experiments were carried out in 100% DMSO, which increases the stability of $O_2^{\bullet-}$.

MATERIALS AND METHODS

Reagents. Cyanidin-3-glucoside and malvidin-3-glucoside were purchased from Extrasynthese (Genay, France). (+)-Catechin, potassium superoxide (KO_2), and dimethyl sulfoxide (DMSO, 99% purity) were bought from Sigma-Aldrich Handels GmbH (Vienna, Austria), and the 18-crown-ether (18C6, 98% purity) was from Merck (VWR International GmbH, Vienna, Austria).

Synthesis of Vinylpyranoanthocyanidin-3-glucoside-catechins (Portisins). Portisins derived from malvidin-3-glucoside (Mv-pt) and cyanidin-3-glucoside (Cy-pt) were prepared through the reaction of anthocyanin−pyruvic acid adducts with catechin and acetaldehyde according to the procedure already reported.[21] The anthocyanin−pyruvic acid adducts were synthesized as described in the literature.[22] The purity of the compounds was checked by LC-MS and NMR analyses.

Radical Scavenging Assay (DPPH). Following the method described in the literature[23] with some modifications, radical activities were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. The tested compound reacts with DPPH and induces a decrease of the absorbance measured at 515 nm, which indicates the scavenging potential of the compounds. Previous control assays were performed with all of the compounds to subtract their contribution at 515 nm. The reaction for scavenging DPPH radicals was performed in a microplate reader of 96-well plates (Biotek Powerwave XS with software KC4). The reaction was carried out on the plate wells with a temperature of 25 °C. A solution of 60 μM DPPH was prepared in methanol. Two

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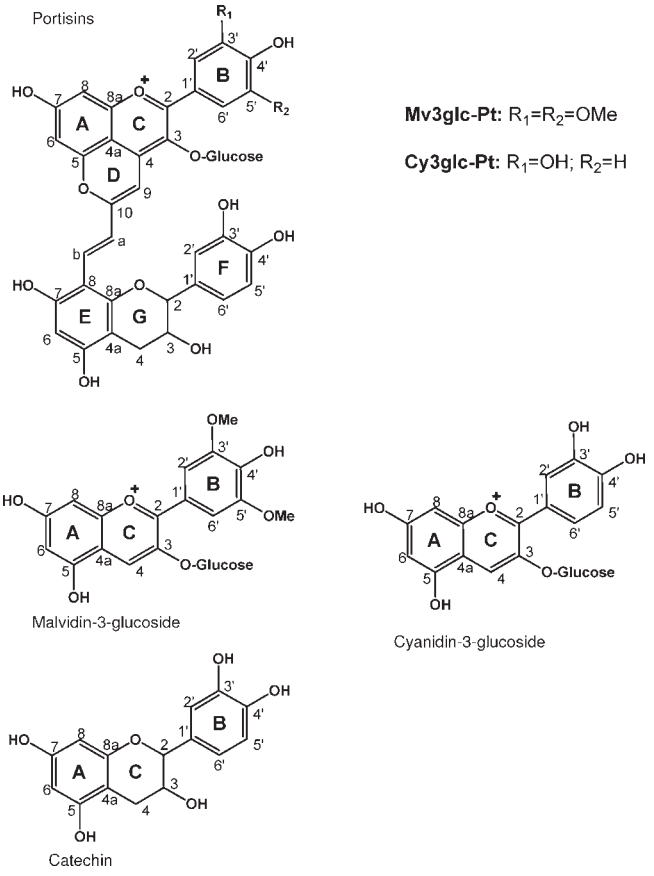

Figure 1. Molecular structures of portisins Mv3glc-pt and Cy3glc-pt, cyanidin-3-glucoside, catechin, and malvidin-3-glucoside.

hundred and seventy microliters of this latter solution was added in each well together with 30 μL of compound. The compounds to be tested were previously dissolved in methanol and used in a final concentration of 10 μM. The decrease in absorbance was measured at 515 nm, at $t = 0$ and every 10 min, during 30 min. For the final results, the 0−20 min reaction time window was used. Antiradical activity was expressed as micromolar Trolox equivalents. The antiradical activity was calculated from the equation determined from linear regression after plotting known solutions of Trolox with different concentrations.

EPR Measurements. Solutions of 1 mM of each portisin, 0.5 mM malvidin-3-glucoside, 0.5 mM catechin, and 0.25 mM cyanidin-3-glucoside were made in H_2O-free DMSO. The $O_2^{\bullet-}$ radical solution (50 mM in DMSO) was prepared in the presence of the crown ether 18C6 according to the method of Valentine et al.[24] Two hundred microliters of the phenolic solutions and 200 μL of the $O_2^{\bullet-}$ solution were gently mixed and immediately transferred into a flat cell for EPR measurement.

EPR spectra were acquired as first derivatives of the microwave absorption using a Bruker EMX CW spectrometer operating at X-band frequencies (9 GHz) and equipped with a high-sensitivity resonator. Microwaves were generated by a Gunn diode, and the microwave frequency was recorded continuously with an in-line frequency counter. One hundred kilohertz modulation frequency (20 kHz for cyanidin-3-glucoside) and a microwave power of 20 mW were used for spectral acquisition. Although at this power there is likely to be partial saturation of the microwave absorption, it was used to maximize the overall signal intensity. The time constant and conversion time were both 41 ms, and spectra were accumulated in 2−22 scans (according to their intensities) using a modulation amplitude in the range 5−10 μT. Spectral parameters were optimized by simulation using Bruker Simfonia software, and g values were calculated by reference to the Bruker standard DPPH, which was used as an external standard ($g = 2.0036$).

LC-MS assays were also performed to ensure that the detected radicals are those primarily formed by abstraction of a single H atom from the initial polyphenols or those formed from oxidation products of the portisins (data not shown).

Table 1. Radical Capacity Assessed by DPPH Assay of Cyanidin (Cy) and Malvidin (Mv) Based Pigments: Anthocyanins Mv3glc and Cy3glc and Portisins Mv3glc-pt and Cy3glc-pt

	Cy (μM Trolox equiv)	Mv (μM Trolox equiv)
anthocyanin	18.23 ± 0.21	15.29 ± 0.31
portisin	23.09 ± 0.44	21.66 ± 0.51

RESULTS AND DISCUSSION

Antiradical Capacity of Anthocyanins and Portisins. The anthocyanin pigments chosen for this study were malvidin-3-glucoside (Mv3glc) and cyanidin-3-glucoside (Cy3glc) and their respective portisins, vinylpyranomalvidin-3-glucoside (Mv3glc-pt) and vinylpyranocyanidin-3-glucoside (Cy3glc-pt). The anthocyanins were obtained commercially, whereas the portisins were synthesized and purified according to the procedure previously described.[21] The portisins studied arise from the reaction of carboxypyranoanthocyanins with vinylcatechin, yielding structures that possess an additional flavanol moiety (Figure 1). The antiradical properties of all these pigments were studied by means of the DPPH assay, which has been systematically used to assay this particular feature in these kinds of compounds.

The results obtained show that the radical scavenging activity of the pigments increased with the number of hydroxyl groups in the structure of the pigment: Mv3glc < Cy3glc and Mv3glc-pt < Cy3glc-pt (Table 1). This trend is very likely associated with the higher scavenging capacity of the catechol group of Cy3glc and agrees with previous literature.[19] By comparison of the results of portisins versus anthocyanins it was found that the former have higher antiradical capacity, probably due to the presence of an additional catechol group in ring F of these pigment structures, thereby increasing the overall antiradical activity. Portisins are thus anthocyanin-derived pigments that possess an increased antiradical capacity. Nonetheless, their antiradical capacity depends also on factors other than only structural features, namely, conformational issues and steric hindrances, which may impair this phenomenon. That is why the radical scavenging of more complex structures such as portisins would never represent the sum of their individual moieties (anthocyanin and flavanol). All of these results were already anticipated and confirmed in previous assays involving these compounds.[20] The main aim of this work was to establish a putative mechanism for the scavenging radical capacity of portisins. For this purpose, an EPR study was conducted.

EPR Study. EPR experiments were carried out with the two anthocyanins and the two portisins studied as well as with catechin because it constitutes a significant structural part of the overall structure of these portisins. Oxidation of the compounds by $O_2^{\bullet-}$ radicals resulted in the generation of relatively stable free radical products that could be observed for >1 h. Free radical stabilization should occur preferentially on the B rings because of the extended conjugated systems in the anthocyanin moieties or

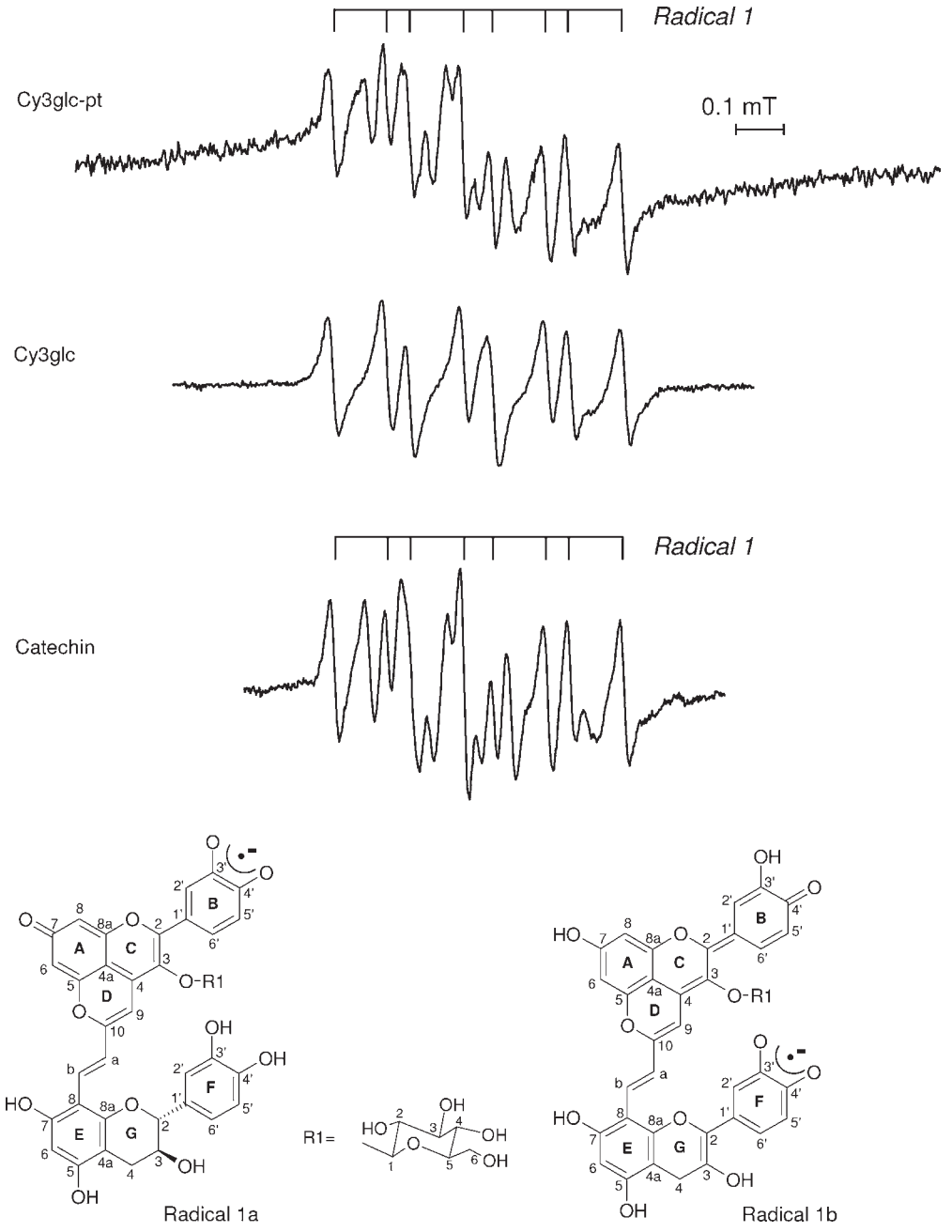

Figure 2. EPR spectra of Cy3glc-pt, Cy3glc, and catechin observed after oxidation with $O_2^{\bullet -}$ along with suggested structures for radicals 1a and 1b.

Table 2. Hyperfine Coupling Constants and *g* Values for the Individual Radicals Detected in Portisins Mv-pt and Cy-pt

radical	$a(^1H)$ (mT)	$a(^1H)$ (mT)	$a(^1H)$ (mT)	$a(^1H)$ (mT)	*g* value
1	0.332	0.160	0.112		2.0051
2	0.125	0.098	0.070		2.0056
3	0.250	0.250	0.225	0.225	2.0053
4	0.203	0.203			2.0051

on the F rings, which have a catechol group (two adjacent OH groups).[25]

The EPR spectrum of Cy3glc-pt after oxidation with $O_2^{\bullet -}$ radicals is characterized by two components (Figure 2). The dominant signal (radical 1) consists of eight peaks with hyperfine coupling (hfc) constants (Table 2) similar to those of the radical produced by oxidation of Cy3glc (Figure 2). Hence, it probably arises from localization of the unpaired electron on the cyanidin moiety on ring B (Figure 2, radical 1a). However, a similar spectrum was also observed when catechin was oxidized by $O_2^{\bullet -}$ radicals (Figure 2). This suggests that oxidation of catechin is likely to result in deprotonation at carbons 2 and 3, yielding a structure similar to that of the cyanidin moiety (Figure 3). As such, we cannot exclude the possibility that part of the spectrum from radical 1 originates from oxidation of the catechin moiety (F ring oxidation) of Cy3glc-pt followed by deprotonation at carbon 2 (Figure 2, radical 1b).

The second signal (radical 2) from oxidized Cy3glc-pt (Figure 4), which was clearly revealed after subtraction of the

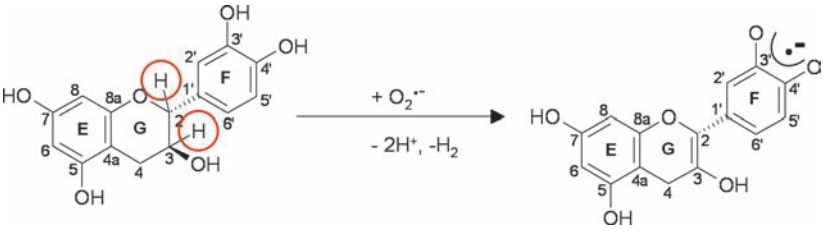

Figure 3. Possible mechanism for the reaction of catechin with $O_2^{\bullet-}$ radicals.

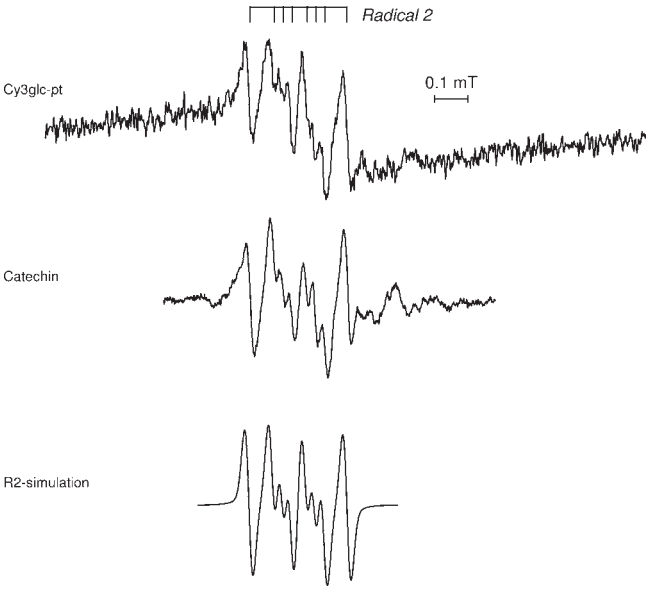

Figure 4. EPR spectra after oxidation with $O_2^{\bullet-}$ of Cy3glc-pt and catechin after subtraction of radical 1. R2-simulation, simulation of the spectrum of radical 2.

spectrum of radical 1, could be simulated with three inequivalent ^{1}H hfc constants (Table 2). Although it has not yet been positively identified, a similar spectrum was revealed when the spectrum of radical 1 was subtracted from that of oxidized catechin (Figure 4). Because this spectrum was not observed when Cy3glc was oxidized, it can be concluded that radical 2 is most likely a reaction product of the oxidized catechin moiety in Cy3glc-pt.

With regard to the EPR spectrum of Mv3glc-pt, three radical components were observed as shown in Figure 5A. One of these corresponds to radical 1b, in which the unpaired electron is localized on the catechin-moiety (F ring) (Figure 5). Hence, as already concluded with Cy3glc-pt, the F ring seems to be an active site for reaction with $O_2^{\bullet-}$ radicals.

After subtraction of the spectrum of radical 1b from the EPR spectrum of Mv3glc-pt, the remaining spectrum (Figure 5A) was dominated by a quintet (radical 3) and a triplet (radical 4). The quintet signal (Figure 5C) could be simulated using two sets of two equivalent ^{1}H splittings with magnitudes similar to those of the *p*-benzosemiquinone radical ($a(^1H)$ = 0.236 mT × 4).[26,27] Although it is not clear how such a radical could be generated from the Mv3glc-pt structure, it seems likely that a derivative of *p*-benzosemiquinone arises from a degradation product of Mv3glc-pt. Unfortunately, this could not be clarified by LC-MS analysis. After subtraction of the quintet signal from Figure 5A, the remaining triplet (Figure 5C) is similar to the spectrum obtained by oxidation of Mv3glc (Figure 5) with $O_2^{\bullet-}$ radicals.

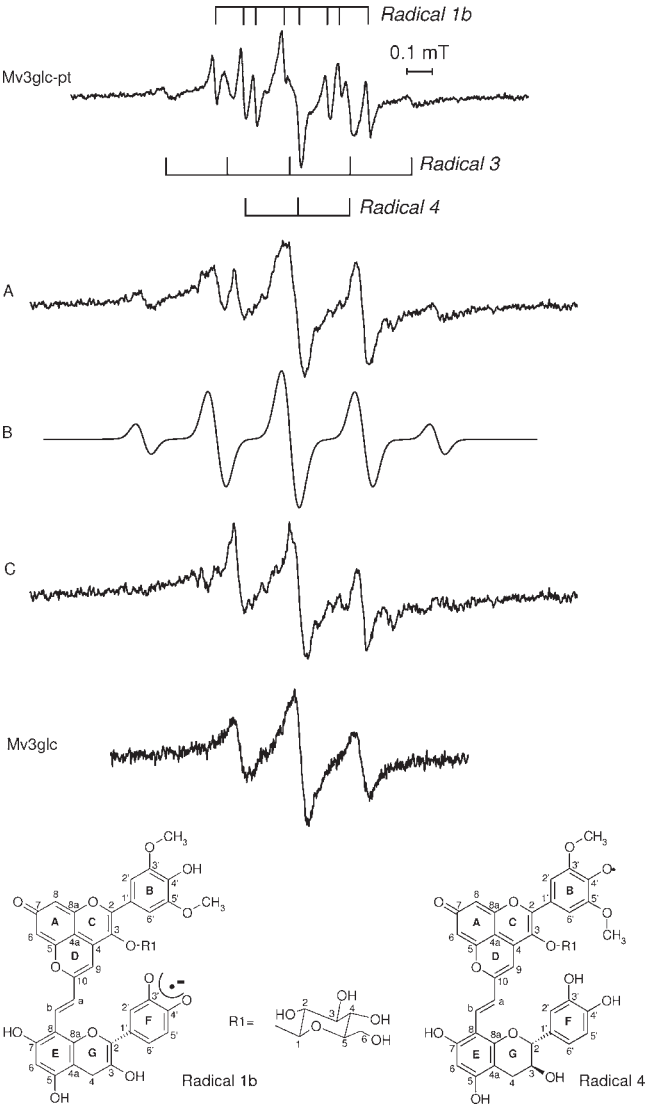

Figure 5. EPR spectra of $O_2^{\bullet-}$ oxidized malvidin-3-glucoside (Mv3glc), Mv3glc-pt, and Mv3glc-pt after subtraction of radical 1 (A). (B) Simulation of the quintet signal in (A). (C) Spectrum A after additional subtraction of the simulated five-peak spectrum (B). Suggested structures for radicals 1b and 4 are also shown.

This suggests that the spectrum arises from a radical in which the unpaired electron is localized on the B ring of Mv3glc-pt (Figure 5, radical 4).

The hfc parameters for all of the radicals described here were confirmed by simulation and are summarized in Table 2. *g* values were all within the range 2.0051−2.0056, which is typical for oxygen-centered free radicals.[28]

Conclusion. The data yielded from the present work show how the enhanced antiradical capacity of portisins, a new family of anthocyanin-derived pigments occurring in red wines, is directly related to the additional flavanol moiety in their overall structure. The EPR experiments performed after reaction of the compounds with $O_2^{\bullet -}$ radicals allowed localizing the position of unpaired electrons in the structure.

These results indicate that localization of the unpaired electron can occur on either ring B or ring F irrespective of the portisin tested. Assignment of spectra to B ring oxidation was confirmed by comparison with results from $O_2^{\bullet -}$ oxidation of malvidin-3-glucoside (in the case of Mv3glc-pt) and cyanidin-3-glucoside (in the case of Cy3glc-pt). Localization of the unpaired electron on the F ring after reaction with $O_2^{\bullet -}$ radicals in both portisins tested was confirmed by comparison of the EPR spectra with the spectrum obtained from the product of a similar reaction with catechin.

The antioxidant chemistry of portisins Mv3glc-pt and Cy3glc-pt is hence complex, and products corresponding to oxidation of both the B and F rings are observed after reaction with $O_2^{\bullet -}$ radicals. Furthermore, the EPR spectra indicate the formation of additional radical reaction products that have not yet been identified. The flavanol moiety thus plays a crucial role in contributing to the antiradical properties of these pigments.

Altogether, these results bring new insights on how portisins may display a much higher antiradical capacity than original anthocyanins, thus contributing to the enhanced antioxidant capacities of red wines.

AUTHOR INFORMATION

Corresponding Author

*E-mail: nbmateus@fc.up.pt. Fax: +351.220402562. Phone: +351.220402659.

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