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Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins

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Anthocyanins are a group of natural occurring pigments responsible for the red-blue color of grapes and many fruits and vegetables. Anthocyanins and derived pigments are of double interest, one technological, as they can be used as natural colorants, and another one due to their implication on human health through their antioxidant activity. Although there are numerous studies regarding the antioxidant activity of grape extracts as well as red wine, the free radical scavenging activity of purified anthocyanins and pyranoanthocyanins is largely unknown. In the present study, the hydroxyl and superoxide anion scavenging activities of anthocyanins and their pyruvic acid adducts were systematically investigated by electron spin resonance spectroscopy and spin trapping. The 3-glucosides of delphinidin, cyanidin, petunidin, pelargonidin and malvidin, and the pyruvic adduct of the 3-glucoside of delphinidin exhibited a potent superoxide anion radical scavenging and, to a lesser extent hydroxyl anion radical scavenging activity. The pyranoanthocyanins of cyanidin, petunidin, malvidin and pelargonidin showed a high capacity to scavenge superoxide anion radicals but did not scavenge hydroxyl radicals. Current data indicate that formation of anthocyanin adducts with pyruvic acid, which may occur during wine ageing or fruit juice processing, decreases the hydroxyl and superoxide anion scavenging and thus could decrease the antioxidant potential of these compounds.

Keywords: Anthocyanins / Antioxidant activity / ESR / Pyranoanthocyanins / Wine

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1 Introduction

Anthocyanins are a group of natural occurring pigments responsible for the red-blue color of many fruits and vegetables. They are glycosides of the anthocyanidins that consist of various polyhydroxy or polymethoxy derivates of 2-phenylbenzopyrylium or flavilium salts [1]. Many red-colored fruits, cereals and vegetables, as well as wine, are the main sources of anthocyanins in the human diet. The intake of anthocyanins in humans has been estimated to be 180–215 mg/day [2] in the United States, which is much higher than other flavonoids (23 mg/day) including quercetin, kaempferol, myricetin, apigenin, and luteolin [3]. Anthocyanins are rapidly absorbed in a very low rate (<1%). They then appear in blood circulation and urine as intact, methylated, glucuro-and/or sulfoconjugate forms. The recovery of anthocyanins and anthocyanins metabolites in urine has been estimated to be in the range 0.004–0.11% [4].

During red wine ageing, there is a loss of anthocyanins and it appears that other pigments, so called vitisins, are formed through the interaction of the original anthocyanins with pyruvic acid [5]. Moreover, other pyranoanthocyanins have been detected in other natural sources such as grape pomace [6], red onion [7], strawberries [8], and more recently in some processed foods such as strawberry and raspberry juices [9]. Pyranoanthocyanins have an additional pyran ring between C4 and the hydroxyl group attached to C5 of the anthocyanin core (Fig. 1). This change in the structure of the anthocyanins molecule has been referred to be responsible for the higher stability compared to that of the original anthocyanins and to influence the red color of wine, fruits and fruit products [10].

Epidemiological studies suggest that a moderate consumption of these anthocyanins may be associated with a protec-

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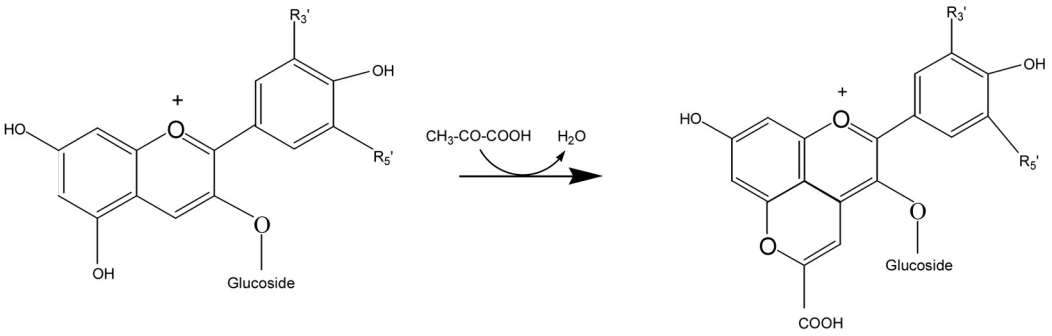

ANTHOCYANINS	PYRANOANTHOCYANINS	R_3'	R_5'
Dp-3-glu	Pyruvic adduct of dp-3-glu	-OH	-OH
Cy-3-glu	Pyruvic adduct of cy-3-glu	-OH	-H
Pt-3-glu	Pyruvic adduct of pt-3-glu	-OH	$-OCH_3$
Pg-3-glu	Pyruvic adduct of pg-3-glu	-H	-H
Mv-3-glu	Pyruvic adduct of mv-3-glu	$-OCH_3$	$-OCH_3$

Figure 1. Reaction of anthocyanins and pyruvic acid to form their corresponding pyranoanthocyanins.

tion against cardiovascular diseases [11], chronic inflammation [12], and cancer [13]. However, although antioxidant properties have been described for wine and fruit extracts, the direct free radical scavenging activity towards reactive oxygen species by the most abundant anthocyanins present in the diet (delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside and malvidin-3-glucoside) has not been systematically studied [14]. Furthermore, even less is known concerning the free radical scavenging activity of some of their corresponding pyranoanthocyanins (pyruvic adducts of delphinidin-3-glucoside, cyanindin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside and malvidin-3-glucoside). The objective of the present work was to investigate the free radical scavenging activity, by means of electron spin resonance, towards reactive oxygen species of anthocyanins in comparison to their pyruvic adducts.

2 Materials and methods

2.1 Samples preparation

2.1.1 Test components

Anthocyanins (delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside and malvidin-3-glucoside) were isolated from different methanol-acid extracts and their corresponding pyranoanthocyanins synthesized from the anthocyanins.

2.1.2 Extracts preparation

Delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside were isolated from red grape skin extracts and cyanidin-3-glucoside from red plum skin extracts. Grape and plum skins were macerated in methanol containing 5% of 1 N HCl; the methanol extracts were evaporated under vacuum and the aqueous phases obtained washed with *n*-hexane to remove liposoluble substances.

Pelargonidin-3-glucoside was isolated from strawberry extract. Strawberries were homogenized in 0.1% HCl in methanol and later filtered through a Buchner funnel under vacuum. After addition of water, the supernatant was concentrated under vacuum to total evaporation of the methanol and the aqueous extract obtained was washed with *n*-hexane to remove liposoluble substances.

2.1.3 Isolation and purification of anthocyanins

Anthocyanins were isolated from the aqueous extracts obtained as described above, by semi-preparative HPLC using a Waters 600 chromatograph. The column was an

Ultracarb ODS, 5 μm, 250 × 10.00 mm (Phenomenex). Detection was carried out at 520 nm. Solvents used were 5% acetic acid (A) and methanol (B) at a flow rate of 3 mL/min. The gradient used to isolate dp-3-gluc, pt-3-glu and mv-3-gluc was 10–15% B over 15 min, isocratic 15% B for 20 min, 15–20% B over 10 min, 20–25% B over 10 min, 25–30% B over 10 min, 30–45% B over 10 min.

Isocratic 10 % B for 5 min, 10–14% B over15 min, 14–15% B over 5 min, 15–18% B over 5 min, 18–35% B over 20 min was established to isolated pg-3-glu and isocratic 10% B for 5 min, 10–15% B for 25 min, isocratic 15% B for 5 min, 15–50% B over 5 min was applied for cy-3-glu.

2.1.4 Synthesis and purification of pyranoanthocyanins

Synthesis of pyranoanthocyanins was performed as described previously [15]. Pyruvic acid was added to pure anthocyanin, dissolved in potassium hydrogen tartrate buffer containing 10% ethanol, in a molar ratio of pyruvic acid to pure anthocyanins of 300:1. The pH was adjusted to 3.7 by addition of Na_2CO_3 and the solution was incubated at 32°C in the dark in the presence of air. After pyranoanthocyanin synthesis, the solution was passed through an Ultracarb ODS, 5 μm, 250 × 10.00 mm (Phenomenex), using 100% of water as solvents. The column was initially washed with water for 15 min at a flow rate of 3 mL/min. The pigments were eluted with 5% acetic acid and methanol using the gradient that is mentioned above for their corresponding anthocyanins.

The identity of the pyranoanthocyanins was confirmed by LC-MS analysis and the kinetic of formation was followed by HPLC analysis as described below.

2.1.5 Anthocyanins and pyranoanthocyanins purity

The purity of anthocyanins and pyranoanthocyanins was tested by HPLC analysis following the method of de Pascual-Teresa *et al*. [16] with slight modifications. Briefly, a Hewlett-Packard 1100 HPLC equipment was used with a quaternary pump and a photodiode array detector. The column was an Aqua® C18, 5 μm (150 × 4.6 mm) (Phenomenex) thermostated at 350C. Solvents were 0.1 % TCA (A) and ACN (B) with the following gradient: isocratic 10% B for 5 min, from 10 to 15% B over 15 min, isocratic 15% B for 5 min, from 15 to 18% B over 5 min, and from 18 to 35% B over 20 min at a flow rate of 0.5 mL/min. The detection was made in the photodiode apparatus, selecting 520 nm as the preferred wavelength.

The identity of the synthesized pyranoanthocyanins was confirmed by HPLC using a dual on-line detection by diode array spectrophotometry and MS (HPLC-DAS-MS). MS spectrometry was performed using a Finningan LCQ equipped with API surface, using an ESI interface. The HPLC system was connected to the probe of the mass spectrometer via the diode array detector cell outlet, using a polyethyletherketone (PEEK) tubing. Both the auxiliary and the sheath gas were a mixture on nitrogen and helium at flow rates of 1.2 and 6 L/min, respectively. The capillary temperature was 195°C and the capillary voltage was 4 V. The MS detector was programmed to perform a series of two consecutive scans: a full scan from 120 to 1500 amu and an MS–MS scan of the most abundant ion in the full mass. The normalized energy of collision was 45%. Spectra were recorded in the positive ion mode.

2.2 Antioxidant capacity: Electron spin resonance spectral studies

2.2.1 General remarks

Electron spin resonance (ESR) spin trapping studies were carried out on a JEOL JES-TE 200 spectrometer (X-band microwave unit). Experiments were conducted in duplicate at 23°C.

2.2.2 Superoxide radical scavenging activity

For the ESR spin trapping study, superoxide scavenging activity of antioxidants was carried out in 600 μL solution in a microtest tube containing 160 μL of 2 mM hypoxanthine solution in 100 mM phosphate buffered solution, 20 μL of 10.5 M DMPO solution, 160 μL of 0.4 U/mL xanthine oxidase solution in re-distilled water, 60 μL of 10 mM DTPA (diehtylenetetramine-N,N,N′,N″,N″-pentaacetic acid) in re-distilled water, 100 μL of re-distilled water and 100 μL of the sample solution. The reaction was started by the addition of the xanthine oxidase solution. After stirring for 5 s, 50 μL of the reaction mixture was transferred to the disposable capillary tube. ESR spectra were measured 2 min after the addition of the xanthine oxidase solution. ESR spectral settings were as follows: central field 325 ± 5 mT; microwave frequency 9.00 GHz; modulation amplitude 0.1 mT; microwave power 8.00 mW; time constant 0.3 s; gain 5.0×10^5; sweep time 0.5 min; and scan width 100 G.

2.2.3 Hydroxyl radical scavenging activity

For the ESR spin trapping study, the Fenton reaction system was carried out in 500 μL aqueous solution in a microtest tube containing 50 μL of 5 mM H_2O_2, 50 μL of 100 mM DMPO (5,5-dimethyl-1-pyrroline-N-oxide), 150 μL of re-distilled water, 150 μL of 50 mM phosphate buffered solution (pH 7.4), 50 μL of the test compound and 50 μL of 0.5 mM freshly prepared ferrous chloride solution. Metal-free water was used instead of test compounds for the control

experiment. The Fenton reaction was started by adding the aliquot of $FeCl_2$ solution. After stirring for 5 s, 50 μL of the reaction mixture was transferred to the disposable capillary tube and the tube was sealed by the small amount of the clay. The ESR spectra were measured 2 min after the addition of $FeCl_2$. ESR spectra setting were as follows: central field 325 ± 5 mT; microwave frequency 9.00 GHz; modulation amplitude 0.2 mT; microwave power 8.00 mW; time constant 0.3 s; gain 5.0×10^5; sweep time 0.5 min; and scan width 100 G.

3 Results and discussion

3.1 Isolation, synthesis and identification

Anthocyanins-derived pigments constitute a group of compounds mostly associated with anthocyanins transformation taking place during the maturation and ageing of red wines, resulting in the formation of more stable pigments that stabilize wine color. Recent data indicate that certain anthocyanin-derived pigments occur, not only in red wine, but also in small amounts in plants and plant products [17].

The isolation of anthocyanin-derived pigments, generally called pyranoanthocyanins, has proved difficult, especially because their levels are much lower than those of original anthocyanins in the natural sources are and because pyranoanthocyanins usually co-elute with their corresponding anthocyanins in the chromatographic conditions normally used in wine analysis [5]. Due to the difficulty of isolating the compounds of interest, the pyranoanthocyanins tested in this study have been synthesized from their corresponding anthocyanins previously isolated from natural sources.

Pyranoanthocyanins are formed through the interaction between the anthocyanins and pyruvic acid. The extent to which these reactions occur has been found to depend on anthocyanin composition, pH, and pyruvic acid concentration. In addition, temperature has an influence oin the formation of these pigments, which are formed most rapidly at high temperature [15]. Taken this into account the formation of each pyranoanthocyanin in the current study was performed in model solutions at a molar ratio of pyruvic acid to pure anthocyanin of 300:1. The pH value (3.7) and temperature (32°C) were controlled and adjusted to optimize the synthesis of the compounds of interest. Due to the co-elution of the anthocyanin and its corresponding pyranoanthocyanin, the kinetic of formation of pyranoanthocyanins was monitored by the spectral analysis recorded from the HPLC diode array detector. The visible maximum of the original anthocyanins ($\lambda_{max} \approx 520$ nm), was shifted to a shorter wavelength ($\lambda_{max} \approx 507$ nm) and another absorption maximum around 370 nm, which is characteristic of C4-substituded anthocyanins, was observed (Fig. 2) [18].

After the pyranoanthocyanin was formed, it was purified by preparative HPLC. The solution containing the products of reaction was passed through an Ultracarb ODS column where the pigments are retained. The column was initially washed with water to eliminate the excess of pyruvic acid that did not react with the anthocyanins. The pigments were then eluted with 5% acetic acid and methanol using an elution gradient as described in the materials and methods section.

The identities of the pyranoanthocyanins synthesized, vitdp-3-glu, vitcy-3-glu, vitpt-3-glu, vitpg-3-glu and vitmv-3-glu were confirmed by LC-MS, which spectra showed (Fig. 3) molecular ions at *m/z* 533, 517, 547, 501 and 561, respectively. In their MS/MS spectra, major fragments appeared at *m/z* 371, 355, 385, 339, 399, respectively (–162 amu, loss of a glucose moiety), corresponding to the aglycons of the corresponding pyranoanthocyanins.

The purities of all anthocyanins and pyranoanthocyanins test components used in this study, were in every case higher than 95% as tested by HPLC analysis.

3.2 Antioxidant activity

3.2.1 Superoxide radical scavenging

Superoxide anion radicals were generated by the xanthine/xanthine oxidase reaction. A typical ESR spectrum of a DMPO–OOH spin adduct is shown in the insert of Fig. 4. All anthocyanins test components exhibited a potent superoxide anion radical scavenging activity, similar to that of quercetin, which due to its well-known free radical scavenging activity is frequently used as positive control in ESR experiments [19, 20]. Anthocyanins were tested in this work at concentration 6.7–33.3 μM. Taking into account the IC_{50}, concentration necessary for causing a 50% of inhibition of the ESR peak height, the superoxide anion scavenging activity decreases in the order mv-3-glu (slightly <13.3 μM) > dp-3-glu (slightly > 13.3 μM) > pt-3-glu (>13.3 μM) > pg-3-glu (≅ 20 μM) > cy-3-glu (>33.3 μM). This ranking is followed when the test compounds were tested at low concentration (6.7 and 13.3 μM). However, when mv-3-glu, pt-3-glu, dp-3-glu and pg-3-glu were analyzed at higher concentrations (20–33.3 μM) they reached a similar potency regarding their superoxide anion scavenging activity (Fig. 4). This finding seems to be of physiological importance since human and animal studies have demonstrated that the absorption rate of anthocyanins depends on the different anthocyanidins and glycoside patterns. For instance, the apparent absorption rate of pg-3-glu is almost eight times higher than that of cy-3-glu [21]. The structural difference among the anthocyanins tested is the substitution in the 3′- and 5′-position in the B ring. Mv-3-glu, dp-3-glu and pt-3-glu, which showed the lowest IC_{50} in

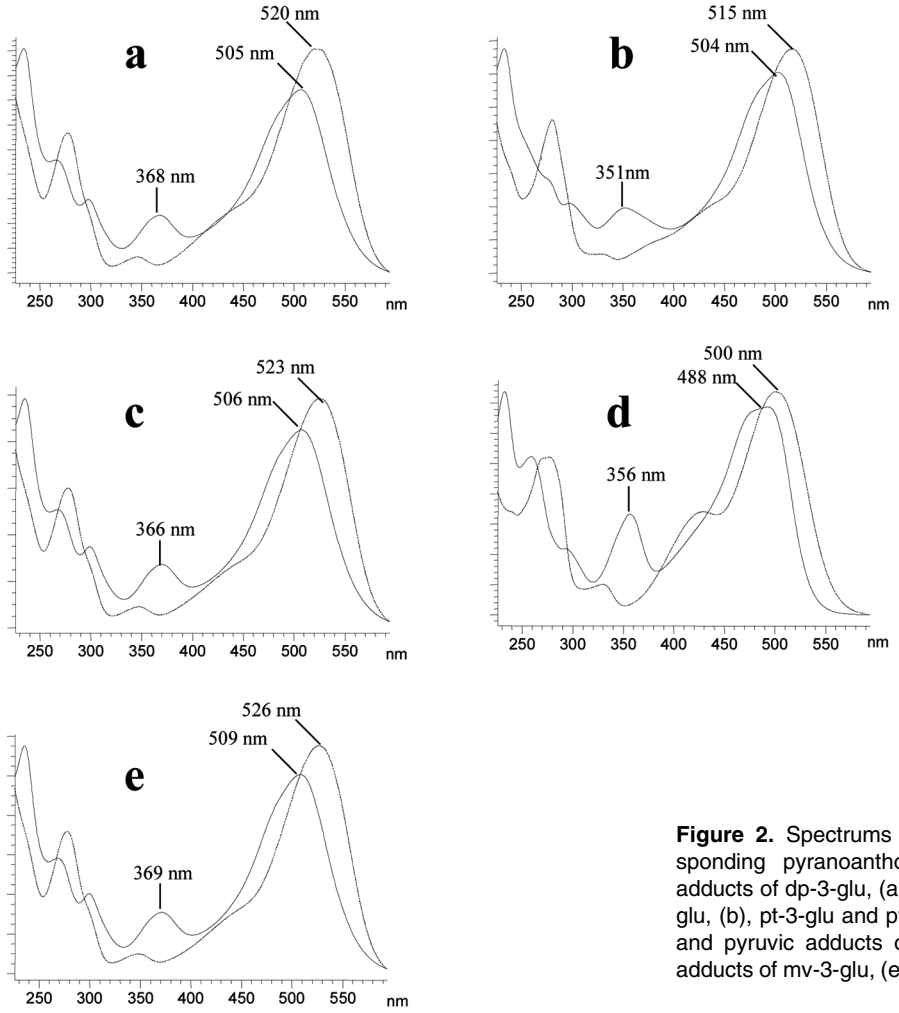

Figure 2. Spectrums of anthocyanins (⋯) and their corresponding pyranoanthocyanins (—): dp-3-glu and pyruvic adducts of dp-3-glu, (a); cy-3-glu and pyruvic adducts of cy-3-glu, (b), pt-3-glu and pyruvic adducts of pt-3-glu, (c), pg-3-glu and pyruvic adducts of pg-3-glu, (d), mv-3-glu and pyruvic adducts of mv-3-glu, (e).

this work, are among the anthocyanins studied that presented both positions occupied. Mv-3-glu, with two methoxyl and one hydroxyl group was the most potent component at all concentrations tested, followed by pt-3-glu, with one methoxyl and two hydroxyl groups in the B ring or by dp-3-glu, the only compound studied that contains the 3′, 4′ and 5′hydroxy groups (gallocatechol structure) in the B ring. The lowest superoxide anion scavenging activity in this assay was that of pg-3-glu, without substitution in 3′- and 5′-position, and cy-3-glu, with only one hydroxyl group in 3′position. This indicates that both, the nature and the rate of substitution of the B ring, are important determinants for the radical scavenging potential of anthocyanins.

Among the pyranoanthocyanins tested the pyruvic adduct of delphinidin-3-glucoside was the most potent scavenger of superoxide radicals ($IC_{50} \cong 33.3$ μM), followed by the pyruvic adducts of pg-3-glu ($IC_{50} \cong 333.$ μM) ≅ pt-3-glu ($IC_{50} > 333.3$ μM), > mv-3-glu and that of cy-3-glu ($IC_{50} >>$ 333.3 μM), which exhibited a lower capacity than the flavonol quercetin (Fig. 5). The relative orders, from greater to lesser superoxide scavenger capacity among the pyranoanthocyanins tested, is the same as their corresponding anthocyanins, with the exception of the pyruvic adducts of pg-3-glu and mv-3-glu, which have their ranking position changed.

Importantly, anthocyanins have a higher superoxide anion radical scavenging capacity than the group of pyranoanthocyanins (Figs. 4 and 5). Only the pyruvic adduct of dp-3-glu exhibited a superoxide anion radical scavenging activity, which was similar to those of the anthocyanins. However, the superoxide anion radical scavenging capacity exhibited by dp-3-glu was higher than that of its corresponding pyruvic adduct. Thus, the incorporation of an additional C_3O_2 group in position 4 and 5 of the molecule decreases the ability of pyranoanthocyanins to scavenge superoxide radicals. This decrease in superoxide anion scavenging activity may be due to the lack of the 5 hydroxyl group in the pyranoanthocyanin molecule, which together with the 7 hydro-

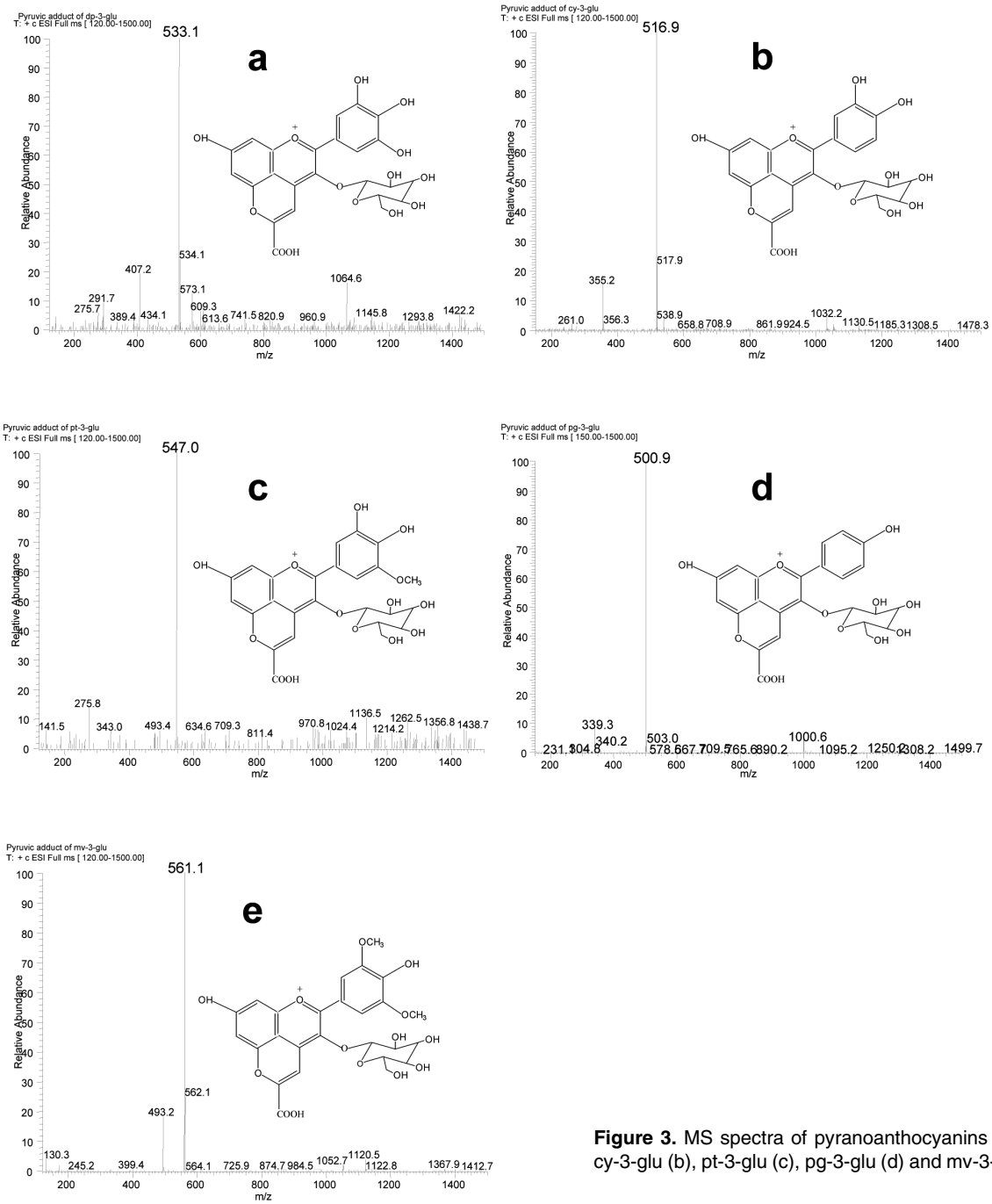

Figure 3. MS spectra of pyranoanthocyanins of dp-3-glu (a), cy-3-glu (b), pt-3-glu (c), pg-3-glu (d) and mv-3-glu (e).

xyl group contributes to the antioxidant activity of anthocyanins [22].

3.2.2 Hydroxyl radical scavenging activity

In the Fenton system containing H_2O_2 and Fe^{2+}, the typical ESR signal of DMPO/OH adduct, consisting of a 1:2:2:1 quartet with splitting constants of aN=aN = 14.9 G, was observed in the presence of DMPO (Fig. 6). Anthocyanins and pyranoanthocyanins were tested at concentration 100—1000 μM. Among the anthocyanins assayed only malvidin-3-glucoside presented a potency lower than the positive control, quercetin. The hydroxyl scavenging activity decreased in the order pg-3-glu > dp-3-glu > pt-3-glu > cy-3-glu > mv-3-glu at the highest concentrations used in this assay (400–1000 μM). However, dp-3-glu was a more potent hydroxyl scavenger than pg-3-glu at the lowest concentrations tested (100–200 μM).

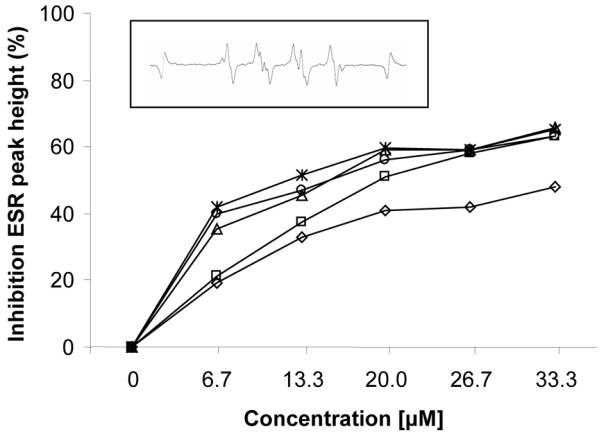

Figure 4. Scavenging effects of anthocyanins on superoxide radical generated by xanthine/xanthine oxidase reaction: dp-3-glu (–○–), cy-3-glu (–◇–), pt-3-glu (–△–), pg-3-glu (–□–), mv-3-glu (–*–). A typical ESR spectrum of DMPO-OOH is shown in the inset. Data are expressed as percentage of inhibition of ESR peak. Values are means of two independent experiments.

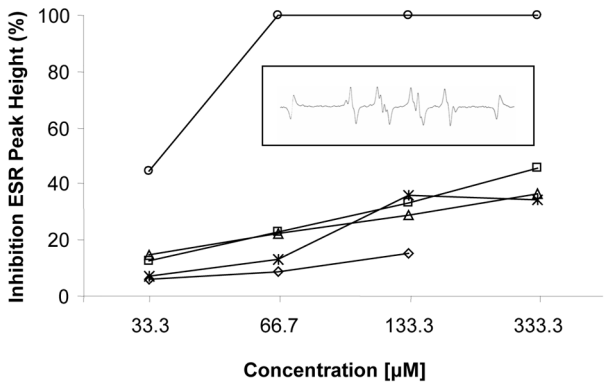

Figure 5. Scavenging effects of adduct of anthocyanins on superoxide radical generated by xanthine/xanthine oxidase reaction: adduct of dp-3-glu (–○–), adduct of cy-3-glu (–◇–), adduct of pt-3-glu (–△–), adduct of pg-3-glu (–□–), adduct of mv-3-glu (–*–). A typical ESR spectrum of DMPO-OOH is shown in the inset. Data are expressed as percentage of inhibition of ESR peak. Values are means of two independent experiments.

Among the pyranoanthocyanins studied, the pyruvic adduct of delphinidin-3-glucoside was the only one which scavenged hydroxyl radicals at all concentrations tested (100–1000 µM). The pyruvic adduct of delphinidin-3-glucoside showed an inhibition of 53% followed by the pyruvic adducts of pelargonidin-3-glucoside (30%) and malvidin-3-glucoside (21%) at 1000 µM. The other pyranoanthocyanins tested (petunidin-3-glucoside and cyanidin-3-glucoside derivatives) did not affect the signal of the DMPO/•OH adduct at concentrations up to 1 mM.

In the current study, all compounds tested showed a more powerful effect in scavenging superoxide anion radical than

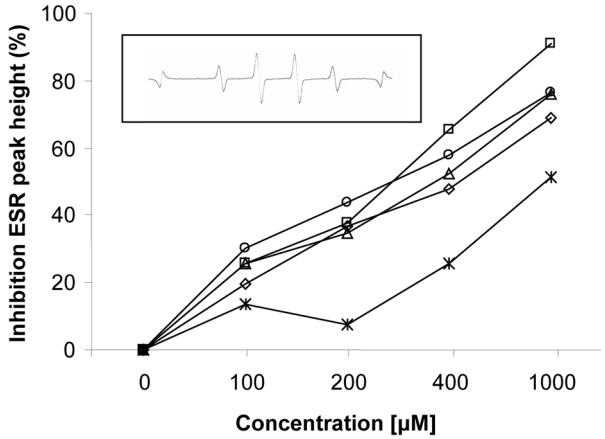

Figure 6. Scavenging effects of anthocyanins on hydroxyl radical generated in the Fenton system: dp-3-glu (–○–), cy-3-glu (–◇–), pt-3-glu (–◇–), pg-3-glu (–□–), mv-3-glu (–*–). A typical ESR spectrum of DMPO-OH is shown in the inset. Data are expressed as percentage of inhibition of ESR peak. Values are means of two independent experiments.

hydroxyl radical. This result is in agreement with data by Nam *et al*. [23], who found that pigmented rice extracts, which contain, among other polyphenols, anthocyanins (glycosides of cyanidin and peonidin), scavenged superoxide anion radicals more effectively than hydroxyl radicals. Some authors have hypothesized that in the case of nasunin, a delphinidin derivative present in eggplant, the hydroxyl scavenging activity is not due to direct scavenging but inhibition of the hydroxyl radical generating system by chelating ferrous ion. However, our data demonstrate that pelargonidin-3-glucoside, which has no ortho-dihydroxy substitution in the B ring, and has been reported to have no metal chelating capacity [24, 25], exhibited similar or even higher activity than delphinidin-3-glucoside and cyanidin-3-glucoside, which possesses gallocatechol and catechol structures, and thus chelates iron [26].

4 Concluding remarks

In summary, glucosides of delphinidin, cyanidin, petunidin, pelargonidin and malvidin, and the pyranoanthocyanin of the glucoside of delphinidin are potent superoxide anion radical scavengers and to a lesser extent hydroxyl anion radical scavengers. The other pyranoanthocyanins tested in this work (pyruvic adducts of cyanidin, petunidin, malvidin and pelargonidin) showed a high capacity to scavenge superoxide radical but a very low, or null in the case of the pyruvic adducts of petunidin and cyanidin, capacity to scavenge hydroxyl anion radicals. Current data indicate that the hydroxyl and superoxide anion scavenging may contribute to the reported antioxidant activity of anthocyanins. However, formation of anthocyanin adducts with pyruvic

acid, which for instance may occur during wine ageing or fruit juice processing, decreases the hydroxyl and superoxide anion scavenging and, thus, could decrease the antioxidant potential of these compounds.

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