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Antioxidant properties of anthocyanidins, anthocyanidin-3-glucosides and respective portisins

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ABSTRACT

The study of the antioxidants properties of three classes of pigments, namely anthocyanidins (cyanidin-Cy, delphinidin-Dp and malvidin-Mv), anthocyanidin-3-glucosides (Cy-3-gluc, Dp-3-gluc and Mv-3-gluc), and portisins (Cy-pt, Dp-pt and Mv-pt) was carried out. The aim was to evaluate the relationship between the structure antioxidant properties of individual anthocyanins and respective derivative pigments. The ability of these compounds to inhibit lipid peroxidation in a liposome membrane system was examined by monitoring oxygen consumption and the antiradical and reducing capacities were determined using the DPPH and FRAP assay, respectively. All compounds tested showed antiradical and reducing properties. These features seemed to increase with the presence of catechol and pyrogallol groups in ring B of anthocyanidin-3-glucosides and respective aglycones. The results obtained for portisins are very likely to be related with their unique structural features. The flavanol moiety of the compounds structure seems to be crucial for the antiradical properties, whilst their reducing ability was only evident for the portisin derived from malvidin. This outcome could be due to some of the structural features of these pigments as they are complex structures and may have several different conformations in solution. The antioxidant protection towards lipid peroxidation increased with the overall hydrophobicity of the compounds. This feature is related to the location of the antioxidant on the liposome surface vs. water phase. Among the pigments tested, portisins showed the higher effect in preventing lipid peroxidation of soybean phosphatidylcholine liposomes, especially the portisin derived from Cy. This outcome could be due to the presence of two *o*-catechol groups in the structure of this compound.

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1. Introduction

Pigments derived from natural sources, display a wide range of colours and are usually regarded as safe. Among those pigments, anthocyanins assume an important role when dealing with natural colourants. These polyphenolic compounds constitute the largest group of water-soluble pigments throughout the plant kingdom. These natural pigments are usually associated with red fruits, but are present also in vegetables, roots and cereals (Mazza & Miniati, 1993).

In addition to their colour features, anthocyanins have more interest due to their possible health attributes, such as a reduced risk of coronary diseases, reduced risk of stroke, anticarcinogen activity, anti-inflammatory effects and improved cognitive behaviour (Cao, Sofic, & Prior, 1997; Clifford, 2000; Prior, 2003; Scalbert & Williamson, 2000; Wang, Cao, & Prior, 1997).

The consumption of anthocyanins reported in the literature is around 200 mg per day in the US (Kühnau, 1976). These values are higher for frequent red wine consumers. However, despite not being the best source of anthocyanins compared to fruit berries, anthocyanins from red wine may complex with other wine components, thereby their consumption levels will be significantly higher than initially expected (Timberlake, 1988). Also, anthocyanins may undergo several chemical transformations in red wine, yielding new classes of anthocyanin-derived pigments. These include, pyranoanthocyanins, carboxypyranoanthocyanins and vinylpyranoanthocyanin-catechins (portisins) that have been detected in red wine (Bakker & Timberlake, 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Mateus, Pascual-Teresa, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2002; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & Freitas, 2003). These compounds are unusual in colours (orange and blue) and they are very stable because of their structural properties. These compounds also show great resistance to colour bleaching by sulfur dioxide, and are intensely coloured at higher pH values,

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compared to their anthocyanin precursors (Oliveira, Santos-Buelga, Silva, Freitas, & Mateus, 2006). These newly-formed pigments appear to be challenging as well as profitable. Several research groups have studied the identification and characterisation of new naturally-occurring pigments from different natural sources, such as red wine, to use them as food colourants. The novel colours of these classes of anthocyanin derivatives have been reported, their antioxidant properties have only been briefly studied. Some investigations have, however, focused on anthocyanin-derived pigments (Faria et al., 2005).

Several methods have been developed to measure the efficiency of dietary antioxidants either as pure compounds or in food matrixes. These methods focus on the different mechanisms of the antioxidant defense system, such as scavenging of oxygen and hydroxyl radicals, reduction of lipid peroxyl radicals, inhibition of lipid peroxidation or chelation of metal ions (Halliwell, Aeschbach, Loliger, & Aruoma, 1995; Halliwell & Whiteman, 2004). Nevertheless, the use of LDL or liposomes constitute a more promising method of assessing antioxidant properties relevant to human nutrition (Storm & Crommelin, 1998), since these systems allow the investigation of the protection of a determined substrate by an antioxidant in a model biological membrane or a lipoprotein.

The aim of this work was to study the antioxidant properties of anthocyanidins (malvidin, delphinidin and cyanidin), respective anthocyanin monoglucosides and portisins, and to assess structural–activity relationships. The antiradical and reducing properties of these pigments were evaluated using DPPH and FRAP assays, respectively. The activity against lipid peroxidation was determined using soybean phosphatidylcholine liposomes as a membrane model system. The extension of membrane lipid oxidation was followed by measuring the oxygen consumption.

2. Materials and methods

2.1. Reagents

AAPH, DPPH, $FeCl_3$, DMSO, Trolox, Hepes, NaCl and soybean L-α-phosphatidylcholine were purchased from Sigma–Aldrich (Madrid, Spain). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka (Madrid, Spain).

2.2. Anthocyanidins and anthocyanidin-3-glucosides

Malvidin, cyanidin and delphinidin were purchased from Extrasynthese® (Genay, France). The anthocyanidin-3-glucosides were isolated from red fruits. Delphinidin-3-glucoside and malvidin-3-glucoside were isolated from red grape skins while cyanidin-3-glucoside was isolated from blackberries (*Rubus fruticosus*). The anthocyanins were extracted with an aqueous solution methanol/water to 50% (v/v) acidulated with HCl for 24 h at room temperature (Oliveira et al., 2006). Then, the extract was filtered by gravity, with nylon membranes, resulting in an extract rich in anthocyanins. The resulting solution was concentrated by rotary evaporator under vacuum at a temperature of 30 °C in water. Samples were purified on a polyamide gel column (Mesh 100–120). The anthocyanidin-3-glucosides fraction was eluted with a methanol 10% (v/v) aqueous solution. The fractions were concentrated in a rotary evaporator under vacuum in water, freeze-dried and stored at −20 °C until further purification.

2.3. Synthesis of vinylpyranoanthocyanidin-3-glucoside-catechins (portisins)

These new pigments were prepared through a reaction of anthocyanin–pyruvic acid adducts with catechin and acetaldehyde according to the procedure reported (Mateus, Oliveira, Santos-Buelga, Silva, & Freitas, 2004). The anthocyanin–pyruvic acid adducts were synthesised as previously described (Oliveira et al., 2006).

2.4. Isolation of anthocyanidin-3-glucosides and portisins by preparative HPLC

The pigments were isolated by preparative HPLC (Knauer K-1001) with a Purospher® C-18 reversed-phase column 250 mm × 25 mm i.d.; detection was carried out at 520 nm using a UV–vis detector (Merck® Hitachi L-2420). The injection volume was 2 ml. The solvents for the isolation of the anthocyanidin-3-glucosides were as follows, A: H_2O/HCOOH (90:10) and B: HCOOH/MeOH/H_2O (10:50:40). The gradient consisted in a linear gradient from 65% to 15% of A in 70 min at a flow rate of 10 ml/min. The column was washed with 100% of B during 20 min and then stabilised with the initial conditions for another 20 min. The solvents for the isolation of the portisins were, A: H_2O/HCOOH (90:10) and B: 2.5% Acetic Acid/MeOH (20:80). The injection volume was 2 ml and the gradient was 40–5% of B in 50 min at a flow rate of 10 ml/min. The column was washed with 100% of B during 20 min and then stabilised with the initial conditions for another 20 min. The purity of all isolated compounds was confirmed by HPLC-DAD and NMR. The isolated compounds were then frozen, freeze-dried and stored at −20 °C until use.

2.5. Radical scavenging assay (DPPH)

Following the method described in the literature (Bondet, Brand-Williams, & Berset, 1997) with some modifications, radical activities were determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a free radical. The tested compound reacted with DPPH and decreased the absorbance measured at 515 nm, which indicated the scavenging potential of the compounds. As all pigments tested absorbed at 515 nm, previous control assays were performed with all the compounds in order to subtract their contribution at this absorbance. 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 hundred and seventy micro liters of this solution was added in each well together with 30 μl of antioxidant. The compounds tested were a final concentration of 10 μm. The decrease in absorbance was measured at 515 nm, at $t = 0$ and every 10 min, for 30 min. For the final analysis, the 0–20 min reaction time range was used. Antiradical activity was expressed as μm Trolox equivalents. The antiradical activity was calculated from the equation determined from linear regression after plotting known solutions of Trolox with different concentrations.

2.6. Ferric reducing antioxidant power (FRAP)

The FRAP assay developed by Benzie & Strain (Benzie & Strain, 1996) was performed with some modifications. The reaction 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 37 °C. In short, FRAP reagent (10 vol of 300 mm acetate buffer, pH 3.6 + 1 vol of 10 mm TPTZ in 40 mm HCl + 1 vol of 20 mM $FeCl_3$) was diluted to one-third with acetate buffer. Two hundred and seventy micro liters of this solution was added in each well together with 30 μl of compound. The blank assay was performed using 270 μl of FRAP reagent and 30 μl of methanol. The compounds to be tested were dissolved in methanol and used in a final concentration of 10 μm. The absorbance at 593 nm was measured in time 0 and 4 min. The results were expressed as Trolox equivalents.

2.7. Liposome preparation

Liposomes were prepared by evaporation to dryness of L-α-phosphatidylcholine (PC) from soybean solution in chloroform with a stream of argon; the film was then left under vacuum over 3 h to remove all traces of the organic solvent. The resulting dried lipid film was dispersed with Hepes buffer (10 mM Hepes, 0.1 M NaCl, pH 7.4), and then the mixture was shaken above the phase transition temperature to produce multilamellar liposomes (MLV). Frozen and thawed MLVs were obtained by repeating the following cycle five times: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 °C. Lipid suspensions were equilibrated at 37 °C for 30 min and extruded 10 times through polycarbonate filters of 100 nm pore size in a 10 ml stainless steel extruder to form large unilamellar vesicles (LUV) (Rodrigues, Gameiro, Reis, Lima, & Castro, 2001).

2.8. Oxygen consumption

Lipid peroxidation of soybean LUVs were induced by peroxyl radicals generated at a constant rate, by thermal degradation of the azo compound AAPH in the presence or absence of antioxidants and followed by measuring the oxygen consumption. The rate of oxygen consumption was measured continuously with a Clark-type oxygen electrode (Hansatech®) provided with an automatic recording apparatus. The reaction mixture containing 1350 μl of Hepes buffer, 150 μl de LUV (340 μM final concentration) and 2 μl of the antioxidant (1 mM initial concentration) tested, dissolved in methanol, was left in a 37 °C thermostated bath for 1 h. This mixture was introduced in a closed glass vessel, protected from light, thermostated at 37 °C, and provided with a stirrer, and the reaction was started after the addition of AAPH (10 mM final concentration). (Porto, Laranjinha, & Freitas, 2003). The induction periods of the compounds were determined graphically from the profiles of oxygen consumption, by the coordinates of the interception of tangents to the inhibited and uninhibited rates of oxidation (Faria et al., 2005). Results were expressed relatively to those obtained with Trolox.

2.9. Statistical analysis

All tests were conducted at least in quadruplicate. Values are expressed as the arithmetic means ± standard deviation. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni test, using GraphPad Prism 5 software, version 5.01, GraphPad Software, Inc. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. General

The antioxidant features of three classes of anthocyanin-derived pigments were tested, including anthocyanidins (cyanidin-Cy, delphinidin-Dp and malvidin-Mv), anthocyanidin-3-glucosides (Cy-3-gluc, Dp-3-gluc and Mv-3-gluc), and portisins (Cy-pt, Dp-pt and Mv-pt). The structural differences of these three anthocyanidins are due to the hydroxylation and methoxylation pattern of ring B (Fig. 1). The anthocyanidin-3-glucosides have a glucose moiety linked at the OH group of ring C and the portisins studied herein arise from the reaction of carboxypyranoanthocyanins with vinylcatechin yielding more complex structures that possess an additional flavanol moiety (Fig. 1). The antioxidant properties of all these pigments were studied using three different in vitro techniques: DPPH assay, FRAP assay and monitoring oxygen consumption during lipid peroxidation of soybean PC liposomes. In addition to these polyphenolic compounds, the antioxidant activity of Trolox (water-soluble analogue of Vitamin E) was evaluated. The results were expressed in terms of ratio between these pigments and Trolox antioxidant capacities.

3.2. DPPH

In general, the radical scavenging activity of the pigments increased with the number of hydroxyl groups present in the structure of the pigment. As shown in Fig. 2, the results obtained for the anthocyanidins and anthocyanidin-3-glucosides revealed that the antiradical capacity increased in the following order: Mv, Mv-3-gluc < Cy, Cy-3-gluc < Dp, Dp-3-gluc. This trend is very likely associated with the higher scavenging capacity of the catechol and pyrogallol groups of Cy and Dp pigments and agrees with previous literature reported (García-Alonso et al., 2005). In general, the DPPH assay results obtained for anthocyanidin-3-glucosides were in accordance with previous observation on the effects of hydroxylation and metoxilation in ring B of these compounds to their radical scavenging ability in aqueous phase (Kähkönen & Heinonen, 2003; Rice-Evans, Miller, & Paganga, 1996; Wang et al., 1997).

In the case of portisins, this trend was not observed. Overall, portisins were found to have higher antiradical capacity than the other pigments, probably due to the presence of an additional catechol group in ring F of these pigments structures. As already referred, flavonoid structures that bear *ortho*-dihydroxyl groups

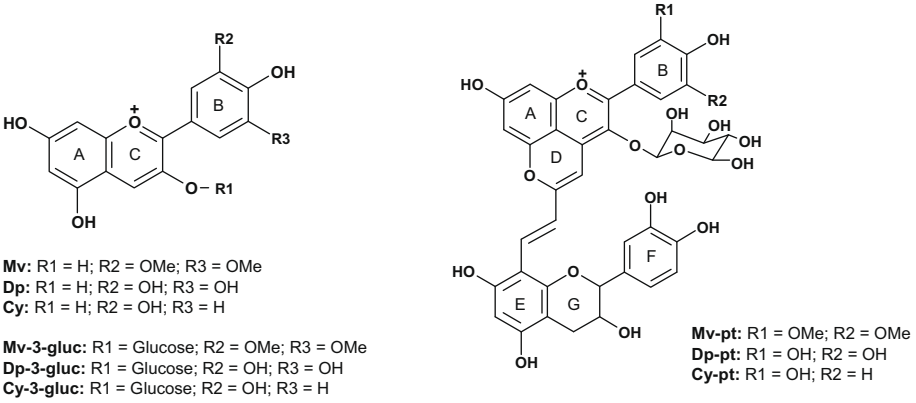

Fig. 1. Structures of malvidin (Mv), cyanidin (Cy), delphinidin (Dp), malvidin-3-glucoside (Mv-3-gluc), cyanidin-3-glucoside (Cy-3-gluc), delphinidin-3-glucoside (Dp-3-gluc), and the portisins of malvidin (Mv-pt), cyanidin (Cy-pt) and delphinidin (Dp-pt).

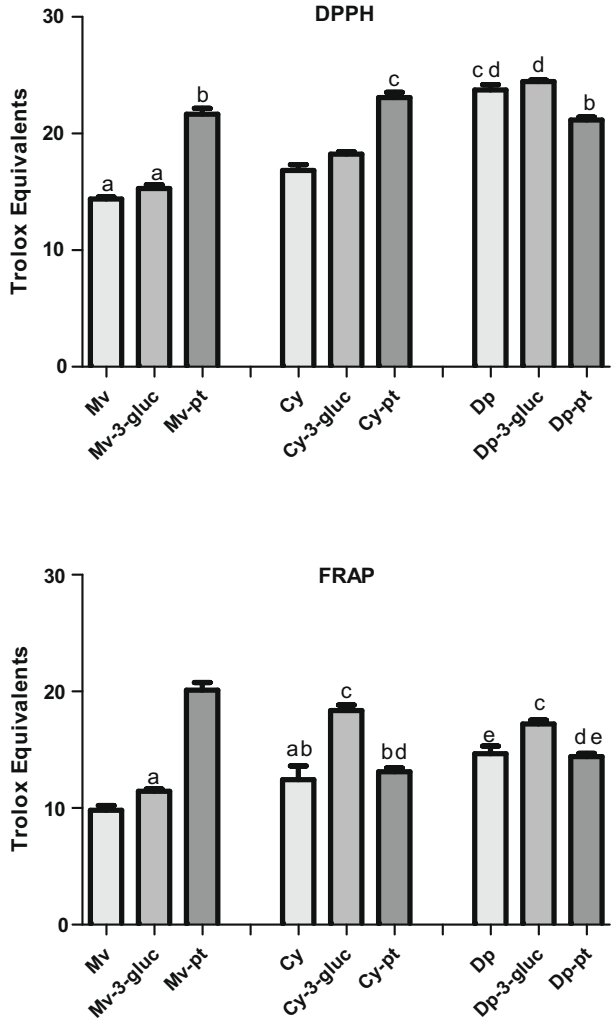

Fig. 2. Radical scavenging activity (DPPH) and reducing capacity (FRAP) of 10 μM malvidin, cyanidin, delphinidin, respective 3-glucosides and portisins (μM Trolox equivalents). Columns represent mean ± standard deviation (SD), $^{*}p < 0.05$. Columns with the same letter do not differ statistically.

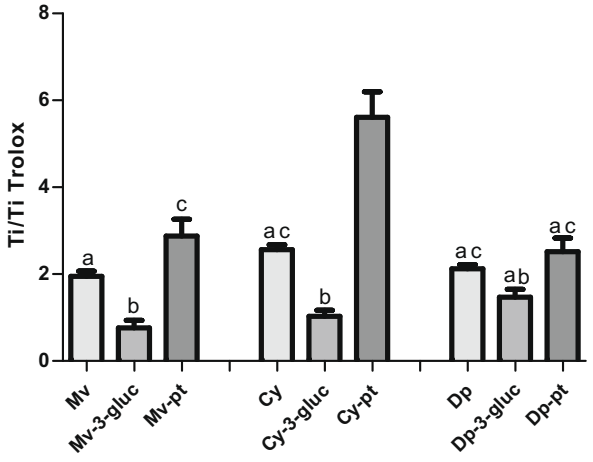

Fig. 3. Inhibition of AAPH-initiated oxidation in soybean PC liposome by 1.25 μM of cyanidin and malvidin-derived pigments measured by oxygen consumption. Results are inhibition times in ratio to Trolox. Columns represent mean ± standard deviations (SD), $^{*}p < 0.05$. Columns with the same letter do not differ statistically.

have been related to display antiradical activity. Surprisingly, the portisin of delphinidin (Dp-pt) was found to have a lower effect than its anthocyanin counterparts (Dp and Dp-3-gluc).

3.3. FRAP

The reducing capacity of the tested pigments were assayed using the FRAP method previously developed by Benzie and Strain (Benzie & Strain, 1996) with modifications. Once again, for anthocyanidins and their corresponding 3-glucosides, the reducing capacity increased with the number of hydroxyl groups in ring B (Mv < Cy ≈ Dp) (Fig. 2). This result is in agreement with previous studies reported (Jordheim, Aaby, Fossen, Skrede, & Andersen, 2007).

Concerning portisins, the trend for the portisins study was inverted, where Mv-pt was the most efficient pigment in terms of reducing capacity. This outcome could be due to the structural features of these pigments as they present more complex structures.

3.4. Oxidation of soybean PC liposomes

The evaluation of the antioxidant capacity of the pigments, against oxidation of soybean PC liposomes, was performed using AAPH as a peroxidation initiator. The antioxidant/antiradical capacity of the pigments was assessed at the initial and propagation stages of oxidation through the measurement of oxygen consumption, as previously described (Faria et al., 2005). The generation of peroxyl radicals from AAPH, induces oxidation by hydrogen subtraction of polyunsaturated acyl chain of the liposomes, resulting in the lipid radicals that carry on the propagation phase (Esterbauer, Striegl, Puhl, & Rotheneder, 1989). The data obtained from the oxygen consumption assays showed that all anthocyanins, anthocyanidins and portisins efficiently scavenged the peroxyl radicals generated in the aqueous phase (compared to the control with no compounds) (Fig. 3). Among the three classes of compounds tested, portisins were the most potent antioxidants in delaying lipid peroxidation, followed by the anthocyanidins and finally the anthocyanidin-3-glucosides. There were no significant differences between cyanidin, malvidin and delphinidin-derived compounds within the same class, except for the portisin of cyanidin (Cy-pt) that displayed a much higher antioxidant capacity.

4. Conclusion

All anthocyanidins, anthocyanidin-3-glucosides and portisins derived from malvidin, cyanidin and delphinidin were shown to have antioxidant properties, by using three different methods.

In the DPPH method, the higher antiradical capacity of cyanidin and delphinidin-derived pigments was already anticipated. The cyanidin and delphinidin-derived pigments have an *ortho*-dihydroxyl group (catechol) and a vicinal trihydroxyl group (pyrogallol), respectively. These structural features are likely to induce a significant radical scavenging capacity of these compounds (Bors, Heller, Michel, & Saran, 1990; Rice-Evans et al., 1996). This trend was not observed for portisins. Indeed, comparing the three portisins studied, there was no major difference in the antiradical capacity among them, thereby suggesting that the common flavanol moiety of the structure may play the primary role in this effect. Previous studies had already shown that the antiradical capacity of similar compounds without the flavanol moiety (replaced by a phenolic moiety) were significantly reduced (data not shown).

The results obtained with the FRAP assay showed that the reducing capacity of these pigments increased with the number of hydroxyl groups in ring B. However, the results obtained with the portisins were unexpected. For these pigments, the reducing power of Dp-pt and Cy-pt were significantly lower than that of

Mv-pt. This may be due to Cy-pt and Dp-pt having two catechol groups, one catechol and one pyrogallol groups, respectively. Therefore, these two pigments have two sites that may chelate ferric ions. Since the FRAP assay includes the presence of ferric ions ($FeCl_3$), those may be more easily chelated by Cy-pt and Dp-pt, which are thus less able to reduce the TPTZ complex (see Section 2). This possible chelation was observed for portisins but not for the other pigments tested. The structural complexity of portisins and their likely occurrence under several conformations in solution, makes the compounds effect difficult to understand. Other phenolic compounds have already been reported to display this behaviour under similar conditions (Paiva-Martins & Gordon, 2005). However, it is interesting to notice that there is a positive correlation between the DPPH and the FRAP assays in the case of malvidin-derived pigments, but not for the other cyanidin and delphinidin-derived pigments (Fig. 4). The lack of relationship between the DPPH and FRAP assays for the cyanidin and delphinidin-derived pigments could thus result from the presence of chelating sites in their structure (catechol and pyrogallol ring B, respectively) likely to chelate ferric ions, thereby influencing the reducing capacities of these compounds towards the TPTZ complex.

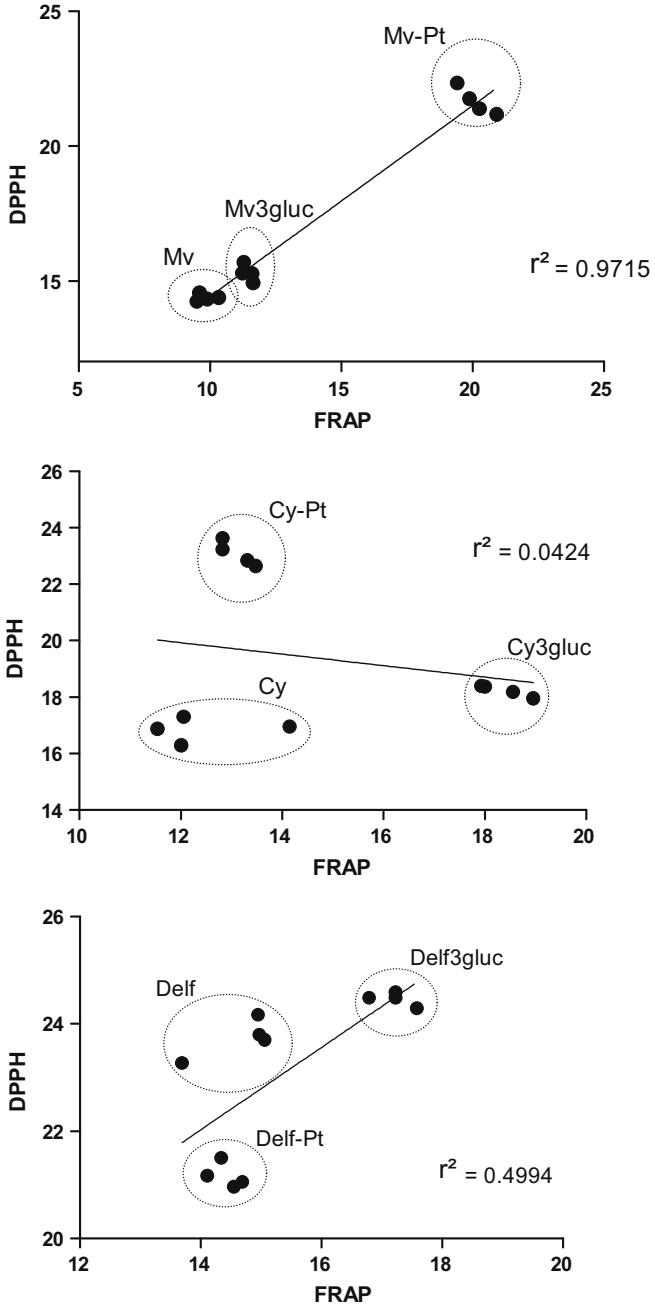

Fig. 4. Correlation plot of DPPH vs. FRAP methods for the compounds tested.

As seen from the results obtained by the oxygen consumption assays, all compounds tested trapped AAPH-derived peroxyl radicals inhibiting the initiation of lipid peroxidation. Interestingly, the antioxidant protection towards lipid peroxidation increased positively with the overall hydrophobicity of the compounds (anthocyanins < anthocyanidins < portisins). This latter feature may be crucial for a strategic location of the compounds in the liposome surface vs. water phase and thus influence their effect towards protection from oxidation. Therefore, in addition to the inherent antioxidant capacity of the tested compounds, if their location is at the liposome surface, they may also quench liposome-derived peroxyl radicals and thus inhibit the chain propagation, at least until exhaustion of the compound. Portisins were the pigments that displayed the greatest protection towards lipid peroxidation. Among these, Cy-pt showed the strongest protecting activity, due to its higher ability to scavenge radicals (as determined by DPPH) in conjunction with its hydrophobicity.

A polyphenol has to comply with two important requisites to be considered an efficient antioxidant: it has to exert its effect in low concentration relatively to the substrate to be oxidised, and it must be stable after exerting its effect (e.g. the resulting radical must be stable) (Halliwell, 1990; Shahidi & Wanasundara, 1992). Concerning this last point, the structure of portisins is likely to increase the overall antioxidant capacity as a result of the extended conjugation of π electrons which could stabilize the radical scavenged throughout the whole structure (Faria et al., 2005).

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