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Antioxidant Activity of Anthocyanins and Their Aglycons

MARJA P. KÄHKÖNEN* AND MARINA HEINONEN

Department of Applied Chemistry and Microbiology, Division of Food Chemistry, P.O.Box 27, FIN-00014 University of Helsinki, Finland

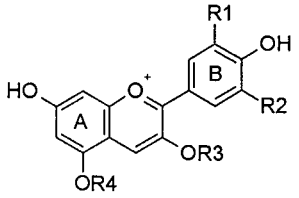
The antioxidant activity of the six common anthocyanidins, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and their glycosidic forms was evaluated in three lipid-containing models [human low-density lipoprotein (LDL) and bulk and emulsified methyl linoleate]. In addition, the radical scavenging activity of the compounds against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was studied. Most anthocyanins and their aglycons acted as strong antioxidants in emulsion and LDL. Many compounds showed an activity comparable to the well-known antioxidants α-tocopherol, Trolox, catechin, and quercetin. In bulk methyl linoleate, anthocyanins and anthocyanidins possessed only a weak antioxidant activity or even oxidation-promoting activity. Depending on the anthocyanidin, different glycosylation patterns either enhanced or diminished the antioxidant power. For the most part, the activities of the glycosides and the aglycons did not differ remarkably in emulsion. In LDL the aglycons showed in general higher activities than the glycosides. In bulk oil, to the contrary, the glycosides were more effective than the aglycons.

KEYWORDS: Anthocyanins; anthocyanidins; antioxidant activity; methyl linoleate; emulsion; human LDL; DPPH

INTRODUCTION

Anthocyanins represent one of the most widely distributed classes of flavonoids in plants. Apart from their coloring effects in fruits, anthocyanins show ability to prevent lipid oxidation in different lipid environments such as human low-density lipoprotein (LDL) in vitro and liposome (*1*) and scavenging activity against various artficially generated free radicals (*2*−*4*). The common aglycon forms, anthocyanidins, found are cyanidin, delphinidin, peonidin, petunidin, malvidin, and pelargonidin. They all have the basic flavylium cationic structure at low pH, and they differ from each other by having different substituents in ring B (**Figure 1**). This structure provides them with the bright red, orange, and blue colors. In plants, anthocyanidins occur as glycosylated forms, anthocyanins. The prevalent sugar moieties are glucose, rhamnose, xylose, galactose, arabinose, and fructose. Both mono- and diglycosides are common, as well as acylated forms. The sugar moiety can be located on carbons 3, 5, 7, 3′, and 5′, the 3- and 5-positions being dominant (*5*).

Anthocyanins may exist in a variety of protonated, deprotonated, hydrated, and isomeric forms, and the relative proportion of these molecules is strongly dependent on pH. The red flavylium cation is dominant at very acidic pH (pH 1−3). In aqueous media, as the pH is raised to 4−5, hydration reactions generate the colorless carbinol pseudo-base, which can further undergo ring opening to the light yellow chalcones, most rapidly at pH 2.5−5 at increased temperatures. The flavylium cation can alternatively be transformed to quinonoidal bases through proton transfer reactions and at pH values between 6 and 7 be further converted to the blue-purple quinonoid anions (*6*, *7*).



Antocyanidin	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

R3, R4 = H or sugar moiety

Figure 1. Structural formulas of common anthocyanins.

These forms may play an important role in the antioxidant action of anthocyanins (*8*, *9*). The completely conjugated structure of anthocyanins that allows electron delocalization results in very stable radical products, which is favorable considering antioxidativite ability (*10*). The degree and position of hydroxylation and methoxylation in the B ring affect their stability and reactivity (*11*) and thereby also antioxidant actions

* Corresponding aurhor (telephone +358 9 191 58 281; fax +358 9 191 58 475; e-mail marja.kahkonen@helsinki.fi).

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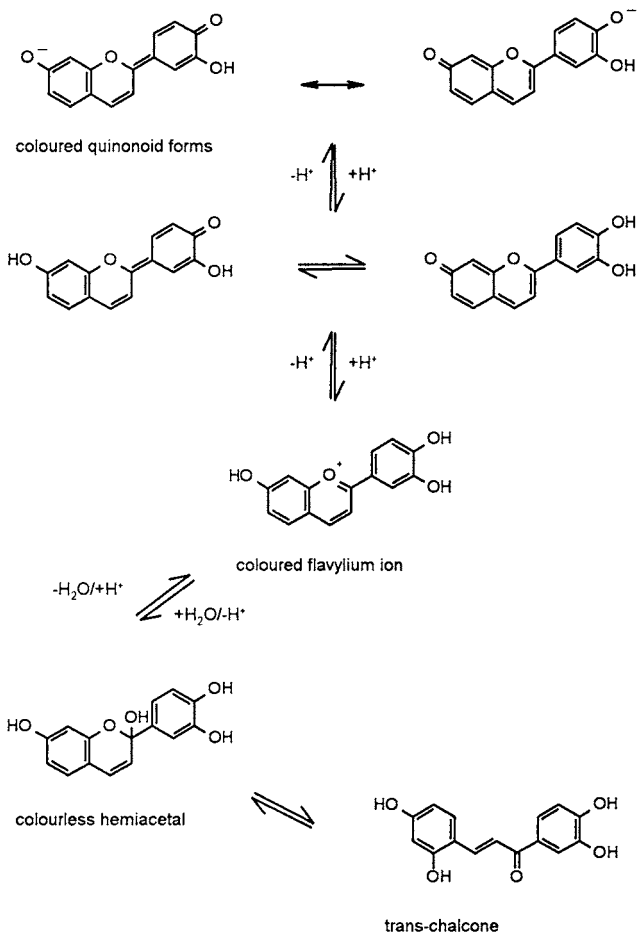

Figure 2. Resonance structures of anthocyanins.

(*1*, *3*). The *O*-diphenol substitution offers a higher stability to the *O*-semiquinone radical formed upon hydrogen donation compared to the stability of simple aryloxyl radixals (*3*, *12*). In addition, the pattern of glycosylation is of importance. In many studies, the antioxidant potentials of anthocyanidins were generally higher than those of the corresponding glycosides (*1*−*3*). Together these structural factors modulate the stability and polarity as well as the ability of anthocyanins to act as free radical scavengers and metal chelators.

Although many papers have been published on the radical scavenging activities of anthocyanins, they have mainly focused on the aglycon forms. Only a few systematic studies on the antioxidant activity of both the aglycon and glycosidic forms in lipid-containing oxidation models have been conducted, probably due to the poor availability and high price of the commercial anthocyanin standards. It is of great importance to study thoroughly the actions of the glycosylated compounds as they are the native forms occurring in plants as well as in plant extracts and other products manufactured without hydrolysis. The role of glycosides in our diets has become even more significant as it was recently suggested that in the human gastrointestinal tract anthocyanins may be absorbed as intact glycosides into the circulation (*13*−*15*). The aim of this study was to determine the antioxidant activity of the common anthocyanidins (6 compounds) and anthocyanins (17 compounds) in two different lipid environments, in emulsified oil (methyl linoleate, MeLo) and in human LDL in vitro. Selected compounds were also tested in bulk oil (MeLo). In addition, the radical scavenging activity of the compounds was evaluated by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test. For comparison we also tested three other flavonoids [(+)-catechin, quercetin, and rutin], three phenolic acids (gallic, caffeic, and chlorogenic acids), and the well-known antioxidants ascorbic acid, α-tocopherol, and Trolox.

MATERIALS AND METHODS

Chemicals. Peonidin, cyanidin 3-arabinoside, cyanidin-3-(xylosylglucose)-5-galactose, cyanidin-3-(coumaroyl-xylosylglucose)-5-galactose, delphinidin 3-glucoside, delphinidin 3-rutinoside (rutinose = rhamnoglucose), peonidin 3-arabinoside, peonidin 3-galactoside, and petunidin 3-glucoside were purchased from Polyphenols (Sandnes, Norway). Cyanidin, delphinidin, malvidin, pelargonidin, peonidin, cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, malvidin 3,5-diglucoside, caffeic acid, (+)-catechin, and chlorogenic acid were purchased from Extrasynthése (Genay, France). Ascorbic acid, gallic acid, quercetin, rutin, and human LDL were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl linoleate was from Nu Check Prep (Elysian, MN). Emultop (partially hydrolyzed soybean lecithin) was a gift from Lucas Meyer GmbH (Hamburg, Germany). α-Tocopherol, copper sulfate, Na_2HPO_4, and formic acid were purchased from Merck (Darmstadt, Germany). All solvents were of HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland), and water was of Milli-Q quality (Millipore Corp., Bedford, MA).

Antioxidant Activity in MeLo Emulsion. The oxidation experiment in a 10% oil-in-water emulsion was carried out using 0.40 g of MeLo and 3.6 mL of 1% soybean lecithin (Emultop) dissolved in Milli-Q water (Millipore Corp.). The tocopherol content of MeLo and Emultop was studied by using HPLC (*16*). MeLo was free of tocopherol, whereas in Emultop some γ- and δ-tocopherol was found, their concentrations being 62 and 40 μg/g, respectively. The anthocyanins, their aglycons, and the reference compounds were dissolved in methanol and added at levels of 50 and 250 μM. Methanolic solutions (100 μL) were pipetted into glass vials (20 mL), and solvent was evaporated under nitrogen. MeLo and the emulsifier solution were added into the vials, and emulsions were prepared by sonicating the solution for 3 min in an ice bath with a U 50 Control Ikasonic sonicator (Janke & Kunkel GmbH & Co. KG, Staufen, Germany). Oxidation was carried out in the dark at 40 °C. Formation of hydroperoxides was followed by measuring the formation of conjugated diene hydroperoxides spectrometrically at 234 nm (*17*). The antioxidant activity was expressed as percent inhibition of formation of MeLo hydroperoxides after 72 h of oxidation. The pH of the emulsified samples ranged between 5.1 and 6.0 during the whole incubation period.

Antioxidant Activity in Bulk Oil. The bulk oil method with MeLo as the oxidizing substrate has been used in previous antioxidant activity studies of plant extracts (*17*, *18*). Selected compounds were solved in methanol and added to MeLo (0.2 g), after which methanol was evaporated under nitrogen. Oxidation of MeLo was carried out in the dark at 40 °C, and oxidation measurements were performed as in the emulsion test. Sample aliquots (10 mg) were taken at the starting point and after 72 h of oxidation. The antioxidant activity was expressed as percent inhibition of formation of MeLo hydroperoxides after 72 h of oxidation.

Antioxidant Activity in Human LDL. Human LDL was diluted to a protein concentration of 0.2 mg/mL using 0.01 M Na_2HPO_4, pH adjusted to 7.4, containing 0.15 M NaCl. Samples were incubated at 37 °C with 10 μM copper sulfate solution and various antioxidant concentrations (final concentrations of 2.5, 10.0, and 25.0 μM), in sealed headspace vials. The compounds were added in ethanolic solution (10 μL), and ethanol was removed by nitrogen flushing. LDL solution (450 μL) was pipetted into the vial, 1340 μL of phosphate buffer and 10 μL of copper sulfate were added, and the vials were sealed and stirred carefully before they were placed into a water bath (37 °C). After 2 h of incubation, the extent of oxidation was determined by measuring the formation of hexanal using static headspace gas chromatography with an Autosystem XL gas chromatograph equipped with an HS 40XL headspace sampler (Perkin-Elmer, Shelton, CT) according to the method of Frankel et al. (*19*).

Radical Scavenging Activity (RSA). The ability of the compounds to act as free radical scavengers against DPPH radical was tested

Table 1. Antiradical and Antioxidant Activities of Anthocyanidins and Anthocyanins

	DPPH[a]	MeLo emulsion[b]		LDL[c]			MeLo bulk[b]
compound[d]	17 μM	50 μM	250 μM	2.5 μM	10 μM	25 μM	50 μM
Pg	31 ± 0	45 ± 0	49 ± 1	8 ± 1	31 ± 4	92 ± 0	–3 ± 0
Cn	33 ± 0	56 ± 5	84 ± 0	–3 ± 1	94 ± 0	98 ± 0	–18 ± 1
Dp	42 ± 1	67 ± 5	83 ± 1	–3 ± 0	94 ± 0	98 ± 0	14 ± 2
Pn	33 ± 0	49 ± 2	84 ± 1	6 ± 0	59 ± 1	95 ± 1	21 ± 15
Pt	10 ± 1	38 ± 2	74 ± 3	–2 ± 1	–7 ± 1	–3 ± 4	–22 ± 7
Mv	24 ± 1	63 ± 1	88 ± 0	24 ± 2	84 ± 1	97 ± 0	–21 ± 3
Pg-3-glu	20 ± 0	44 ± 3	68 ± 0	–17 ± 1	–23 ± 2	–2 ± 1	33 ± 2
Cn-3-glu	32 ± 1	52 ± 2	83 ± 0	–9 ± 1	92 ± 0	92 ± 0	20 ± 1
Dp-3-glu	42 ± 1	51 ± 4	70 ± 1	–5 ± 1	90 ± 1	93 ± 0	18 ± 15
Pn-3-glu	26 ± 1	43 ± 1	47 ± 3	–5 ± 9	7 ± 1	97 ± 0	
Pt-3-glu	23 ± 1	60 ± 4	87 ± 2	–20 ± 1	59 ± 5	83 ± 0	33 ± 10
Mv-3-glu	26 ± 1	82 ± 0	90 ± 1	–17 ± 1	–13 ± 1	14 ± 1	–13 ± 1
Cn-3-gal	25 ± 0	47 ± 1	85 ± 0		86 ± 1	89 ± 0	
Pn-3-gal	20 ± 1	–6 ± 4	88 ± 1		–8 ± 2	97 ± 0	
Mv-3-gal	22 ± 0	25 ± 3	77 ± 2		91 ± 2	98 ± 0	
Cn-3-ara	26 ± 0	50 ± 5	76 ± 0		98 ± 0	99 ± 0	
Pn-3-ara	6 ± 0	20 ± 6	72 ± 3		–19 ± 1	94 ± 0	
Cn-3-rut	25 ± 0	21 ± 6	78 ± 4		11 ± 3	77 ± 0	
Dp-3-rut	32 ± 2	–57 ± 13	47 ± 6		98 ± 0	98 ± 0	
Cn-3,5-diglu	21 ± 0	40 ± 2	77 ± 1		11 ± 1	53 ± 2	
Mv-3,5-diglu	14 ± 0	49 ± 0	73 ± 0		10 ± 0	25 ± 2	
Cy-3-(xyl-glu)-5-gal	22 ± 0	37 ± 7	94 ± 3		–4 ± 1	98 ± 0	
Cy-3-(coum-xyl-glu)-5-gal	26 ± 0	38 ± 4	74 ± 1		98 ± 0	97 ± 1	

Expressed as [a]radicals scavenged (%) after 4 min of reaction time, $n = 3$; [b]inhibition (%) of MeLo hydroperoxide formation after 72 h of oxidation, $n = 2$; and [c]inhibition (%) of hexanal formation after 2 h of oxidation, $n = 3$. [d] Pel, pelargonidin; Cn, cyanidin; Dp, delphinidin; Mv, malvidin; Pn, peonidin; Pt, petunidin; gal, galactoside; glu, glucoside; diglu, diglucoside; ara, arabinoside; rut, rutinoside; xyl, xylosyl; coum, coumaroyl.

spectrophotometrically with a λ15 UV−vis spectrophotometer (Perkin-Elmer, Norwalk, CT) by measuring the disappearance of the absorbance at 517 nm after the addition of antioxidant solution. In a cuvette, 2950 μL of 0.1 mM methanolic DPPH solution was mixed with 50 μL of a 1 mM antioxidant solution to start the reaction. The result is expressed as the percentage of radicals scavenged after 4 min of reaction time.

Statistical Analysis. The results were processed by using one-way variance analysis (ANOVA). Differences at $p < 0.05$ were considered to be significant. In addition, simple regression analysis was performed to seek for relationships between different tests. The computer program employed was Statgraphics Plus for Windows version 3.0.

RESULTS

Antioxidant Activity in Oil-in-Water Emulsion and Bulk Oil. The activity of anthocyanidins in preventing the oxidation of emulsified methyl linoleate at a 50 μM addition level decreased in the order delphinidin > malvidin > cyanidin > peonidin > pelargonidin > petunidin (**Table 1**). At the 250 μM level, the differences between malvidin, cyanidin, delphinidin, and peonidin were not statistically significant. Pelargonidin was clearly the weakest antioxidant at the higher concentration. Glycosylation altered the activity order. At the 250 μM level, the antioxidant activity of the 3-glucosides decreased in the order malvidin > petunidin > cyanidin > delphinidin > pelargonidin > peonidin. The differences between malvidin, petunidin, and cyanidin 3-glucosides were not statistically significant. In comparison with the aglycons, the activity of the glycosides either increased (petunidin and pelargonidin), remained unchanged (malvidin and cyanidin), or decreased (delphinidin and peonidin). The quality of the sugar substituent affected the activity as well. There was no statistically significant difference between the cyanidin 3-glucoside and the corresponding galactoside, but the arabinoside was less efficient than the other monoglycosides at the 250 μM level. The activity of malvidin 3-galactoside was significantly lower than that of the 3-glucoside. No clear trend could be seen with the monoglycosides of peonidin, as at the lower concentration the 3-galactoside and 3-arabinoside were less efficient than the 3-glucoside, but the opposite could be seen at the higher concentration. Cyanidin and malvidin 3,5-diglucosides were less active than the corresponding monoglucosides, and similarly the rutinosides of cyanidin and delphinidin were less active than the monoglucosides. The triglycoside tested, cyanidin-3-(xylosylglucose)-5-galactose, was less active than the monoglucoside at the lower concentration, but the most active anthocyanin at the higher concentration. Acylation of the molecule to cyanidin-3-(coumaroylxylosylglucose)-5-galactose lowered the activity only at the higher concentration tested. Among the reference antioxidants tested, α-tocopherol and its water-soluble analogue Trolox, as well as quercetin, showed superior activity (**Table 2**). Rutin, caffeic acid, and (+)-catechin possessed also high activities, being approximately as efficient as the most active anthocyanins. Cholorogenic acid showed only moderate activity, and ascorbic acid was totally inactive; that is, it had no effect on the hydroperoxide formation. In bulk methyl linoleate, two anthocyanidins possessed a weak antioxidant effect (peonidin and delphinidin), whereas the others showed pro-oxidant activity (**Table 1**). However, the action of all the monoglucosides except malvidin 3-glucoside was antioxidative, the activity being higher

Table 2. Antiradical and Antioxidant Activities of Selected Phenolics, Ascorbic Acid, α-Tocopherol, and Trolox

	DPPH[a]	MeLo emulsion[b]		LDL[c]		MeLo bulk[b]
compound	17 μM	50 μM	250 μM	10 μM	25 μM	50 μM
catechin	30 ± 0	73 ± 1	78 ± 2	95 ± 1	99 ± 0	90 ± 2
quercetin	34 ± 0	77 ± 4	95 ± 0	96 ± 1	97 ± 0	98 ± 0
rutin	37 ± 0	79 ± 1	89 ± 0	91 ± 0	93 ± 0	20 ± 2
gallic acid	62 ± 1	57 ± 1	82 ± 1	96 ± 1	97 ± 0	98 ± 0
caffeic acid	35 ± 0	80 ± 0	87 ± 0	94 ± 1	95 ± 4	92 ± 1
chlorogenic acid	38 ± 1	35 ± 0	65 ± 0	7 ± 3	80 ± 2	90 ± 1
ascorbic acid	29 ± 0	3 ± 7	–4 ± 1	96 ± 2	–10 ± 4	–1 ± 2
α-tocopherol	31 ± 1	88 ± 2	96 ± 0	–9 ± 1	–9 ± 3	98 ± 0
Trolox	35 ± 1	78 ± 1	96 ± 0	94 ± 0	95 ± 0	98 ± 0

[a–c] See **Table 1**.

than that of the aglycon. In comparison, nearly all reference antioxidants showed strong antioxidant activity, the exceptions being rutin with a weak activity and the inactive ascorbic acid.

Antioxidant Activity in Human LDL. The activity of anthocyanidins in preventing the oxidation of human LDL at the 10 and 25 μM addition levels decreased in the order delphinidin = cyanidin > malvidin > peonidin > pelargonidin > petunidin (**Table 1**). At the 25 μM addition level the differences between cyanidin, delphinidin, malvidin, and peonidin and between peonidin and pelargonidin were not statistically significant. At the lowest level (2.5 μM), only malvidin, pelargonidin, and peonidin reduced the extent of hexanal formation. Petunidin showed slight pro-oxidant activity, that is, enhanced the formation of hexanal at all tested concentrations.

Overall, glucosylated forms were less active than free forms in LDL suspensions (**Table 1**). The most dramatic differences were measured between malvidin and petunidin aglycons and their respective monoglucosides. There was only slight alteration between the activity of the monoglycosidic forms of cyanidin, whereas the diglycosylated forms showed clearly lower activities. Malvidin 3,5-diglucoside and delphinidin 3-rutinoside, on the contrary, inhibited LDL peroxidation more efficiently than the monoglucosidic forms, although the antioxidant activity of malvidin 3,5-diglucoside was low. All of the anthocyanin monoglucosides showed pro-oxidant activity at the lowest addition level tested (2.5 μM). At the 10 μM level, cyanidin 3-glucoside was highly antioxidative, followed by delphinidin, petunidin, and peonidin 3-glucosides. Peonidin 3-glucoside was the best monoglucosidic oxidation inhibitor at the 25 μM level, followed by delphinidin and cyanidin glucosides. Malvidin 3-glucoside was pro-oxidative at the middle level, together with pelargonidin 3-glucoside, which promoted hexanal formation at all concentrations tested. The triglycoside, cyanidin-3-(xylosylglucose)-5-galactose, was slightly pro-oxidative at the 10 μM level but highly active at the 25 μM level, whereas its acylated derivative, cyanidin-3-(coumaroylxylosylglucose)-5-galactose, showed high antioxidant activity at both concentrations.

Most reference antioxidants effectively prevented the oxidation of LDL. α-Tocopherol showed slight pro-oxidative action as against Trolox it exhibited high activity at both concentrations. Chlorogenic acid was inactive at the lower concentration but quite active at the higher level. Ascorbic acid acted in a contradictory manner, as it showed excellent antioxidant activity at the 10 μM level but pro-oxidant activity at the 25 μM level.

Radical Scavenging Activity. Compared to the reference antioxidants, anthocyanins and anthocyanidins possess similar or slightly lower radical scavenging activity in the DPPH test. Only gallic acid showed a remarkably higher scavenging activity. Among the aglycons tested, delphinidin possessed the highest activity, followed by cyanidin and peonidin, pelargonidin, malvidin, and petunidin (**Table 1**). The monoglucosides of cyanidin, delphinidin, and malvidin were practically as active as their aglycons, whereas the activities of peonidin and pelargonidin 3-glucosides were lower than the activity of the corresponding aglycons. Petunidin 3-glucoside showed a higher response than petunidin. Cyanidin, peonidin, and malvidin galactosides were weaker scavengers than the glucosides, the decrease being 15−23%. The activity of cyanidin 3-arabinoside was 19% lower than the activity of cyanidin 3-glucoside, but peonidin 3-arabinoside had only 23% of the activity of peonidin 3-glucoside. The 3,5-diglucosides of cyanidin and malvidin showed significantly lower activities than the corresponding monoglucosides. The cyanidin and delphinidin rutinosides were both less active than the glucosides. Acylation of the cyanidin triglucoside improved the radical scavenging activity slightly, the improvement being statistically significant.

Correlation between Tests. On the basis of the simple regression testing, there was a statistically significant relationship between the DPPH and the LDL test results. When the DPPH results are compared to the 10 μM anthocyanin level in the LDL test, the relationship is moderate ($r = 0.66$ and $r^2 = 44\%$, $p < 0.01$). However, the relationship at the higher (25 μM) tested level is weak ($r = 0.46$ and $r^2 = 21\%$, $p < 0.01$). The DPPH test results and the bulk MeLo test results did not correlate with each other; neither was there any statistically significant relationship between the DPPH and emulsion tests or between the three tests in lipid models at the 90% confidence level.

DISCUSSION

Anthocyanins and their aglycons, with some exceptions, are powerful antioxidants in emulsified methyl linoleate and in suspensions of human LDL but relatively inactive or even pro-oxidants in bulk methyl linoleate. Their activity in emulsion and LDL is comparable to that of catechin, the flavonols quercetin and rutin, and gallic, caffeic, and chlorogenic acids. Furthermore, some anthocyanins and their aglycons seem to attain the activity of the well-known antioxidants α-tocopherol and Trolox and surpass clearly the antioxidant power of the paradoxically acting ascorbic acid.

Part of the antioxidative effects of anthocyanins in lipid-containing models can be explained by their radical scavenging activity, but as the present results clearly demonstrate, the hydrogen-donating ability of antioxidants in a solvent model does not necessarily indicate their activity in a lipid environment. According to a study in which antioxidant activities of plant extracts with phenolic compounds were evaluated (*20*), the results from the DPPH assay corresponded well with other radical assays, such as Fremy's salt and galvinoxyl assays, in which stable radicals are used as targets. Furthermore, the DPPH test showed trends similar to the lipid oxidation assay in bulk methyl linoleate. No such correlations could be observed with anthocyanins and their aglycons in this study. Neither were there statistically significant correlations between the DPPH and emulsion tests. Thus, it can be assumed that in more complex lipid systems, several other factors, including metal chelation properties, interactions with emulsifier or proteins, and distribution between the oil and the water phase, play an important role in the antioxidant action (*21*). There was, however, a moderate linear relationship between the DPPH test and the LDL test at the 10 μM level, the relationship between the DPPH test and the LDL test at the 25 μM level being relatively weak. The radical scavenging properties of anthocyanins assessed by the DPPH test seem to be more important explanatory factors in the LDL model than in the oil models. Naturally, considering that the reactive radicals in the lipid models are the peroxyl, alcoxyl, hydroxyl radicals, etc., it is not possible to come to definite conclusions on the basis of the DPPH assay.

In general, the DPPH assay results are in accordance with previous observations on the effect of hydroxylation and methoxylation in ring B to radical scavenging ability in aqueous phase (*2*, *3*, *22*). Those anthocyanidins lacking the *O*-diphenyl structure in the B ring (malvidin, pelargonidin, petunidin, and peonidin) had lower efficiency toward the DPPH radical compared to cyanidin and delphinidin. Peonidin, having a methoxy group in the 3′-position in addition to a hydroxy group in the 4′-position, was more active than pelargonidin. As reported by Fukumoto and Mazza (*22*), the third hydroxyl group

in the B ring enhanced the activity, as delphinidin with hydroxyl groups in the 3′-, 4′-, and 5′-positions was significantly more effective than cyanidin with hydroxyl groups in 3′- and 4′-positions. This enhancement was not found in the studies of Wang et al. (*2*) and Rice-Evans et al. (*3*). In the lipid models the advantage of the third hydroxyl group could not be detected.

In both the present study and the study by of Satué-Gracia et al. (*1*), the activity order of anthocyanidins in preventing the oxidation of human LDL at the 10 μM addition level was the same regarding cyanidin, delphinidin, malvidin, and pelargonidin. Malvidin had lower activity at the 10 μM level compared to cyanidin and delphinidin, and it was the best inhibitor of hexanal formation also at the low concentration (2.5 μM), followed by pelargonidin and peonidin. The high activity of cyanidin and delphinidin containing the 3′,5′-*o*-diphenol group in copper-initiated oxidation of LDL may be attributed to metal chelation, but the better activity of malvidin, peonidin, and pelargonidin at low concentration must be explained by other factors, such as polarity. Malvidin and peonidin may have better access to the lipid peroxidation site due to the methoxy group(s) in the B ring. The polarity of pelargonidin is also lower than that of cyanidin and delphinidin, having more hydroxyl groups. The effect of polarity was demonstrated in an LDL oxidation assay with flavonols, in which metmyoglobin was used instead of copper (*23*). The lipid peroxidation was retarded more by luteolin than by more hydrophilic quercetin, kaempferol, or rutin, but when copper ions were used as the initiator, kaempferol was more active than luteolin.

Because LDL contains endogenous antioxidants, synergism with the well-known chain-breaking antioxidant, α-tocopherol, cannot be disregarded as a possible antioxidative mechanism. Efficient hydrogen donors, cyanidin and delphinidin, can spare tocopherols from consumption by reverting the tocopheryl radical directly back to tocopherol or by scavenging aqueous radicals near the membrane surface, preventing them from attacking tocopherol (*3*, *24*). Another factor affecting the antioxidant action is interaction with the LDL proteins. Anthocyanins may bind with the apolipoprotein B and thus prevent the copper catalyst from binding into LDL, or anthocyanin−protein binding may promote the access of anthocyanins to the lipids (*1*, *23*). The observed inactivity of the added α-tocopherol indicates the inability of the lipophilic compound to reach the lipid core of the LDL particle in this model during such a short incubation period. It should also be noted that the actual antioxidant concentrations in human blood may be much lower than the concentrations used in this study, and on that account the antioxidant actions in vivo may be unequal. In any case, the LDL assay is informative on the effect of anthocyanins on lipid oxidation in a protein-containing environment at neutral pH, in conditions that are relevant for many food systems as well.

Due to the tocopherol content of the emulsifying agent Emultop, the total tocopherol content in the emulsion is ∼10 ppm calculated on the basis of the MeLo concentration. Therefore, it is possible that synergism between these tocopherols and the added compounds may be one important reaction pathway in the model. Further work is needed to establish the nature and intensity of this interaction between anthocyanins and tocopherols. Even though no metal ion initiator was added to emulsion samples, it is conceivable that the metal chelation properties of anthocyanins are also involved in the system, as transition metal ions are naturally present in trace amounts in every experiment. However, the pH of the emulsion (5.1−5.5) should be unfavorable for anthocyanin−metal complexation, as the *o*-dihydroxyl groups are mainly un-ionized (*1*). We assume that oxidation is initiated by the traces of hyroperoxides initially present in MeLo, and thus the main inhibitory reactions would be toward lipid peroxyl radicals. The effectiveness of malvidin and peonidin over cyanidin and delphinidin supports this assumption.

As the pH in the LDL assay was adjusted to 7.4, it is obvious that the equilibrium of the pH-dependent forms of anthocyanins converts from the flavylium cation to favor the quinonoid forms. Therefore, and also on the basis of the observed bluish color of the LDL suspensions, it can be assumed that it is the quinonoid forms of anthocyanins that show the antioxidant activity in LDL in vitro and possibly also in vivo. The formation of chalcones is very slow at neutral pH (8), and therefore they probably do not accumulate in high amounts during the relatively short incubation time used in the LDL assay. In the emulsified oil, however, the lower pH value (5.1−6.0) together with the long incubation time in elevated temperature can result in a quite rapid transformation of the flavylium cation into the colorless pseudo-base and, at least to some extent, to the formation of chalcones (*15*). Consequently, it is possible that the antioxidant action of anthocyanidins in the emulsion is actually due to the activity of pseudo-base and chalchone forms.

This study confirms that in addition to the effect of the different functional groups in the B ring, different glycosylation patterns may modify the antioxidant and antiradical activity of anthocyanidins significantly and that the extent and direction of the change depends on the aglycon. However, this effect is very much dependent on the methods used. It has been postulated that the maximal radical scavenging activity for flavonoids is achieved if there is a free hydroxyl group in ring C (*3*, *12*). This may explain the higher radical scavenging activity of many of the aglycons compared to glycosides in radical scavenging tests. Wang et al. (*2*) observed that glycosylation of anthocyanidins affected the antioxidant capacity measured with the oxygen radical absorbance capacity (ORAC) assay. Glycosylation of cyanidin to cyanidin 3-glucoside increased the activity, but glycosylation of malvidin to the corresponding 3-glucoside decreased the activity, and pelargonin and its 3-glucoside had similar responses. In the study by Fukumoto and Mazza (*22*), the DPPH radical scavenging activities of the monoglucosides of cyanidin, pelargonidin, and peonidin were lower than activities of the aglycons, whereas in the β-carotene bleaching method the monoglycosides of malvidin, pelargonidin, and peonidin showed higher activities than the aglycons. In the present assay, monoglycosylation of cyanidin did not diminish much either the radical scavenging activity toward DPPH radical or the antioxidant activity in LDL or emulsion. The converse was the case with pelargonidin, for which Wang et al. (*2*) did not observe a significant effect. In the DPPH assay, the 3-glucosylated forms of peonidin and pelargonidin were less active than the aglycon forms. According to Rice-Evans et al. (*3*), a free hydroxyl group in the C ring is not important to anthocyanidins lacking the dihydroxy structure in the B ring. This seems to be valid also for peonidin but not for malvidin in LDL. In lipid-containing models, the role of the hydroxyl in the C ring may be only part of the explanation. Glycosylation increases the polarity of the compound, thus affecting the access of the antioxidant to the lipid phase. This is supported by the observation that the diglycosides of cyanidin (3,5-diglucoside and 3-rutinoside) were less active in LDL than the corresponding 3-monoglucosides. Similarily, Fukumoto and Mazza (*22*) found that the antioxidant activities of cyanidin and pelargonidin 3,5-diglucosides in the β-carotene bleaching

method were lower than the activities of the corresponding aglycons. However, in this study a contradictory result was observed with malvidin 3,5-diglucoside, which showed slightly higher activities than the monoglucosides in LDL. Malvidin 3,5-diglycoside differed from other diglycosides also in the β-carotene bleaching study (*22*) by being nearly as active as the aglycon.

In emulsion, the antioxidant efficiency of petunidin and malvidin increased along with glycosylation. In bulk MeLo, the 3-glucosides of cyanidin, pelargonidin, petunidin, and delphinidin showed antioxidant activity, whereas their aglycons acted as pro-oxidants. This is in accordance with the phenomenon called the "polar paradox", that is, hydrophilic antioxidants are more efficient in bulk oils than lipophilic antioxidants. It has been claimed that the phenomenon is due to the ability of hydrophilic compounds to accumulate into the interface of oil and air, whereas lipophilic compounds dilute into the oil phase. In oil-in-water emulsion systems, hydrophilic antioxidants dilute into the water phase and therefore they are usually less efficient than the lipophilic antioxidants that accumulate into the interfaces of oil droplets and water (*25*). However, as discussed above, there are several other factors contributing to the action of antioxidants in emulsions. In conclusion, anthocyanidin are potent antioxidants, and it is evident that the change in the antioxidant action that results from the differences in glycosylation is very much dependent on the assay used.

ABBREVIATIONS USED

LDL, low-density lipoprotein; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MeLo, methyl linoleate; HPLC, high-performance liquid chromatography; *r*, correlation coefficient; r^2, coefficient of determination.

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