DOI:10.1021/jf900841b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Antioxidant Activity of Wine Pigments Derived from Anthocyanins: Hydrogen Transfer Reactions to the DPPH Radical and Inhibition of the Heme-Induced Peroxidation of Linoleic Acid

Pascale Goupy,[†] Ana-Belen Bautista-Ortin,[‡] Helene Fulcrand,[‡] and Olivier Dangles*,[†]

[†]UMR408 Sécurité et Qualité des Produits d'Origine Végétale, INRA, Site Agroparc, Domaine St-Paul, F-84914 Avignon, France, and [‡]UMR1083 Sciences pour l'œnologie, INRA, 2 Place Viala, F-34060 Montpellier, France

The consumption of red wine can provide substantial concentrations of antioxidant polyphenols, in particular grape anthocyanins (e.g., malvidin-3-*O*-β-D-glucoside (**1**)) and specific red wine pigments formed by reaction between anthocyanins and other wine components such as catechin (**3**), ethanol, and hydroxycinnamic acids. In this work, the antioxidant properties of red wine pigments (RWPs) are evaluated by the DPPH assay and by inhibition of the heme-induced peroxidation of linoleic acid in acidic conditions (a model of antioxidant action in the gastric compartment). RWPs having a **1** and **3** moieties linked via a CH_3-CH bridge appear more potent than the pigment with a direct **1**−**3** linkage. Pyranoanthocyanins derived from **1** reduce more DPPH radicals than **1** irrespective of the substitution of their additional aromatic ring. Pyranoanthocyanins are also efficient inhibitors of the heme-induced lipid peroxidation, although the highly hydrophilic pigment derived from pyruvic acid appears less active.

KEYWORDS: Anthocyanin; polyphenol; wine; antioxidant; DPPH; lipid peroxidation; heme

INTRODUCTION

Anthocyanins and their derivatives formed by reaction with other wine components are the main pigments in red wine. Anthocyanins have been shown to possess beneficial in vitro properties related to the protection against pathologies involving oxidative stress, such as cardiovascular diseases, cancer, neurodegeneration, inflammation, and viral infection (*1*, *2*). In particular, anthocyanins are potent antioxidants (*3*, *4*) because of their ability to quickly reduce oxidizing species while being converted into stabilized aryloxyl radicals. Moreover, anthocyanins having a catechol nucleus, e.g., cyanidin derivatives, can chelate transition metal ions potentially involved in the development of oxidative stress (*5*). In young red wine, the main anthocyanin is malvidin-3-*O*-β-D-glucoside (**1**), 3-*O*-β-D-glucosides of cyanidin, delphinidin, petunidin, and peonidin making more minor contribution. Moreover, in mildly acidic conditions, each anthocyanin is in fact a mixture of colored and colorless forms as a result of acid−base and hydration−tautomerization equilibria (*6*). Upon wine aging, anthocyanins under their colored or colorless forms can react with other wine components including ethanal (an oxidation product of ethanol), proanthocyanidins, and 4-hydroxystyrenes (formed by microbial decarboxylation of the corresponding 4-hydroxycinnamic acids) to give a complex distribution of new pigments collectively designated as anthocyanin-derived pigments (*6*−*8*).

The antioxidant properties of red wine pigments are poorly documented and the subject of the present study. Recent nutritional data suggest that the gastrointestinal tract is the most pertinent site for the antioxidant action of poorly bioavailable dietary antioxidants such as most polyphenols (*9*). In addition, common polyphenols were shown to inhibit the peroxidation of dietary lipids in simulated gastric conditions (*10*, *11*) and to prevent the intestinal absorption of cytotoxic lipid peroxidation products in humans (*12*).

Consequently, for evaluating the antioxidant action of red wine pigments (RWPs), we have investigated the inhibition of the heme-induced peroxidation of linoleic acid in acidic conditions as a simple chemical model showing the protection against a possible diet-induced form of oxidative stress in the gastric compartment. For comparison, the intrinsic ability of wine pigments to transfer H atoms to the DPPH radical is quantitatively evaluated in methanol.

MATERIALS AND METHODS

Materials. Horse heart metmyoglobin ($MbFe^{III}$, type II, MW ≈ 17 600 g mol^{-1}), linoleic acid, Tween-20 (polyoxyethylenesorbitan monolaurate), catechin (**3**), and DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from Sigma-Aldrich. All these products of the highest quality available (95−99%) were used without purification. Malvidin-3-*O*-β-D-glucoside (**1**) chloride and cyanidin-3-*O*-β-D-glucoside (**2**) chloride were

*To whom correspondence should be addressed. Telephone: +33-490 14 44 46. Fax: +33-490 14 44 41. E-mail: Olivier.Dangles@univ-avignon.fr.

 Published on Web 06/15/2009

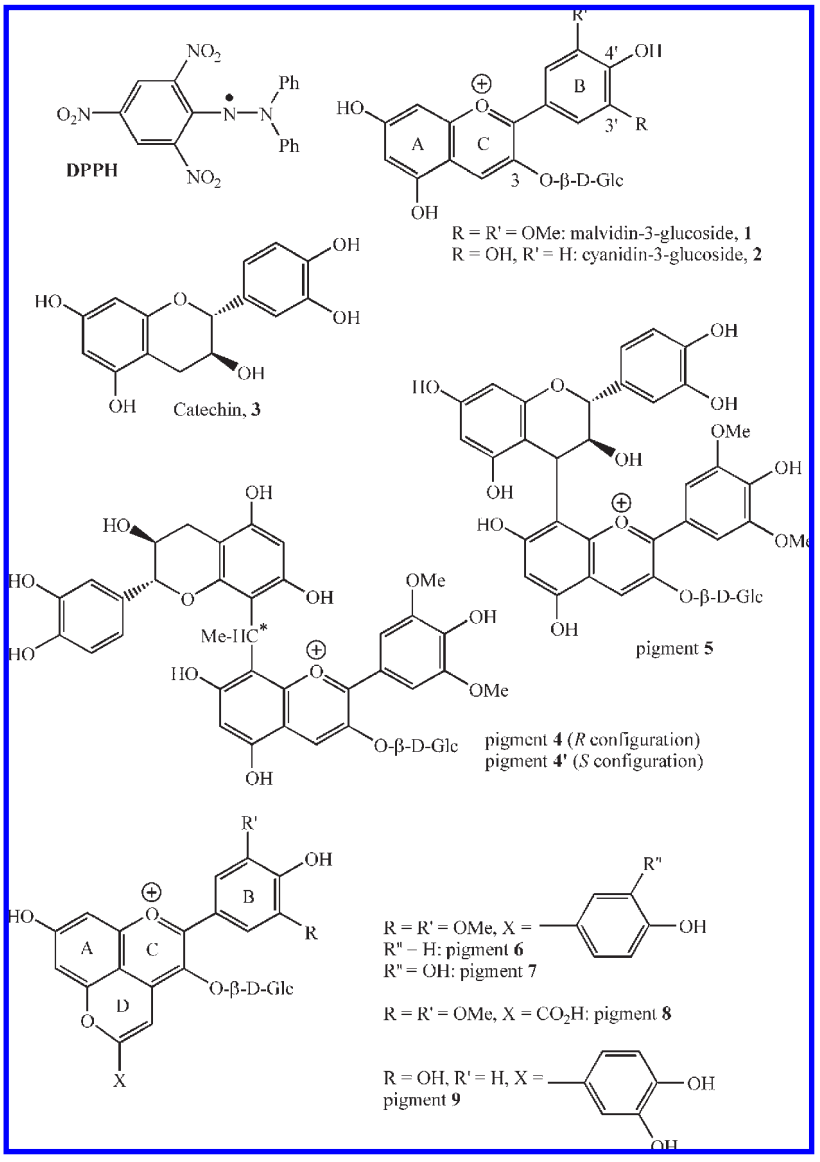

Figure 1. Chemical structures of DPPH and of the polyphenols investigated.

obtained from Extrasynthese (Genay, France). The phosphate (10 mM, pH 6.8) and acetate (10 mM, pH 4) buffers were prepared from Millipore Q-plus water and passed through a column of Chelex-100 chelating resin (Bio-Rad) to remove contaminating free metal ion traces. All solvents used were of analytical grade.

Red Wine Pigments. RWPs (**Figure 1**) were prepared via hemisynthesis from the native anthocyanins according to already published protocols for pigments **4**, **4'**, **5**, **6**, and **8** (*6*, *13*−*15*). Pyranoanthocyanins **7** and **9** were synthesized from 3,4-dihydroxystyrene and anthocyanins **1** and **2**, respectively. 3,4-Dihydroxystyrene was prepared via a Wittig reaction adapted from the literature (*16*). Then, **7** and **9** were synthesized in the same way as **6** and purified by semipreparative HPLC (*6*). The chemical nature and purity of the **7** and **9** were checked by HPLC with UV−visible absorption and mass detection. The reaction yield of the two pyranoanthocyanins was approximately 12%.

Millimolar stock solutions of wine pigments were prepared in MeOH acidified by 0.3% (v/v) of 12 M HCl.

Absorption Spectra. Spectra were recorded on a Hewlett-Packard 8453 diode-array spectrometer equipped with a quartz cell (optical path length, 1 cm) thermostated by a water bath. Magnetic stirring in the cell at a constant rate (1000 rpm) was ensured by a Variomag stirrer (Telemodul 20C).

Hydrogen Abstraction by DPPH. The H atom transfer reactions from an antioxidant to DPPH were investigated by UV−visible spectroscopy at 25 °C. The experimental conditions used were adapted from an already published procedure (*17*): a total of 2 mL of a freshly prepared 2×10^{-4} M solution of DPPH in MeOH was placed in the spectrometer cell, and a total of 25−125 μL of a freshly prepared 10^{-3} M solution of the antioxidant in 0.3% HCl in MeOH was added. The UV−visible spectra were recorded at 600 nm (to avoid possible interference with the pigment absorption band) every 0.5 s over 1−2 min for the determination of rate constants and stoichiometries. Kinetic runs over 15 min were used for the determination of total stoichiometries (see text for details). The molar absorption coefficient of DPPH at 600 nm was estimated to be 5190 M^{-1} cm^{-1} (assuming a purity of 95%).

Inhibition of the Metmyoglobin-Induced Peroxidation of Linoleic Acid. The experimental conditions used were adapted from an already published procedure (*18*, *19*). Metmyoglobin ($MbFe^{III}$, 17.6 mg) was dissolved in 50 mL of phosphate buffer (10 mM, pH 6.8). Its concentration was standardized at 10 μM using ε = 7700 M^{-1} cm^{-1} at 525 nm. An amount of 2 mL of a 2 mM Tween-20 solution in acetate buffer (10 mM, pH 4) was placed in the spectrometer cell, and an amount of 20 μL of a freshly prepared 70 mM solution of linoleic acid in MeOH + 20 μL of the wine pigment solution was added. The final concentrations of wine pigment were in the range 0.2−2 μM. At time zero, an amount of 20 μL of the $MbFe^{III}$ solution was added to the sample under constant magnetic stirring, and the UV−visible spectra were recorded at 234 nm at regular time intervals (10−20 s). Each experiment was duplicated. For reliable comparison between antioxidants, peroxidation experiments have to start from near constant A (234 nm) values reflecting close initial concentrations of lipid hydroperoxides.

Table 1. H-Atom Transfers from Polyphenols to DPPH (MeOH, 25 °C)[a]

antioxidant	DPPH/antiox molar ratio	n	k_1 (M^{-1} s^{-1})
1	4	1.86 (±0.02)	867 (±1)
	6	2.31 (±0.17)	860 (±38)
	8	2.97 (±0.21)	887 (±80)
	12	3.81 (±0.03)	1011 (±14)
2	4	2.78 (±0.07)	1399 (±74)
	6	3.66 (±0.16)	1451 (±26)
	8	4.97 (±0.64)	1295 (±74)
3	6	2.32 (±0.23)	1072 (±76)
	8	2.08 (±0.11)	1269 (±31)
	12	2.16 (±0.13)	1267 (±119)
1 + **3** (1:1)	6	1.14 (±0.06)	869 (±70)
	8	1.07 (±0.06)	1104 (±60)
	12	1.53 (±0.09)	1519 (±43)
4	4	1.01 (±0.07)	676 (±45)
	6	1.04 (±0.02)	1024 (±110)
	8	1.32 (±0.19)	1311 (±179)
4′	4	1.78 (±0.29)	538 (±32)
	6	3.20 (±0.67)	523 (±71)
	8	3.86 (±0.59)	742 (±48)
5	4	2.47 (±0.09)	3925 (±47)
	6	2.36 (±0.11)	5882 (±138)
	8	2.52 (±0.02)	5542 (±141)
6	4	2.92 (±0.02)	1384 (±54)
	6	3.58 (±0.24)	1486 (±59)
	8	4.43 (±0.19)	1568 (±28)
	12	5.52 (±0.10)	1687 (±63)
7	4	3.23 (±0.06)	1119 (±28)
	6	4.26 (±0.22)	1222 (±16)
	8	6.39 (±0.62)	1232 (±19)
	12	8.26 (±0.66)	1376 (±54)
8	4	2.65 (±0.07)	1071 (±17)
	6	3.38 (±0.16)	1310 (±56)
	8	4.93 (±0.20)	1303 (±107)
	12	5.89 (±0.20)	1365 (±219)
9	4	2.75 (±0.30)	1096 (±67)
	6	4.11 (±0.42)	1111 (±37)
	8	4.37 (±0.36)	1296 (±51)

[a] Rate constants and stoichiometries calculated from the curve-fitting of the A (600 nm) vs time curves (n = 3).

Data Analysis. The curve-fittings of the absorbance vs time plots and all calculations and simulations were carried out on a PC using the Scientist program (MicroMath, Salt Lake City, UT). For the DPPH assay, Beer's law and sets of differential kinetic equations (*17*) with initial conditions on concentrations were input in the model. Curve-fittings were achieved through least-squares regression and yielded optimized values for the parameters (kinetic rate constants, stoichiometries). Values reported (**Tables 1** and **2**) for a given antioxidant at a given DPPH-antioxidant molar ratio are mean values of three experiments for the rate constants and stoichiometry n (fast step) and of two experiments for the total stoichiometry n_{tot}. Standard deviations are reported. The curve-fitting procedures typically gave good (>0.99) to excellent (>0.999) correlation coefficients. Consequently, the standard deviations due to the curve-fittings were low and were not taken into account.

RESULTS AND DISCUSSION

The main RWPs studied in this work (**Figure 1**) are derived from malvidin-3-*O*-β-D-glucoside (**1**), the main anthocyanin in grape and young red wine. In its nucleophilic hemiketal form, **1** can be involved in electrophilic aromatic substitutions with catechin carbocations (released from acidolysis of proanthocyanidins) or with ethanal and catechin (**3**) to respectively form pigment **5** and pigments **4** and **4′** (two epimers). In its electrophilic flavylium form, **1** can also react with wine nucleophiles such as 4-hydroxystyrenes, pyruvic acid (under its minor enol form) to form the so-called pyranoanthocyanins **6**−**8** (*7*,*8*). Cyanidin-3-*O*-β-D-glucoside (**2**) and one of its pyrano derivatives (**9**) were also considered because **2**, although much less abundant than **1** in wine, could be an intrinsically more potent antioxidant because of its catechol nucleus. Two antioxidant assays were used: the reduction of DPPH radical (*17*) and the inhibition of the metmyoglobin-induced peroxidation of linoleic acid (*18*, *19*).

Table 2. H-Atom Transfers from Polyphenols to DPPH (MeOH, 25 °C)[a]

antioxidant	DPPH/antiox molar ratio	n_{tot}
1	20	11.26 (±0.08)
2	29.8	17.04 (±0.03)
3	20	4.86 (±0.03)
1 + **3** (1:1)	20	14.04 (±0.10)
4	29.4	14.56 (±0.03)
4′	30.1	14.61 (±0.18)
5	30	7.16 (±0.08)
6	30.5	16.70 (±0.10)
7	31.9	17.91 (±0.42)
8	27.2	16.83 (±0.38)
9	30.7	16.50 (±0.48)

[a] Total stoichiometries calculated from the amplitude of the A(600 nm) vs time curves (n = 2).

Hydrogen-Donating Activity. The DPPH radical (**Figure 1**) is widely used for quickly assessing the ability of polyphenols to transfer labile H atoms to radicals, a likely mechanism of antioxidant protection. DPPH displays a very broad visible absorption band with a maximum at 517 nm in MeOH and can be reduced by polyphenols to the corresponding colorless hydrazine (DPPH-H) (*20*). However, since anthocyanins are characterized by visible absorption bands extending beyond 500 nm, it was considered safer to monitor the reduction of DPPH at 600 nm, i.e., at a wavelength where anthocyanins do not significantly absorb light.

In the DPPH assays, antioxidants are typically characterized by their EC_{50} value, i.e., the antioxidant concentration necessary to reduce half the initial DPPH concentration. This static parameter can be related to the total antioxidant stoichiometry n_{tot} (number of DPPH radicals reduced per antioxidant molecule) but unfortunately offers no kinetic information. In this work, we applied a more informative mathematical treatment (*17*) and based on the simplification that an antioxidant of stoichiometry n can be approximated to n independent antioxidant subunits AH that all transfer a single H atom to DPPH with the same second-order rate constant k. Following the addition of a potent antioxidant, the general trend is that the visible absorbance of DPPH quickly decays over a few minutes as a result of the transfer of the n most labile H atoms of the antioxidant (fast step, **Figure 2**). The absorbance vs time curves can be satisfactorily analyzed for different DPPH/antioxidant molar ratios to extract parameters n (**Table 1**) and k. Moreover, considering the bimolecular reaction between DPPH and the hypothetical antioxidant subunit AH, the initial rate of DPPH consumption may be written as $R_0 = kCc_0 = kncc_0$ (c_0, initial DPPH concentration, c, initial antioxidant concentration; $C = nc$, initial concentration of antioxidant subunit AH). Assuming that R_0 is governed by the transfer to DPPH of the first and most labile H atom of the antioxidant (rate constant k_1), we also have $R_0 = k_1cc_0$. Hence, k can be identified with k_1/n. It can be noted that parameters n and k have a somewhat

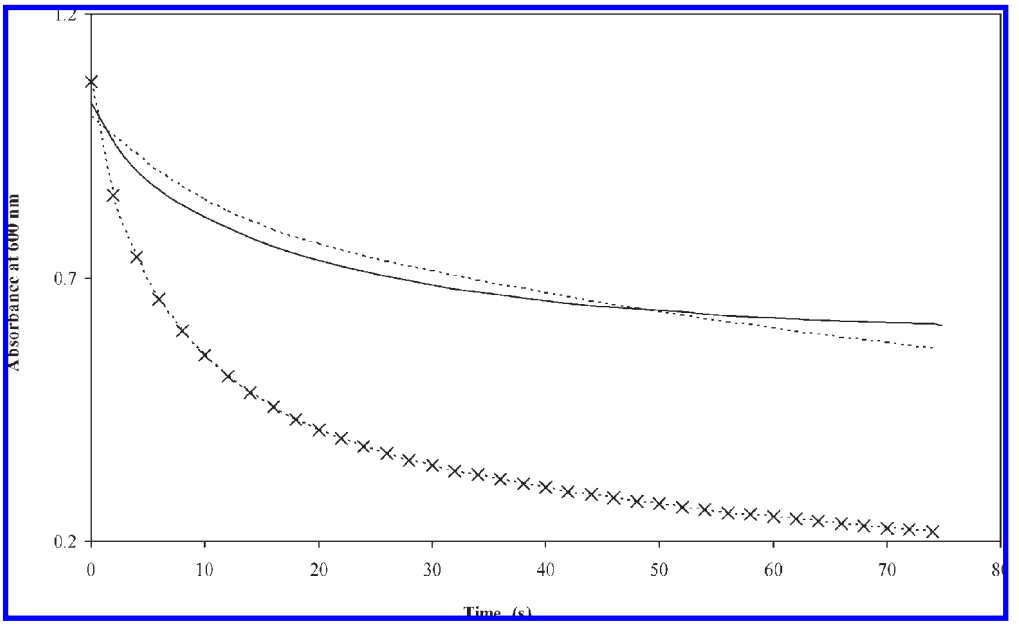

Figure 2. Decay of the visible absorbance (600 nm) of a 0.2 mM DPPH solution in MeOH following the addition of the following red wine polyphenols (25 °C): **1** (dotted line), **3** (full line), **5** (cross and dotted line) (DPPH/antioxidant molar ratio = 6).

arbitrary character, since they depend on the period of time selected for the calculation (T_{calc}), which itself is based on the visual appreciation of the fast step. As such, the n value increases with T_{calc} whereas the k values decreases as a result of slower H-transfers making a larger contribution. However, it is comforting to verify that the kn product is approximately independent of T_{calc} (data not shown) and of the initial DPPH/antioxidant molar ratio (with the exception of **4** and the **1** + **3** mixture; see **Table 1**), thereby providing a reasonable estimate for k_1. In addition, by use of this approach, a k_1 value can be estimated even when a fast step in the DPPH consumption is not easily defined (e.g., pigment **4**; see **Figure 2**).

The fast step is typically followed by a much slower decrease of the visible absorbance featuring the residual H atom donating ability of the antioxidant oxidation products. Hence, experiments extending over 15 min were used for the determination of the total stoichiometry (n_{tot}) of the antioxidant from the total amplitude of the absorbance vs time curves (*17*) (**Table 2**). Overall, k_1 and n_{tot} come up as the most significant kinetic parameters for the measurement of the intrinsic H atom-donating activity. It must be noted that the determination of n_{tot} requires an initial DPPH/antioxidant molar ratio high enough (say, about twice as high as n_{tot}) to exhaust the H-donating activity of the antioxidant and allow the accurate determination of the residual DPPH concentration.

Upon radical scavenging, polyphenols having a 1,2-dihydroxy substitution on their B-ring (catechol nucleus) are converted into semiquinones, which are stabilized by a combination of electronic and intramolecular hydrogen bonding effects. Hence, those polyphenols typically emerge as more potent antioxidants in structure−activity relationships (*17*, *21*). However, in the DPPH assay, **2** ($k_1 \approx 14 \times 10^2$ M^{-1} s^{-1}) and **3** ($k_1 \approx 12 \times 10^2$ M^{-1} s^{-1}), which possess a catechol group, are only slightly more reactive than **1** ($k_1 \approx 9 \times 10^2$ M^{-1} s^{-1}). By contrast, the anthocyanins, especially **2**, display much higher total stoichiometries ($n_{tot} > 10$) than **3** ($n_{tot} \approx 5$). Hence, it can be concluded that, irrespective their B-ring substitution, anthocyanins experience multistep oxidation processes while catechin more rapidly evolves toward stable oxidation products with no residual H-donating activity. This is consistent with flavanols and anthocyanins having very different oxidation pathways, the former leading to mixtures of dimers and oxidized dimers (*22*) and the latter forming degradation products as a result of C-ring cleavage and/or B-ring elimination (*23*). The n_{tot} value of the equimolar **1** + **3** mixture is only slightly lower than the sum of the n_{tot} values of **1** and **3**, which is consistent with an almost additive H-donating activity of both flavonoids.

As for the specific wine pigments, the following observations can be outlined:

(a) On the basis of the k_1 value, pigment **5** ($k_1 \approx 5 \times 10^3$ M^{-1} s^{-1}), which displays **1** and **3** units directly bound to each other, is more reactive than **1** and **3**. By contrast, pigments **4** and **4′**, in which the same units are now bound through a CH−CH_3 linkage, are roughly as reactive as **1** and **3**.

(b) Pyranoanthocyanins derived from **1** (k_1 in the range (11−16) $\times 10^2$ M^{-1} s^{-1}) appear slightly more reactive than the parent anthocyanin. Surprisingly, no clear influence of the groups originating from the entering nucleophile (phenol and carboxyl groups) can be evidenced, in agreement with the H-transfer reaction mainly involving the phenolic OH groups originating from the anthocyanin. In particular, the introduction of a catechol group (**7** vs **6** or **8**) brings no advantage. Pigment **9** is slightly less reactive than the parent anthocyanin **2**. As already observed with the native anthocyanins (**1** vs **2**), the influence of the B-ring substitution (cyanidin vs malvidin) is marginal as evidenced by **7** and **9** having roughly the same k_1 values.

(c) The total stoichiometry n_{tot} shows that the coupling of **1** and **3** through a methylmethine linkage (pigments **4** and **4′**) does not allow scavenging of more DPPH radicals than an equimolar mixture of **1** and **3** ($n_{tot} \approx 14$). It can also be noted that the two epimers cannot be distinguished by the total number of DPPH radicals they are able to reduce. This observation suggests that the same oxidation pathway is followed by pigments **4** and **4′**, which is reasonable. By contrast, the direct biaryl linkage between **1** and **3** (pigment **5**) decreases n_{tot} by a factor 2 ($n_{tot} \approx 7$). Hence, despite its higher reactivity at the onset of the reaction (higher k_1), **5** is more rapidly converted into inert oxidation products than its analogues with a methylmethine linkage. The observation that the antioxidant hierarchy depends on the selected parameters (n_{tot} or k_1) emphasizes the need to characterize antioxidants as completely as possible and validate our multiparameter approach over more global estimations based on IC_{50} values only.

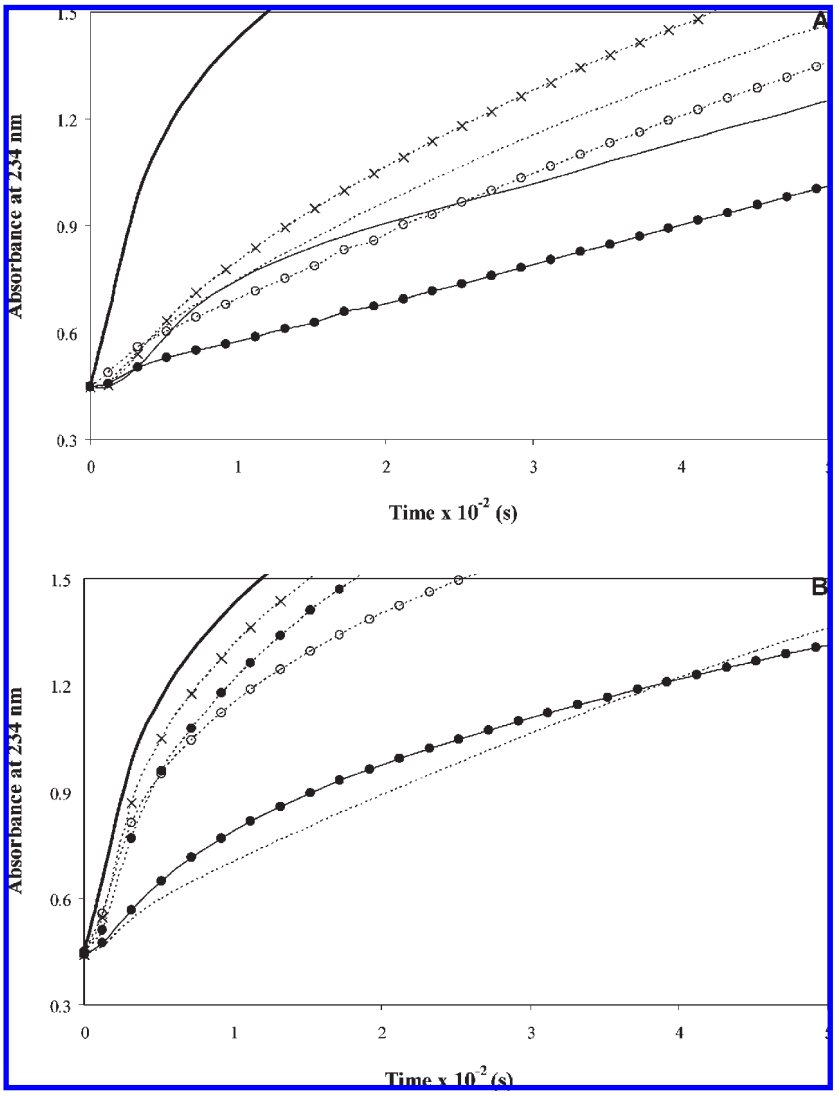

Figure 3. Accumulation of conjugated dienes during the peroxidation of linoleic acid (0.7 mM) induced by metmyoglobin (100 nM) in a pH 4 acetate buffer containing 2 mM Tween-20 and red wine polyphenols (1 μM) (37 °C): (**A**) no antioxidant (full bold line), **1** (dotted line), **3** (full line), **4** (full circle and full line), **4′** (unfilled circle and dotted line), **5** (cross and dotted line); (**B**) no antioxidant (full bold line), **1** (dotted line), **2** (full circle and dotted line), **6** (full circle and full line), **7** (unfilled circle and dotted line), **8** (cross and dotted line).

(d) Irrespective of their substitution on the D-ring, pyranoanthocyanins derived from **1** ($n_{tot} \approx 17$) are able to scavenge more DPPH radicals than **1** ($n_{tot} \approx 11$). This trend is not observed with the pyranoanthocyanin derived from **2** (pigment **9**). Along their oxidative degradation, **2** and **9** reduce ~17 DPPH equivalents, as if the additional catechol group of **9** did not participate in the H-transfers. Overall, the H-donating activities of the pyranoanthocyanins appear very close. As already observed with k_1, no structure−activity relationship can be outlined in the pyranoanthocyanin series. In particular, we have no evidence for the participation of the additional phenolic nuclei stemming from 4-hydroxystyrene metabolites in the H-donating activity.

The antioxidant activity of common anthocyanidin-3-*O*-glucosides and the corresponding carboxypyrano derivatives has been recently investigated via their capacity to reduce Fe^{III} into Fe^{II} (FRAP assay) (*24*). The results, expressed as Trolox equivalents, can be translated in total stoichiometry by assuming that Trolox (the water-soluble analogue of α-tocopherol) can transfer two electrons to Fe^{III} while being converted into inert oxidation products. Thus, the total stoichiometry (or total number of Fe^{III} ions reduced per pigment molecule) is about 6 and 9.5 for **1** and **2**, respectively, and 8.5 and 10 for the carboxypyranoanthocyanins derived from **1** and **2**, respectively. Although the total stoichiometry for electron transfer to Fe^{III} is lower than that for H-atom transfer to DPPH, the trend is the same, i.e., **2** ≥ **8** > **1**.

Inhibition of the Metmyoglobin-Induced Peroxidation of Linoleic Acid in Acidic Emulsions. Although the bioavailability of the wine pigments is essentially undocumented, it is quite unlikely that these bulky hydrophilic polyphenols be more readily absorbed from the gastrointestinal (GI) tract than the native anthocyanins, which themselves are only poorly bioavailable as judged from plasma concentrations (native forms + conjugates) remaining in the range 1−10 nM after absorption of red wine (*25*, *26*). Thus, the GI tract itself comes up as the most likely site for an in vivo antioxidant action of wine pigments. On the other hand, the GI tract can be subjected to various forms of oxidative stress initiated by dietary pro-oxidant species such as nitrite, heme iron, hydrogen peroxide, and hydroperoxides derived from polyunsaturated fatty acids (PUFAs) (*9*). In particular, the presence of heme iron and PUFAs in the diet, combined with the acidity of the gastric compartment, can create the conditions for fast lipid autoxidation processes (*10*, *11*) with subsequent formation of potentially toxic lipid hydroperoxides and carbonyl compounds (*27*). Consequently, some lipid autoxidation products could reach the

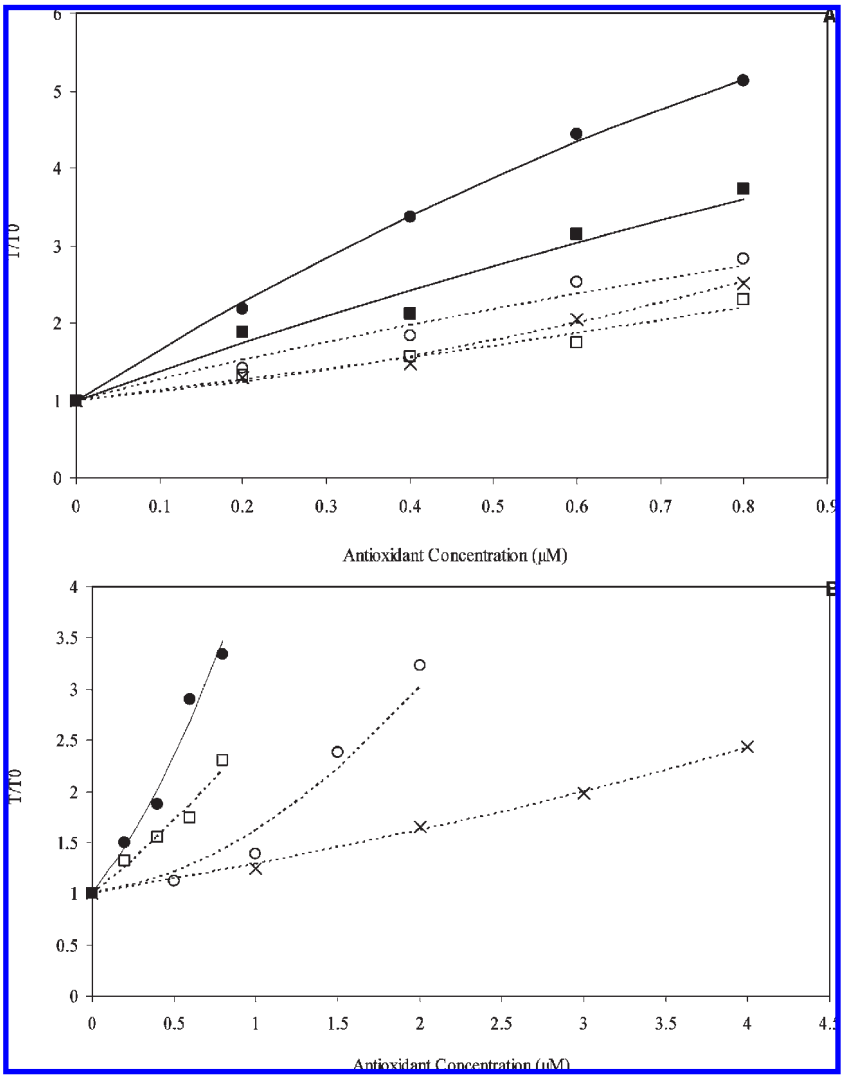

Figure 4. Peroxidation of linoleic acid (0.7 mM) induced by metmyoglobin (100 nM) in a pH 4 acetate buffer containing 2 mM Tween-20 (37 °C): (**A**) **1** (unfilled square), **3** (full square), **4** (full circle), **4′** (unfilled circle), **5** (cross); (**B**) **1** (unfilled square), **6** (full circle), **7** (unfilled circle), **8** (cross) (n = 2).

blood circulation as components of minimally modified LDL particles, which are involved in the early events of atherosclerosis (*28*). Interestingly, it was demonstrated that a moderate intake of red wine during a lipid-rich meal actually lowers the plasma concentration of minimally modified LDL particles (*29*). On the basis of these recent nutritional data, we consider that investigating the inhibition by polyphenols of the heme-induced lipid peroxidation in acidic medium provides the most pertinent and straightforward assay for testing the antioxidant activity of these dietary antioxidants.

In this work, the wine pigments were assayed for their ability to inhibit the metmyoglobin-induced peroxidation of linoleic acid (LH) in a pH 4 Tween-20 solution (*19*). The pH value is aimed at modeling the acidity conditions of the gastric compartment during the early phase of digestion (*30*) when oxidative processes are most likely to take place. At pH 4, hematin ($HtFe^{III}$), the heme cofactor of metmyoglobin, is released as a result of protein denaturation. Hematin is then activated to its hypervalent iron oxo form ferrylhematin ($HtFe^{IV}$=O) by the lipid hydroperoxides (LOOH), including the traces contaminating linoleic acid at the onset of the autoxidation process. Ferrylhematin (or a partially oxidized form) is the likely initiator of lipid peroxidation (*19*). When formation of the conjugated dienes (CDs, mainly LOOH) is monitored at 234 nm, no significant induction period is observed and the initial peroxidation rate (R_p) is maximal (**Figure 3**). Then, R_p tends to decrease as a result of (a) LOOH cleavage gradually making a more important contribution and (b) the release of non-heme iron due to extensive heme degradation. Non-heme iron is still able to initiate the peroxidation process via the homolytic cleavage of LOOH. The wine antioxidants essentially lower the initial peroxidation rate and the rate of the subsequent phase of slower peroxidation (**Figure 3**). With pigments **4** and **4′**, the two steps cannot be distinguished and the peroxidation process simply consists of the slow quasi-linear accumulation of lipid hydroperoxides.

During the inhibited peroxidation, the time T needed to accumulate a given CD concentration after addition of $MbFe^{III}$ (e.g., that corresponding to a 0.7 increase in the absorbance of the conjugated dienes with respect to its value at time zero) is approximately a quadratic function of the antioxidant concentration C (**Figure 4**): $T/T_0 = 1 + aC + bC^2$, T_0 being the time needed to accumulate the same CD concentration in the absence of antioxidant. By defining the IC_{50} parameter as the antioxidant concentration leading to $T = 2T_0$, we readily obtain $IC_{50} = [(a^2 + 4b)^{1/2} - a]/(2b)$. Hence, by fitting the T/T_0 vs C plots against the quadratic law, we obtain parameters a and b, from which IC_{50} can be calculated as well as the time period needed to accumulate the

given CD concentration in the presence of an antioxidant concentration equal to 1 μM (T_1) or 0.5 μM ($T_{0.5}$) (**Table 3**). The IC_{50} parameter is used for a quantitative assessment of the relative antioxidant efficiency. The lower the IC_{50} value, the more effective is the antioxidant.

In this model, the native anthocyanins, especially **2**, appear less efficient than **3** despite their intrinsic higher capacity to scavenge radicals (higher n_{tot} value in the DPPH assay). This trend could point to the highly hydrophilic anthocyanins being essentially distributed in the aqueous phase, whereas catechin may be located in higher proportion at the interface of linoleic acid−Tween-20 micelles where autoxidation is initiated. The methylmethine-bridged pigments (**4** and **4′**) are roughly as potent as **3** and appear more efficient than the biaryl pigment (**5**), in agreement with the DPPH assay. It must, however, be noted that comparisons between the two assays are complicated by the fact that the distribution of each pigment among colored and colorless forms (the latter resulting from reversible solvent addition on chromophores) is likely to differ between a pH 4 aqueous solution (lipid peroxidation assay) and MeOH (DPPH assay).

Table 3. Inhibition of the Metmyoglobin-Induced Peroxidation of Linoleic Acid (pH 4, 37 °C)[a]

antioxidant	IC_{50} [b] (μM)	$T_{0.5}/T_0$ [c]	T_1/T_0 [d]	r [e]
1	0.68	1.70	2.57	0.991
2	1.74	1.25	1.53	0.998
3	0.27	2.74	4.10	0.989
4	0.15	3.89	5.77	0.9995
4'	0.41	2.18	3.04	0.991
5	0.60	1.77	3.14	0.995
6	0.39	2.34	4.36	0.995
7	1.33	1.22	1.62	0.989
8	3.01	1.14	1.28	0.999
9	1.38	1.32	1.69	0.998

[a] Parameters are deduced from the curve-fitting of T/T_0 vs antioxidant concentration (see text and **Figure 4**). [b] IC_{50}: antioxidant concentration for a doubling of the time needed to accumulate a given concentration of conjugated dienes (CD). [c] $T_{0.5}$: time needed to accumulate a given CD concentration in the presence of a 0.5 μM antioxidant. T_0: time needed to accumulate the same CD concentration in the absence of antioxidant. [d] T_1: time needed to accumulate a given CD concentration in the presence of a 1 μM antioxidant. [e] Correlation coefficient.

Since the wine-specific pigments **4**, **4′**, and **5** actually display two flavonoid nuclei instead of only one for **1** and **3**, it is more significant to compare their antioxidant efficiency at half the concentration of their precursors, i.e., for a fixed concentration of flavonoid nuclei. The time needed to accumulate a given CD concentration in the presence of a 1 μM **1** or **3** (T_1) was thus compared to the time needed to accumulate the same CD concentration in the presence of a 0.5 μM **4**, **4′**, and **5** ($T_{0.5}$) (**Table 3**). The ranking is as follows: T_1(**3**) ~ $T_{0.5}$(**4**) > T_1(**1**) ~ $T_{0.5}$(**4′**) > $T_{0.5}$(**5**). As already observed in the DPPH test, linking catechin and an anthocyanin through a methylmethine bridge only weakly lowers the antioxidant activity whereas the direct linkage between the two flavonoid nuclei appears more damaging. Unexpectedly, while pigments **4** and **4′** can reduce the same total number of DPPH radicals, the former is a better inhibitor of the heme-induced peroxidation of linoleic acid (**Figures 3** and **4**, Table **3**).

In the pyranoanthocyanin series, only **6** comes up as a significantly better inhibitor of lipid peroxidation than the parent anthocyanin (**1**). By contrast, the highly hydrophilic pyranoanthocyanin derived from pyruvic acid (**8**) is much less active. Since all the pyranoanthocyanins investigated in this work are roughly as effective at reducing the DPPH radical, it can be inferred that differences in their capacity to inhibit lipid peroxidation essentially reflect their partition in the aqueous vs lipid phase.

Since **1** is better than **2** and since **7** is as efficient as **9**, it can be concluded that a cyanidin nucleus is not a more potent antioxidant unit than a malvidin nucleus, which is unexpected. Similarly, **6** is a better inhibitor of lipid peroxidation than **7** despite the catechol nucleus of the latter. It is thus unlikely that catechol groups are able to bind Fe^{III}, thereby promoting its release from the heme, since this would have certainly resulted in a stronger

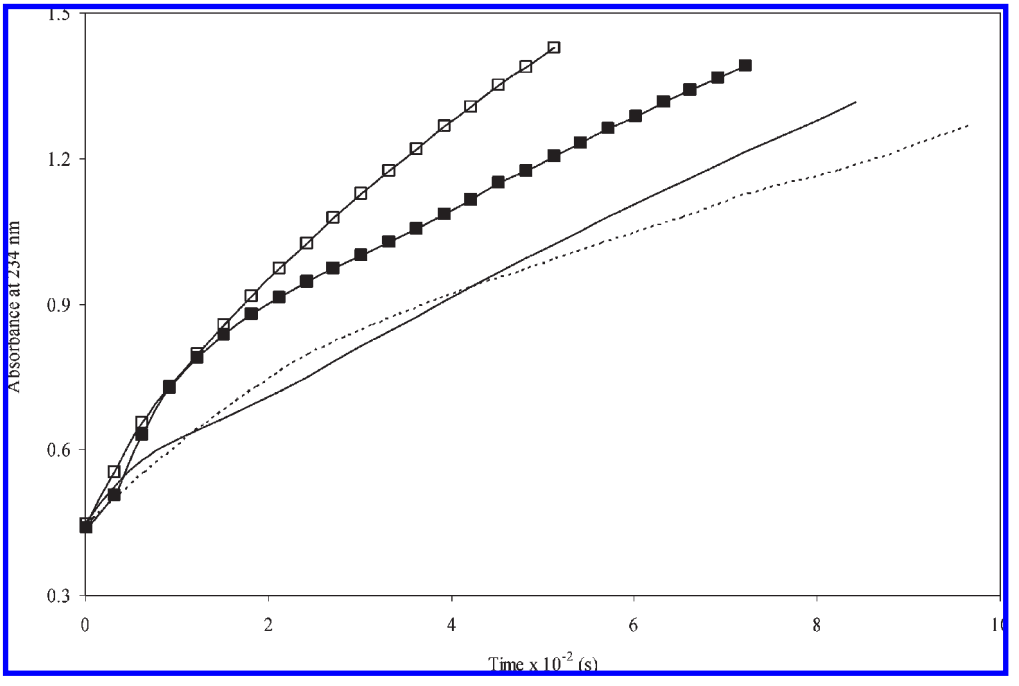

Figure 5. Accumulation of conjugated dienes during the peroxidation of linoleic acid (0.7 mM) induced by metmyoglobin (100 nM) in a pH 4 acetate buffer containing 2 mM Tween-20 and the following red wine polyphenols (1 μM) (37 °C): in the presence of malvidin-3-*O*-β-D-glucoside (**1**, unfilled square) or catechin (**3**, full square) or both (experimental data, full line and simulation assuming additivity, dotted line).

inhibition at the onset of the peroxidation process. Consistently, the addition of **3** or cyanin (cyanidin 3,5-*O*-β-D-diglucoside) to a pH 4 solution of metmyoglobin (flavonoid−heme molar ratio = 10) does not alter the Soret band of the cofactor (data not shown). The DPPH assay has already revealed that catechol groups do not confer a significant advantage in radical scavenging. The adverse effect observed in the inhibition of heme-induced peroxidation could be related to the presence of low metal concentrations contaminating the Tween-20 surfactant. Considering the very low antioxidant concentrations used in this study ($\leq$1 μM) and the high affinity of the catechol groups for hard metal ions, it cannot be excluded that a large fraction of wine pigments bearing catechol groups be deactivated via metal ion binding. It must also be stressed that similar situations may be encountered in the GI tract where loosely bound dietary metal ions may be present in significant concentration. It can also be noted that **2** was found less potent than **1** at inhibiting the peroxidation of linoleic acid in SDS micelles (*31*) and the autoxidation of oil-in-water emulsions (*4*).

From our previous works, it is clear that polyphenols are only modest chain-breaking antioxidants, as evidenced by the lack of well-defined lag phase or induction period during inhibited peroxidation (*18*, *19*, *32*). In fact, their inhibitory properties can be ascribed to their capacity to reduce the initiating species rather than the propagating peroxyl radicals. In this work, RWPs are thus proposed to act by reducing the partially oxidized form of ferrylhematin ($HtFe^{IV}$=O) back to $HtFe^{III}$−OH. In the final phase of slower peroxidation, the peroxidation rate was lower than in the absence of antioxidant (**Figure 3**). This observation suggests that the peroxidation induced by non-heme iron is still inhibited by the wine antioxidants or, more probably, by some of their oxidation products with a residual antioxidant activity.

At pH 4, **1** and **2** are mixtures of colored and colorless forms with the colorless hemiketal making the larger contribution. As for the wine pigments (pyranoanthocyanins, methylmethine-bridged pigments), they are typically much less prone to the water addition step that leads to the colorless forms (a likely consequence of their higher tendency to form noncovalent dimers) (*6*). Consequently, they can be regarded as a mixture of cationic and neutral colored forms in acid−base equilibrium. Besides the higher steric hindrance of the RWPs, such changes in their structural distribution can also affect their lipophilic−hydrophilic balance and antioxidant activity.

The peroxidation curve obtained with a combination of **1** (1 μM) and **3** (1 μM) as inhibitor was not significantly different from the theoretical curve constructed from the curves involving a single antioxidant by assuming additivity (**Figure 5**). Hence, no synergism could be demonstrated. By contrast, a strong synergistic effect between **1** and **3** (interpreted by a recycling of **1** by **3**) was observed in the inhibition of the diazo-initiated peroxidation of linoleic acid in micelles (*31*).

A moderate consumption of red wine has long been considered as cardioprotective even though this hypothesis is still controversial (*33*). Wine polyphenols are actually potent (if only poorly bioavailable) antioxidants, which could efficiently reduce oxidizing species in the GI tract and, more specifically, inhibit the peroxidation of dietary PUFAs in the gastric compartment. The wine-specific pigments formed during wine aging (pyranoanthocyanins, anthocyanin-catechin coupling products) retain a strong H-donating capacity, as evidenced by the DDPH-scavenging test. This strong reducing activity is confirmed in the more relevant situation of the heme-induced lipid peroxidation in mildly acidic solutions. These results suggest a potential protective action of wine pigments against oxidative stress in the gastric compartment.

ACKNOWLEDGMENT

We gratefully thank Pr. M. Taillefer (ENSCM, Montpellier, France) for his collaboration in the 3,4-dihydroxystyrene synthesis.

LITERATURE CITED

(1) Lila, M. A. Anthocyanins and human health: An in vitro investigative approach. *J. Biomed. Biotechnol.* **2004**, *5*, 306–313.
(2) Prior, R. L.; Wu, X. L. Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Res.* **2006**, *40*, 1014–1028.
(3) Brown, J. E.; Kelly, M. F. Inhibition of lipid peroxidation by anthocyanins, anthocyanidins and their phenolic degradation products. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 66–71.
(4) Kahkonen, M. P.; Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. *J. Agric. Food Chem.* **2003**, *51*, 628–633.
(5) Moran, J. F.; Klucas, R. V.; Grayer, R. J.; Abian, J.; Becana, M. Complexes of iron with phenolic compounds from soybean nodules and other legume tissues: prooxidant and antioxidant properties. *Free Radical Biol. Med.* **1997**, *22*, 861–870.
(6) Duenas, M.; Salas, E.; Cheynier, V.; Dangles, O.; Fulcrand, H. UV−visible spectroscopic investigation of the 8,8-methylmethine catechin-malvidin 3-glucoside pigments in aqueous solution: Structural transformations and molecular complexation with chlorogenic acid. *J. Agric. Food Chem.* **2006**, *54*, 189–196.
(7) De Freitas, V.; Mateus, N. Chemical transformations of anthocyanins yielding a variety of colours (Review). *Environ. Chem. Lett.* **2006**, *4*, 175–183.
(8) Rentzsch, M.; Schwarz, M.; Winterhalter, P. Pyranoanthocyanins—an overview on structures, occurrence, and pathways of formation. *Trends Food Sci. Technol.* **2007**, *18*, 526–534.
(9) Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?. *Am. J. Clin. Nutr.* **2005**, *81*, 268S–276S.
(10) Kanner, J.; Lapidot, T. R. The stomach as a bioreactor: dietary lipid peroxidation in the gastric fluid and the effects of plant-derived antioxidants. *Free Radical Biol. Med.* **2001**, *31*, 1388–1395.
(11) Lapidot, T.; Granit, R.; Kanner, J. Lipid hydroperoxidase activity of myoglobin and phenolic antioxidants in simulated gastric fluid. *J. Agric. Food Chem.* **2005**, *53*, 3391–3396.
(12) Gorelik, S.; Ligumsky, M.; Kohen, R.; Kanner, J. A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. *FASEB J.* **2008**, *22*, 41–46.
(13) Fulcrand, H.; Cameira dos Santos, P. J.; Sarni-Manchado, P.; Cheynier, V.; Favre-Bonvin, J. Structure of new anthocyanin-derived wine pigments. *J. Chem. Soc., Perkin Trans. 1* **1996**, 735–739.
(14) Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments yielded by reactions between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401–1407.
(15) Salas, E.; Atanasova, V.; Poncet-Legrand, C.; Meudec, E.; Mazauric, J.; Cheynier, V. Demonstration of the occurrence of flavanol−anthocyanin adducts in wine and in model solutions. *Anal. Chim. Acta* **2004**, *513*, 325–332.
(16) Hakansson, A. E.; Pardon, K.; Hayasaka, Y.; de Sa, M.; Herderich, M. Structures and colour properties of new red wine pigments. *Tetrahedron Lett.* **2003**, *44*, 4887–4891.
(17) Goupy, P.; Loonis, M.; Dufour, C.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615–622.
(18) Vulcain, E.; Goupy, P.; Caris-Veyrat, C.; Dangles, O. Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: Action in the aqueous vs. lipid phase. *Free Radical Res.* **2005**, *39*, 547–563.
(19) Goupy, P.; Vulcain, E.; Caris-Veyrat, C.; Dangles, O. Dietary antioxidants as inhibitors of the heme-induced peroxidation of linoleic acid: mechanism of action and synergism. *Free Radical Biol. Med.* **2007**, *43*, 933–946.
(20) Sanchez-Moreno, C. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.* **2002**, *8*, 121–137.

(21) Wolfe, K.; Liu, R. H. Structure−activity relationships of flavonoids in the cellular antioxidant activity assay. *J. Agric. Food Chem.* **2008**, *56*, 8404–8411.
(22) Guyot, S.; Vercauteren, J.; Cheynier, V. Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry* **1996**, *42*, 1279–1288.
(23) Dangles, O.; Fargeix, G.; Dufour, C. Antioxidant properties of anthocyanins and tannins: a mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1653–1663.
(24) Jordheim, M.; Aaby, K.; Fossen, T.; Skrede, G.; Andersen, O. M. Molar absorptivities and reducing capacity of pyranoanthocyanins and other anthocyanins. *J. Agric. Food Chem.* **2007**, *55*, 10591–10598.
(25) Garcia-Alonso, M., Minihane, A.-M.; Rimbach, G.; Rivas-Gonzalo, J. C.; de Pascual-Teresa, S. Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. *J. Nutr. Biochem.*, in press.
(26) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.
(27) Cohn, J. S. Oxidized fat in the diet, postprandial lipaemia and cardiovascular disease. *Curr. Opin. Lipidol.* **2002**, *13*, 19–24.
(28) Lapointe, A.; Couillard, C.; Lemieux, S. Effects of dietary factors on oxidation of low-density lipoprotein particles. *J. Nutr. Biochem.* **2006**, *17*, 645–658.
(29) Natella, F.; Ghiselli, A.; Guidi, A.; Ursini, F.; Scaccini, C. Red wine mitigates the postprandial increase of LDL susceptibility to oxidation. *Free Radical Biol. Med.* **2001**, *30*, 1036–1044.
(30) Tyssandier, V.; Reboul, E.; Dumas, J. F.; Bougteloup-Demange, C.; Armand, M.; Marcand, J.; Sallas, M.; Borel, P. Processing of vegetable-borne carotenoids in the human stomach and duodenum. *Am. J. Physiol.* **2003**, *284*, G913–G923.
(31) Rossetto, M.; Vanzani, P.; Mattivi, F.; Lunelli, M.; Scarpa, M.; Rigo, A. Synergistic antioxidant effect of catechin and malvidin 3-glucoside on free radical-initiated peroxidation of linoleic acid in micelles. *Arch. Biochem. Biophys.* **2002**, *408*, 239–245.
(32) Roche, M.; Dufour, C.; Mora, N.; Dangles, O. Antioxidant activity of olive phenols: mechanistic investigation and characterization of oxidation products by mass spectrometry. *Org. Biomol. Chem.* **2005**, *3*, 423–430.
(33) De Lange, D. W. From red wine to polyphenols and back: A journey through the history of the French Paradox. *Thromb. Res.* **2007**, *119*, 403–406.

Received March 12, 2009. Revised manuscript received May 20, 2009. Accepted May 25, 2009.