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Free radical scavenging effect of anthocyanins in red wines

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Abstract

Free radicals are extremely harmful to living organisms in that, they attack different constituents of the cell, leading to acceleration of the ageing process and sometimes even its destruction, or if the DNA is affected, irreversible malfunctions. It is now widely recognised that the phenolic compounds of wine have very high free radical scavenging potential. The aim of this paper is to determine which of these phenolic compounds are responsible for the strong free radical scavenging potential of red wines. In order to do so, a red wine was fractionated into phenolic fractions. After extraction and purification of these compounds from the wine, we have measured their free radical scavenging activities using an enzymatic method. The anthocyanic fraction showed a high free radical scavenging power in relation to the other tannic fractions. In order to explain this phenomenon, some pure anthocyanins were studied and a relationship between their free radical scavenging activity and their molecular structure was suggested. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: French paradox; Phenolic compounds; Anthocyanins; Radical scavenger

1. Introduction

Free radicals are highly reactive metabolites which oxidise the constituents of the cell, and in particular the membrane, thus accelerating its ageing and destruction (Ames, 1983; Pryor, 1976–1982; Pryor, Tamura, Dooley, Premovic & Church, 1983). An excess of free radicals causes accelerated ageing as well as lesions of the tissues, thus disturbing the balance of the organism. These imbalances have been related to cardiovascular diseases, inflammation and skin problems induced by solar radiation, etc. The harmful action of the free radicals can, however, be blocked by substances known as free-radical “scavengers”, which detoxify the organism. Current research into free radicals has confirmed that food, rich in antioxidants, play an essential role in the prevention of cardiovascular diseases (Fuhrman, Lavy & Aviram, 1995; Renaud & de Lorgeril, 1992), cancers (Dragsted, Strube & Larsen, 1993; Wood et al., 1982)

and neurodegenerative diseases, the most well-known of which are Parkinson's and Alzheimer's diseases (Okuda, Yoshida & Hatano, 1992), as well as inflammation (Lietty, Cristoni & Picci, 1976) and problems caused by cell and cutaneous ageing (Ames, Shigena & Hagen, 1993). An adequate intake of these free radical scavengers (flavonoids, anthocyanins...) is necessary for efficient prevention of these degenerative disorders (Kanner, Franel, granit, German & Kinsella, 1994).

We have already demonstrated that red wine has great antioxidant potential, due to the phenolic compounds (tannins and anthocyanins) which are present in sufficient quantities to ensure optimal free radical scavenging activity of the compounds and even combined action between them leading to a synergic effect of these polyphenols (Saint-Cricq de Gaulejac, Provost & Vivas, 1999). The protective effects of red wines on the hepatic cells in the livers of rats which were bombarded with free radicals (generated by irradiation) have also been shown during immunochemical tests (Saint-Cricq de Gaulejac, Provost & Vivas, 1999). Therefore, we are now trying to determine which of these phenolic compounds are most likely to provide a protective effect for

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free radical action. The objective of this study is to analyse the protective effects of the polyphenols in order to select the most efficient molecules for pharmaceutical use. On the other hand, this study will enable us to approach the oxidoreduction mechanisms in wines, because radical reactions of polyphenols are generally a source of oxidoreduction phenomena in red wines.

With this objective in mind, the polyphenols in wine were extracted and purified before measuring their free radical scavenging activity (% inhibition of O_2^-). In order to do so, a red wine was fractionated using the Glories method (Glories, 1978) into 5 phenolic fractions, the tannins and anthocyanins present in these fractions were measured and, finally, their anti-radical capacity, expressed as the concentration required to inhibit 50% of the O_2^- radicals, was measured using an enzymatic method (HPX-XOD system) (Hodgson & Fridovich, 1976).

2. Materials and methods

2.1. Chemical products

The xanthine oxidase (XOD) and the hypoxanthine (HPX) were obtained from Sigma Aldrich Chimie (Saint-Quentin Fallavier, France). The anthocyanins (malvidin chloride, malvidin-3-mono-glucoside, cyanidin chloride, cyanidin-3-mono-glucoside, delphinidin chloride, peonidin chloride, peonidin-3-mono-glucoside) were bought from Extrasynthèse SA (Genay, France).

2.2. Fractionation and isolation of the phenolic compounds of red wine

According to the method defined by Glories (1978), the phenolic compounds of wine can be fractionated into 5 groups of phenolic families:

- Tannins-polysaccharides, tannins-salts (TP-TS)
- Very condensed tannins (vcT)
- Procyanidins and catechins (P-C)
- Tannin-anthocyanin complexes (T-A)
- Free anthocyanins (fA)

These fractions were recovered, solvent was evaporated, then the fractions were dissolved in H_2O before measuring their phenolic content and testing their free radical scavenging activity.

2.3. Isolation of free anthocyanin fractions of grape skins

One hundred berries of selected grapes were picked to recover the free anthocyanin fraction. Skins were separated from grapes, freeze-dried, grown to a powder and subjected to homogenization for 2 min in a blender with

100 ml of hydroalcoholic solution (ethanol 12% and HCl to adjust pH at 3.2). The medium was agitated during the next 6 h and then centrifuged (20 min, 4000 g). The supernatant was filtered to eliminate insoluble fractions (Millipore filter, 0.45 μ m). Skin extracts obtained were passed through a PVPP column. Non-phenolic constituents were washed by H_2O (150 ml). Fraction of free anthocyanins were eluted by MeOH acidified by 5% of HCOOH (150 ml) and collected. Solvent was evaporated and the fractions were dissolved in 100 ml of H_2O to analyze their free radical scavenging activity.

2.4. Quantification of the phenolic compounds

2.4.1. Total anthocyanins

This method is based on the discoloration of the anthocyanins when reacted with an excess of SO_2 (Ribéreau-Gayon & Stonestreet, 1965). As certain T-A combinations are not discoloured (the TAT), the results obtained are higher than the actual values.

2.4.2. Total tannins (LA) and estimating the corrected tannins (T)

This measurement is based on the ability of proanthocyanidins to be transformed into anthocyanidins in an acid medium at 100°C (Ribéreau-Gayon & Stonestreet, 1966). All the tannins are converted during this reaction, including those combined with other phenolic compounds. The measurements were corrected (Glories, 1988, results not published) in order to take into account the errors that can be attributed to some of these combinations. The quantifications are based on the measurement of OD at 470, 520 and 550 nm of the samples before and after acid hydrolysis at 100°C. The differences between the wavelengths of the (heated) sample and (unheated) control are noted at specified wavelengths. The corrections made to the measurement value of the tannins include:

- (a) Calculation of the ΔOD_{570c} from the measured ΔOD_{470} (m):

$$\Delta OD_{570c} = 0.715 \times \Delta OD_{470m}$$

If the value of ΔOD_{570c} obtained is lower than that measured at this wavelength, we keep the measured ΔOD_{470} value. If, on the contrary, the value of ΔOD_{570} obtained is higher than that measured at this wavelength, we keep the value measured at 570 nm so as to obtain, according to the same equation, the calculated ΔOD_{470} .

(b) With the measured value of ΔOD_{470} in the first case, or the calculated ΔOD_{470} in the second case, we calculate the value of ΔOD_{520c} according to this equation: $\Delta OD_{520c} = 1.1 \times \Delta OD_{470}$

(c) We obtain the value of the corrected proanthocyanidic tannins T:

$$T(\text{g/l}) = \text{OD}_{520\text{c}} \times 15.7$$

2.5. Estimation of free radical scavenging activity: hypoxanthine–xanthine oxidase enzymatic system specific to superoxide radicals: $\text{O}_2^{\cdot-}$ (Hodgson & Fridovich, 1976)

The hypoxanthine–xanthine oxidase (HPX-XOD) system generates superoxide $\text{O}_2^{\cdot-}$ radicals. At pH 7.4, these $\text{O}_2^{\cdot-}$ radicals reduce the nitroblue tetrazolium to a blue formazan product ($\text{max}\lambda_{560}$). In the presence of free radical scavengers (such as the phenolic compounds), the formation of the blue formazan is proportionally reduced (λ_{560} decreases). λ_{560} is thus proportional to the production of $\text{O}_2^{\cdot-}$ radicals. The nitroblue tetrazolium (NBT, 10^{-3} M) was prepared in a TRIS buffer (T, HCl 0.05 M pH7.4). The hypoxanthine solution (H, 5×10^{-3} M) and the xanthine oxidase solution (XOD, 1.67 u/ml) were prepared in the same buffer. The tested compounds (pure anthocyanins, wines, fA fractions) were dissolved in H_2O . All the spectrophotometric data were recorded on a Kontron Uvikon 810 spectrophotometer. Disposable cuvettes ($1 \times 1 \times 4.5$ cm) from Muller Ratiolab (Dreieich, Germany) were used for absorption measurements. The cuvette O contained 2400 μl of Tris buffer (T), 100 μl of solution NBT and 500 μl of solution H. The cuvette 1 contained 2300 μl of T, 100 μl of NBT, 500 μl of H and 100 μl of solution XOD. The cuvette 2 enclosed 2200 μl of T, 100 μl of NBT, 500 μl of H, 100 μl of XOD and 100 μl of sample. In each case, the reduction in absorbance was determined at 560 nm every minute for 10 min. The gradient of cuvette 1 (P_1) represents the maximum effect of superoxide $\text{O}_2^{\cdot-}$ anions ($\approx 100\%$), whereas the gradient of cuvette 2 (P_2) represents the effect of these $\text{O}_2^{\cdot-}$ free radicals trapped by the tested compounds. The quantity of residual superoxide $\text{O}_2^{\cdot-}$ anions can thus be calculated and is expressed by the relation:

$$\text{residualO}_2^{\cdot-} = (P_2 - P_0)/(P_1 - P_0) \times 100$$

The free radical scavenging activity may be defined by the quantity of antioxidant necessary to inhibit 50% of the $\text{O}_2^{\cdot-}$ radicals generated by the XOD-HPX system [efficient concentration = EC_{50} (mol/l of phenolic compounds)]. The activity of XOD can be considered as constant since the inhibition of its activity is independent of the reagents and products involved in this reaction. Indeed, XOD activity decreases during time and significantly after 30 min. So, it is advisable to remake the XOD solution every third samples. In the phenolic fraction case, the results can be expressed in equivalent

malvidin-mG (the most important anthocyanin in the anthocyanic fractions issuing from wines or grapes) in order to compare their scavenging activity with those of some pure anthocyanic molecules.

3. Results and discussion

3.1. Determination of the phenolic compounds responsible for free radical scavenging activity in a red wine

A red Merlot wine (1996) that was fermented in vats (RWv) was fractionated according to the Glories method into 5 fractions: tannins-polysaccharides–tannins-salts (TP–TS); very condensed tannins (vcT); procyanidins and catechins (P–C); tannin-anthocyanin complexes (T–A); free anthocyanins (fA). The vinification in vats ensures the absence of the phenolic compounds likely to be released into the wine by the wood, such as ellagitannins or lignin fractions, and simplifies the fractionation of the wine and the determination of the free radical scavengers. These five groups of compounds were separated and the tannins and anthocyanins present were measured before estimating their free radical scavenging capacity (% inhibition of $\text{O}_2^{\cdot-}$). The results of these measurements and the free radical scavenging capacity, measured by the XOD-HPX system, are shown in Table 1. It can immediately be noticed that the anthocyanic fA fraction, corresponding to the group of free anthocyanins which are not combined with the tannins, is the most efficient in scavenging free radicals ($\approx 91\%$ of $\text{O}_2^{\cdot-}$ radicals inhibited by this fraction). We can, thus, conclude that the free anthocyanins are the strongest anti-radical polyphenols in red wines, all the more since the results of the analysis (Table 1) clearly show that this fA fraction is of the lowest concentration (289 mg/l) in relation to the other essentially tannic fractions (around 1 g/l).

In order to generalize these results, we have realized the same experiment on different red wines. The results obtained are reported in Table 2. So, we can note that, whatever the origin of wines and the nature of vine, the distribution of anti-radical activity, within the different phenolic fractions, stay approximately the same. Namely, the fA fraction is the more active to trap $\text{O}_2^{\cdot-}$ radicals, followed by the vcT fraction, then by the TP–TS fraction, the P–C fraction and the T–A fraction is the less efficient. So, this classifying is independent of vine choice and wine origin.

3.2. Contribution of anthocyanic molecular structure at their free radical scavenging potential

First, we investigated the $\text{O}_2^{\cdot-}$ scavenging effect of some pure anthocyanins shown in Fig. 1 These phenolic compounds were tested with the HPX-XOD system, in a series of concentrations from 1.5 to 400 mg/l. We

observed that these molecules scavenged $O_2^{\cdot-}$ in a dose-dependent manner and the concentrations of anthocyanins causing 50% decrease of $O_2^{\cdot-}$ (EC_{50} values) were determined and are listed in Table 3. These high reactivities observed to superoxide radicals that were observed can be explained by the fragility of the structure of the anthocyanin molecules (oxonium ion, Fig. 1), thus making them more rapidly oxidizable on opening of the C ring. A hypothetical reaction diagram is proposed in Fig. 2, but we should point out that these arguments concerning reaction mechanisms have been formulated for the superoxide $O_2^{\cdot-}$ anion, that is to say for the negatively-charged free radicals which are likely to react first and principally with the (positively-charged) oxonium of the anthocyanins. In this case, the pathway A, corresponding to the attack of the flavylum cation (C ring) may be more probable. The results can thus not be extrapolated to other species of uncharged free radicals such as $^{\circ}OH$, $^{\circ}OOH$. However, the anthocyanins in grapes and wines from *Vitis vinifera* are only in their heteroside monoglucoside forms (Ribéreau-Gayon, 1973), having a glucose molecule attached to the hydroxyl function of the C_3 carbon of the oxygenated heterocyclic ring C. We thus took the precaution of checking to see if it is the glucose that was responsible

for the strong free radical scavenging activity of wine anthocyanins. These experiments showed that the glucose, in the XOD-HPX system only shows free radical scavenging potential at concentrations above 100 g/l, which is never the case in dry wines. Moreover, at concentrations similar to those of the anthocyanins (300 mg/l), glucose is even a pro-oxidant. Thus, the anti-radical activity of the fA fraction of wines is, therefore, due to the anthocyanins and not to the glucose moiety attached to them. We likewise compared the anti-radical activity of the malvidin chloride (aglycone) to that of the malvidin-3-mono-glucoside (3mG) (Fig. 3). It was noticed that the malvidin-3mG always has greater anti-radical activity than malvidin aglycone. This goes against earlier results, given that the aglycone has an additional hydroxyl function and that the glucose in the 3mG is not active on $O_2^{\cdot-}$ radicals. We can nevertheless explain this high difference in conformational states of malvidin-3mG and malvidin aglycone. Malvidin-3mG certainly adopts a conformation which is different to that of malvidin aglycone, due to the presence of the glucose, and thus may be predisposed to oxidation. We can assume that the benzene ring B of the malvidin chloride fixes onto the oxygenated heterocyclic ring C, so blocking the oxonium function of that heterocycle and thus the radical attack at that point, and then, we know that in our conditions, the oxidation reaction is essentially situated in C ring. In contrast, the oxidation

Table 1

Results of measurement of the phenolic compounds in the different fractions of a red wine (Merlot, 1996) aged in vats

	Phenolic fractions	[Corrected tannins] g/l	[Anthocyanins] mg/l	% Inhibition of $O_2^{\cdot-}$
1	fA	—	289	90.9
2	vcT	1036	—	87.4
3	TP,TS	527	—	69.5
4	P-C	95	—	67.2
5	T-A	95	67	60.1
6	Total	1753	356	86.0

Table 2

Scavenging activity of the phenolic fractions of different red wines

Red wines (1996)		$O_2^{\cdot-}$ Scavenging effect					
		RW ^a	TP, TS ^b	vcT ^b	P-C ^b	T-A ^b	fA ^b
1	Assembled vines, Bordeaux, France	91	3	2	4	5	1
2	Assembled vines Bourgogne, France	79	4	2	3	5	1
3	Merlot, Saint-Emilion, France	86	3	2	4	5	1
4	Cabernet Sauvignon, Saint-Emilion, France	92	3	1	5	4	2
5	Cabernet Franc, Saint-Emilion, France	87	3	2	4	5	1
6	Cabernet Sauvignon, California, USA	74	3	2	4	5	1
7	Pinot noir, California, USA	79	4	1	3	5	2
8	Tempranillo, Rioja, Spain	73	3	2	5	4	1
9	Cabernet sauvignon, Stellenbosh, South-Africa	88	3	1	4	5	2
10	Xynomavro, Goumenissa, Greece	69	4	2	3	5	1
Mean		81.8	3.3→3	1.7→2	3.9→4	4.8→5	1.3→1

^a Percentage of inhibited $O_2^{\cdot-}$ radicals.

^b 1-The more efficient fraction towards $O_2^{\cdot-}$ radicals; 2- the second efficient fraction; 3-the third efficient fraction; 4-the fourth efficient fraction; 5-the less efficient fraction.

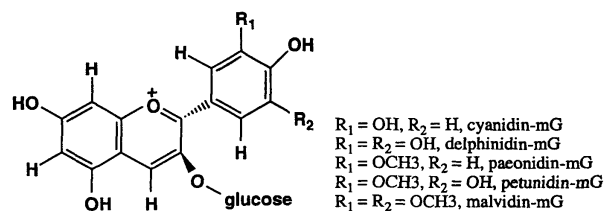


Fig. 1. Structure of grape and wine anthocyanins.

reactions of the malvidin-3mG would in fact appear to be facilitated by the attack of the heterocyclic ring C sterically: the presence of glucose would flatten the benzene ring B of this molecule, disengaging the C ring and facilitating the radical attack on oxonium (pathway

Table 3
Concentrations of pure anthocyanins and fA fractions causing a 50% decrease of $O_2^{\cdot-}$, EC_{50} values in an HPX-XOD system

Compounds	EC_{50} (mg/l) ^a	EC_{50} (μm/l) ^a
<i>Pure anthocyanins</i>		
Delphinidin	170 ± 4 (6)	565 ± 13 (6)
Cyanidin	250 ± 9 (6)	877 ± 31 (6)
Cyanidin-3mG	325 ± 11 (6)	724 ± 24 (6)
Peonidin	404 ± 21 (5)	1351 ± 43 (5)
Peonidin-3mG	517 ± 23 (5)	1117 ± 32 (5)
Malvidin	380 ± 7 (5)	1155 ± 21 (5)
Malvidin-3mG	412 ± 12 (5)	837 ± 24 (5)
<i>fA fractions of grape skins^b</i>		
Cabernet Sauvignon	97 ± 4 (6)	197 ± 8 (6)
Cabernet Franc	104 ± 5 (5)	210 ± 10 (5)
Merlot	99 ± 4 (5)	201 ± 8 (5)
<i>fA fractions of red wines^b</i>		
Cabernet Sauvignon	109 ± 7 (5)	221 ± 14 (5)
Cabernet Franc	114 ± 8 (6)	231 ± 16 (6)
Merlot	102 ± 5 (5)	207 ± 10 (5)

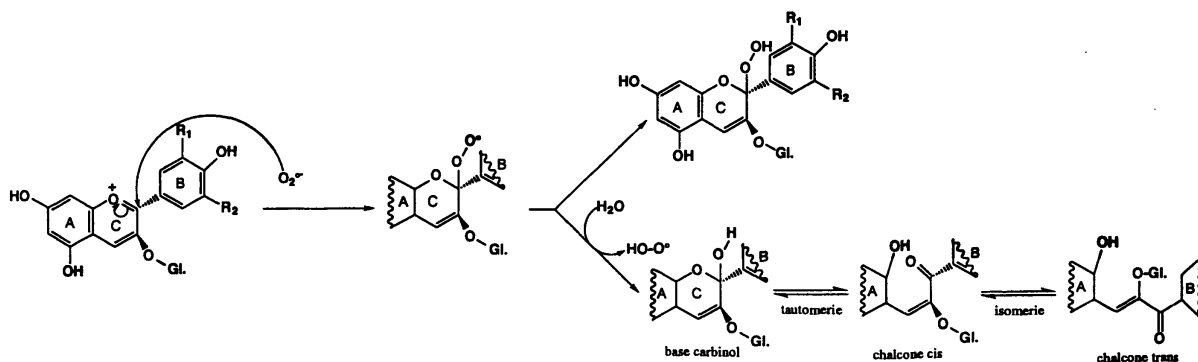
^a Numbers represent means ± SD of results from 4 to 6 (numbers in brackets) different preparations.

^b EC_{50} (μm/l) in equivalent Malvidin-mG.

A). The calculations of molecular modelization currently under way are likely to confirm these hypotheses. Nevertheless, we noted (Table 3) that the different substitutions between anthocyanins have an influence upon their ability to trap $O_2^{\cdot-}$ radicals, although the radical reactions essentially intervenes on oxonium. Indeed, we observed a lesser efficiency for malvidin and peonidin which could be due to the presence of methoxy groups in the lateral ring bringing about a small sterical cluttering.

On the other hand, the same experiment realized on free anthocyanin fractions of skins and wines (fA) showed a clearly more important effectiveness for these anthocyanic fractions in relation to pure anthocyanin molecules. Indeed, anthocyanin distribution in skins and wines (mixing of five anthocyanins) is such that the $O_2^{\cdot-}$ scavenging action of grape and wine is more efficient than that of only one anthocyanin-mG in the same concentration. This phenomenon can explain itself by synergistic effects between anthocyanins. In support of this theory is the finding that fA fractions of skin and wine extracts have a stronger $O_2^{\cdot-}$ scavenging activity (low EC_{50} values cf. Table 3) than pure anthocyanin solutions. It is interesting to note that some analogous results have been obtained about dimer procyanidins of seed and wine extracts (Saint-Cricq de Gaulejac, Provost & Vivas, 1999). Experiments on another active oxygenated species produced by the Fenton reaction, and on the stable DPPH° radical which is in the market, are currently under way in our laboratory in order to extend our results.

1^{er} pathway: attack of the cycle C



2^{er} pathway: attack of the cycle B

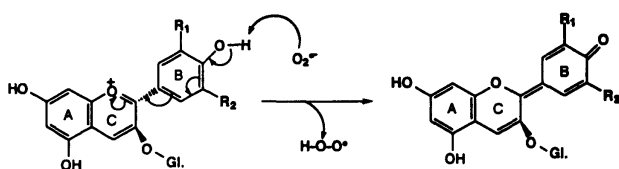


Fig. 2. Diagram of the different hypothetical pathways of oxidation of the anthocyanins by the superoxide $O_2^{\cdot-}$ radical. As the radical is negatively charged, pathway A (attack of the flavylium cation) may be more probable.

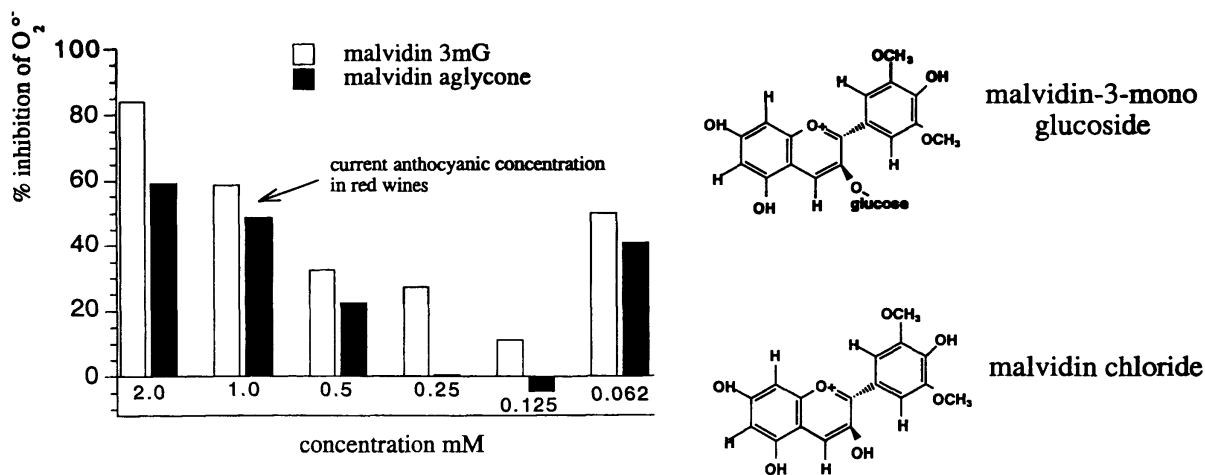


Fig. 3. Comparison between malvidin chloride and malvidin-3-mono-glucoside in aqueous solution.

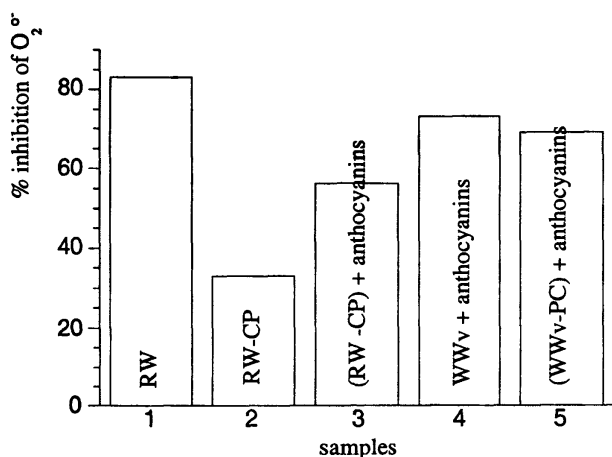


Fig. 4. Free radical scavenging activity (expressed as a percentage of inhibition of $O_2^{\bullet -}$ radicals) of the different samples: 1- RW: 1996 red wine containing 409 mg/l of anthocyanins (measured by discoloration with sodium bisulphite). 2- RW-PC: RW treated with active carbon (loss of all phenolic compounds). 3- (RW-PC) + anthocyanins: (RW-PC) to which 409 mg/l of anthocyanins were added. 4- WWv + anthocyanins: WWv to which 409 mg/l of anthocyanins were added. 5- (WWv-PC) + anthocyanins: (WWv-PC) to which 409 mg/l of anthocyanins were added.

3.3. The participation of anthocyanins in the free radical scavenging activity of a red wine

To visualise the effect of anthocyanins in the free radical scavenging activity of a red wine, wine RWv, which contains a total of 409 mg/l of anthocyanins (measured by discoloration with sodium bisulphite), was treated with active carbon in order to remove all its phenolic content (RWv - PC). The free radical scavenging activity of RWv and RWv - PC are shown in Fig. 4. We can note that RWv has a free radical scavenging potential of 83%. Once the phenolic compounds have been removed, it loses a large part of its scavenging

potential, thus demonstrating that the essential part of the free radical scavenging power of wines can be attributed to their phenolic compounds. Secondly, we can see (Fig. 4) that the addition of 400 mg/l of anthocyanins (extracted from the skins of Merlot grapes) to the treated RWv (RWv - PC) leads to an increase of 70% in this activity in comparison with the control wine (RWv). We can thus demonstrate the importance of the role of the anthocyanins in the free radical scavenging activity of red wines. Likewise, we checked that the addition of this concentration of anthocyanins to a white wine fermented in vats (WWv) led to a rate of inhibition of $O_2^{\bullet -}$ radicals quite close to that of the control wine (RWv). We can thus conclude that the anthocyanins are in large part responsible for the scavenging activity of red wines.

4. Conclusion

Over the last few decades, scientists have confirmed certain observations relating to the French Paradox phenomenon, by carrying out many epidemiological studies (Doll, 1990; Gey, 1990). They have shown that moderate consumption of red wine reduces the risk of cardiovascular diseases and mortality rates due to coronary ischaemia by 25 to 60%. Certain phenolic substances found specifically in the non-alcoholic fraction of the wines behave as antioxidants, limiting the oxidation of the lipids and thus bringing about the effects of protection of the heart. The essential property of these phenolic compounds is their ability to capture the free radicals which are very harmful, attacking the different constituents of the cell and thus leading to its destruction or to irreversible malfunctions (Ames, 1983; Pryor, 1976-1982). The phenolic compounds conserved in the wine could thus act to prevent the degenerative diseases such

as cancers or neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, which to a large extent, involve free radical attacks on the cells.

This study has enabled us to define which phenolic compounds are most likely to participate in this anti-radical protection. We have thus demonstrated the importance of the anthocyanins in red wines, which participate in this notion of the "French Paradox". These molecules have been most often studied for their participation in the colour of red wines (as a pigment). However, they remain rarely cited for their participation in the free radical scavenging potential of red wines, usually attributed to procyanidic tannins, although several studies on free radical scavenging properties of anthocyanins previously existed in the literature (Larson, 1988).

Moreover, this study has enabled us to show a "synergistic effect" between anthocyanins bringing about a stronger O_2^- scavenging activity for anthocyanic fractions of wines and skins compared with the pure anthocyanin molecules. We must now try to explain this phenomenon which is also observed about procyanidic tannins of seed and wine extracts. . . On the other hand, an experiment is currently underway in our laboratory to study the behaviour of these anthocyanic scavengers directly on cells and, from the pharmaceutical point of view, it would be interesting to study and improve the penetration of these compounds into the cells, in order to ensure that they act as efficiently as possible.

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