Free Radical Research, Vol. 35, pp. 933–939
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Stable Free Radicals as Ubiquitous Components of Red Wines

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Accepted by Professor J.V. Bannister

(Received 17 November 2000; In revised form 20 March 2001)

A stable ESR signal, centred at $g = 2.0037 \pm 0.0002$, characterised by a single resonance and assignable to a free radical, was found in all the bottled red wines, both commercial and experimental, that we have examined. The radical concentration was calculated to be in the range of 5–82 nM. After exposure of the wines to air for a few minutes a two fold increase of the ESR signal, followed by a slow decrease with time, was observed. The intensity of ESR signal in experimental red wines, was found to increase with the ageing of the wines and was strictly correlated to the total content of polyphenols. The formation of semiquinone radicals of polyphenols is suggested as one possible mechanism leading to the presence of stable free radicals in red wines.

Keywords: Wine; Free radicals; Polyphenols; ESR

INTRODUCTION

Free radicals are usually rather unstable species because of the high reactivity. As a consequence the ESR signals of relatively reactive free radicals can be observed in solution for a prolonged period of time only in the absence of species that can react with them or when they are the product of equilibrium between reduced and oxidised species as in the case of living polymers and of semiquinone radicals, respectively. In the case of living polymers very reactive radicals are generated in aprotic solvents and, under favourable experimental conditions, may exist

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indefinitely.[1] Semiquinone radicals ($Q^{-\cdot}$) may generate rather stable ESR signal since they are in equilibrium with their parent compounds, the quinones (Q) and the reduced form (QH_2), according to the equation:

$$Q + QH_2 \rightleftarrows 2Q^{-\cdot} + 2H^+ \quad (1)$$

In this case the intensity of the ESR signal of the semiquinone radical, depends on the equilibrium constant and on the presence of alternative mechanisms leading to its disappearance.[2]

The presence of a free radical signal in red wines has been reported by Troup *et al.* in 1994.[3] However, these authors observed the free radical signal at liquid nitrogen temperature only and after the red wines were cold evaporated to about 1/10 of their volume. These radicals were hypothesised to be associated with the phenolics because the ESR signal from the residue of red wine treated with 10% (w/v) polyvinyl–pyrrolidone was reduced by about 80%. However, the lack of an ESR signal in the starting wine, the contact with the atmospheric oxygen during the procedure of concentration and transfer of wine in the ESR cell and the low temperature at which the ESR spectra were recorded leave some doubts on the presence of free radicals in wine and on the free radical generation pathway. In fact oxidation reactions due to the contact with molecular oxygen may lead to the generation of the free radicals while the low temperature may shift the equilibria involving free radicals or may stabilise them.

Aim of this work was to demonstrate the presence in all the red wines of significant amounts of stable free radicals and to correlate the radical concentration to wine characteristics.

MATERIALS AND METHODS

Reagents

The reagents were purchased from Fluka (Buchs, Switzerland) and were of the highest available quality. 2,2,6,6-tetramethylpiperidinoxyl was obtained from Sigma (St. Louis, USA). Aqueous solutions were prepared with doubly distilled water and, when necessary, were passed through a column of Chelex-100 (Biorad, Richmond, USA), to minimise the concentration of heavy metal ions.

The 2,2′-azobis[2-(2-imidazolin-2-yl)propane] (ABIP) was a kind gift of Wako Chemicals (Germany).

Wines

Commercial bottled wines were obtained from a local commercial winery. In order to study the effects of the concentration of polyphenols and of the ageing on the level of free radicals, 16 experimental monovarietal red wines, produced in the vintages 1993 and 1998, were used. The wines were obtained from four grape cultivars, having different amounts of polyphenols,[4] in the experimental winery of Istituto San Michele all'Adige (IASMA) by a traditional skin-contact technique, then subjected to natural malolactic fermentation, settled, filtered, bottled five months after the production and stored at 10–12°C.

ESR Measurements

The wines were transferred by a short transfer-line from the bottle into a quartz flat cell inserted in the ESR cavity. The cell was flushed with argon and the transfer was carried out by applying a positive pressure of nitrogen over the bottled wine.

A Bruker X band (9.8 GHz) ER 200D spectrometer, equipped with a TE standard cavity, was employed. Typical instrument settings were as follows: modulation frequency 100 KHz, modulation amplitude 4 $Gauss_{pp}$, microwave power 20 mW.

All spectra were normalised for accurate calculation using manganese oxide as internal

standard. In particular a small sealed vial containing MnO doped CaO was fixed on the exterior surface of the quartz flat cell. The absolute concentration of radicals was calculated by calibration of the manganese signal using 2,2,6,6-tetramethylpiperidinoxyl as concentration standard. All the measurements were performed at 25°C, during the summer 2000.

Total Polyphenols

Polyphenol concentration was measured by the reduction of phosphotungstic and phosphomolybdic acids (Folin–Ciocalteu's reagent) to blue pigments by phenols in alkaline solution,[5,6] and expressed as gallic acid.

RESULTS AND DISCUSSION

ESR Signal in Bottled Commercial Wines: Signal Analysis

Samples of commercial bottled wines were examined by ESR at their normal concentration. The samples of wine were directly transferred from the bottle into the ESR cell avoiding any contact with the atmospheric oxygen in order not to perturb possible redox equilibria among different species settled in the bottle. In all the samples of red wine we found an ESR signal, centred at $g = 2.0037 \pm 0.0002$, characterised by a single resonance and by 3.14 $Gauss_{pp}$ linewidth, assignable to a free radical, see Fig. 1. The same signal, characterised by a variable intensity, was found in all the tested red wines, whether aged on oak cask (barrique) or not. No detectable free radical signal was found in the white wines that we examined. In Table I we reported the free radical concentration measured by ESR in various commercial wines (standard error <6%), together with the optical density at 520 nm due to anthocyanins. A significant correlation between these two variables was found ($P < 0.01$). The concentration of the free radicals in wine appears comparable or even higher to that measured in some biological fluids, such as plasma, which was ascribed to ascorbyl radical.[7] The radical signal of wine was always overlaid to a very broad signal, due to the presence of aqueous Mn^{2+}, see Fig. 1 inset, the concentration of which, by integration of the ESR signal, was found to be in the range of 3–12 μM. The Mn^{2+} concentrations are in agreement with the concentration of manganese measured in a large set of Italian red and white wines by inductively coupled plasma-optical emission spectroscopy (ICP-OES).[8] The presence of the Mn^{2+} signal both in red and white wines is not surprising since this ion is known to play an important role in plant physiology.[9]

No hyperfine structure was observed in the red wine signal when the ESR instrumental conditions were similar to those which permitted to acquire the spectrum of the ascorbyl radical with a resolution better than 0.16 Gauss (modulation amplitude 0.1 $Gauss_{pp}$, microwave power 20 mW, scan rate 0.4 mGauss/s, time constant 50 s). Since the acquisition was performed in the liquid phase, the inhomogeneous

TABLE I Free radical concentration and absorbance at 520 nm in commercial red wines

Wine	Year	Country	A_{520}*	Radical (nM)
Valpolicella Sup.	1995	Italy	0.478	15.9
Barolo	1995	Italy	0.692	24.7
Chianti Classico	1995	Italy	0.405	18.0
Chateau Reynon	1997	France	0.555	12.8
Chateau la Capere	1997	France	0.503	12.5
Bourgogne	1997	France	0.478	5.2
Teofilo Reyes	1997	Spain	0.812	14.8
Coonawarra Shiraz	1995	Australia	0.587	21.0
Shiraz Cabernet	1996	Australia	0.624	21.0
Sangiovese	1994	USA	0.464	9.7
Cabernet Sauvignon	1993	USA	0.882	24.0
Mocsenyi	1997	Hungary	0.691	20.3
Malbec	1999	Argentina	0.703	15.6
Pinot Noir	1998	Chile	0.264	5.7

* Absorbance values measured at 520 nm with an optical path length of 0.2 cm. No free radical signal was detected in the following commercial white wines: Vin d' Alsace (France), Weissburgunde (Germany) and Prosecco (Italy).

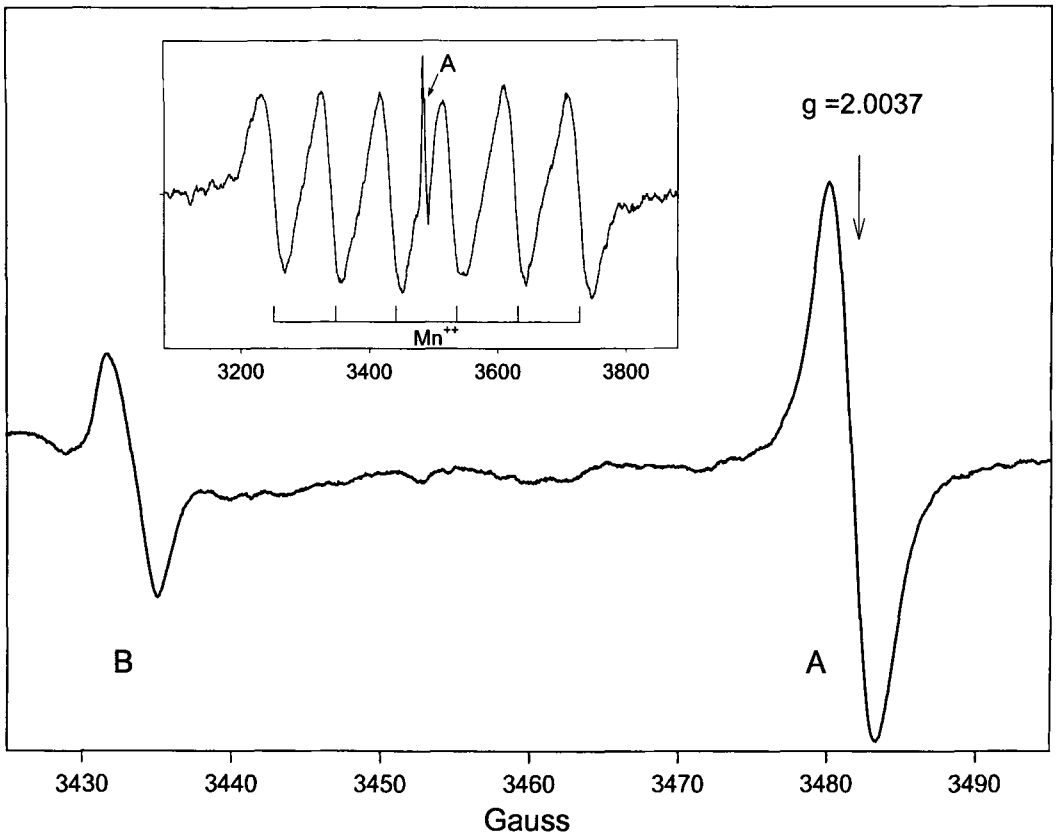

FIGURE 1 ESR spectrum of a bottled red wine. ESR signal of a bottled red wine (Teroldego) (A) under anaerobic conditions ($g = 2.0037 \pm 0.0002$). ESR signal of MnO (CaO doped by MnO) used as a standard (B). The instrumental parameters were: modulation amplitude 4 Gauss, microwave power 20 mW, scan rate 0.08 Gauss/s, time constant 10 s. Inset: Full ESR spectrum of the wine Teroldego showing the signal of the free radical (A) superimposed to the spectrum of aqueous Mn^{++} ion present in the wine. The instrumental parameters were as above except the scan rate was 0.82 Gauss/s.

broadening effects due to random displacement of the frequency[10] or to effects related to anisotropy of g factor can be excluded. Therefore it appears that the lack of hyperfine structure of the signal could be due to: (i) the presence of a variety of radical species so that the observed signal is the summation of the single resonances or (ii) the short lifetime of the quantum states between which transitions are tacking place, due to spin–lattice relaxation.[11] However, increasing the microwave power up to 100 mW, the saturation sets in, see Fig. 2, indicating that the spin–lattice relaxation is slow.

Stability of the ESR Signal

Addition of sulphur dioxide, a common additive of wine, up to 2 mM (128 mg/l) had relatively low effect on the intensity of the ESR signal. The stability of the ESR signal demonstrated that this species is not involved in the formation or removal of the free radicals present in red wines.

Addition of Mn^{++} up to 30 μM, that is about three fold the mean concentration of Mn^{++} present in the red wines we have examined, or sequestering this ion by Chelex 100, brought no significant change of the intensity of the free radical signal. This result indicated that the Mn^{++}

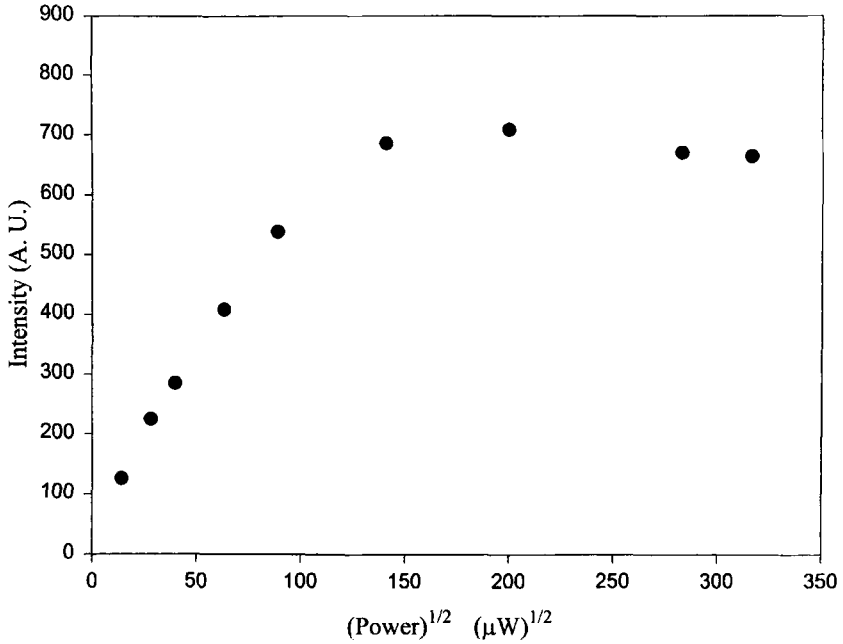

FIGURE 2 Power saturation of the radical signal present in red wine. Instrumental parameters were as in Fig. 1.

ion is not correlated to the presence of free radicals in the red wines.

The effect of molecular oxygen on the intensity of ESR signal was studied, because in preliminary experiments we observed an increase of the ESR signal when bottled red wines were exposed to air. According to the literature, the concentration of molecular oxygen in bottled wine is negligible after three months of storage.[12] The wine samples were equilibrated with atmospheric oxygen by 5 min vortication. After this treatment the intensity of the ESR signal increased by about a factor of two while no significant change of other signal characteristics was observed. This abrupt change of the intensity is followed by an exponential decrease of the radical concentration to a level about 60–80% of the value found under anaerobic conditions, being the decay time constant of the order of 1 h (see Fig. 3). The change of the level of radical concentration following the exposure to atmospheric oxygen can be due to the possible displacement of the equilibria involving the free radicals, and to the activation of oxidation processes. This behaviour is interesting because oxygenation of the red wines before drinking is usually carried out to improve the wine sensorial characteristics.

Since the observed free radical signal appears reasonably associated to the polyphenols present in red wines, four types of grape cultivars (Teroldego, Cabernet, Merlot and Schiava) were chosen for their different content of polyphenols. For each type of cultivar two sets of grape obtained from different IASMA vineyards were independently processed and bottled in 1993 and 1998. The mean value of the free radical level found in the four types of wine, together with the concentration of polyphenols, are reported in Table II. From this table it appears the relative high concentration of the free radicals in some of the examined red wines, (close to 100 nM in the case of the Teroldego wine), and the large variability of the signal (more than one order of magnitude). The lowest values were observed in the vintage for the Schiava having the lowest concentration of red pigments. As expected, the concentration of free radicals correlated strictly

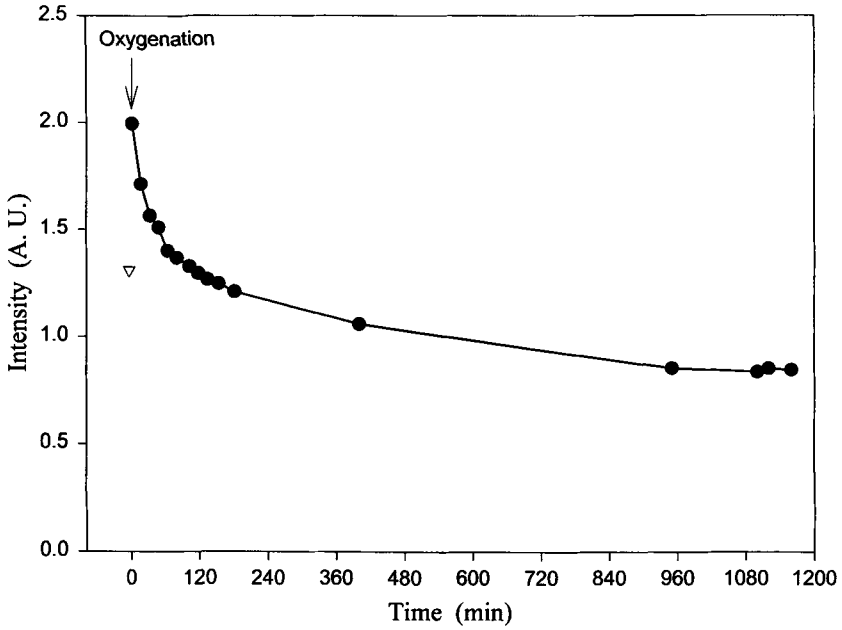

FIGURE 3 Effect of molecular oxygen on the ESR signal of wine. After the recording of ESR signal of the wine under anaerobic conditions (▽), the wine was equilibrated for 5 min with atmospheric oxygen. Instrumental parameters were as in Fig. 1.

with the value of polyphenols ([radical] (nM)=3.32+0.0038 [polyphenols] (mg/l), $r = 0.93$). Furthermore when each type of wine is considered separately, it appears a strong increase of the radical concentration (from a minimum of two fold to a maximum of about 3.5-fold) increasing the ageing (1993 and 1998), see Table II.

TABLE II Free radical concentration and polyphenol content of differently aged wines produced from various grape cultivars

Wine	Year	Radical* (nM)	Total polyphenols† (mg/l)
Teroldego	1993	81.5	2839
	1998	22.8	1467
Cabernet	1993	50.0	2165
	1998	17.1	1803
Merlot	1993	35.6	1566
	1998	16.1	1481
Schiava	1993	18.3	1238
	1998	7.0	919

*Mean value obtained from two samples of wine produced from grapes coming from different IASMA vineyards. The standard deviation was always below 12%.
† Expressed as gallic acid.

To obtain information on the nature of the free radicals we observed in red wines, we added 5 mM 1,4-benzoquinone to 5 mM gallic acid solution, buffered with tartaric acid at pH 3.8, that is the average pH of the wines we have studied. Operating under anaerobic conditions we observed a relatively strong and persistent (days) single line ESR signal at $g = 2.0046$, characterised by a 2.90 $Gauss_{pp}$ linewidth. No ESR signal was observed when, operating under similar conditions, we added 1,4-benzoquinone to 1,4-dihydroxybenzene, or in the presence of 1,4-benzoquinone or gallic acid alone. These experiments strongly indicated that semiquinones of gallic acid and 1,4-benzoquinone are formed in the redox equilibrium between these species.

This result, together with the fast increase of the ESR signal when the wines are exposed to atmospheric oxygen, suggest that the free radical signal that we observed in red wines is due to redox equilibria involving the red wine polyphenols, leading to the formation of stable semiquinone radicals.

In conclusion we have observed that long-living free radicals, probably generated from polyphenols, are present in red wine at a concentration comparable to that of ascorbyl radical in some biological systems. Beyond to the possible influence of these species on the wine characteristics (i.e. we observed an increase of radical concentration with ageing), we should take into consideration the possible role of the long-living polyphenol intermediates in the beneficial effects of red wine. In this regard, work is in progress to achieve a better understanding of the mechanisms involved in the formation of the stable free radicals that we observed in red wines and of their reactivity toward more dangerous species.

Acknowledgements

We thank G. Nicolini for providing the experimental wines. This work was in part supported by Provincia Autonoma di Trento, Consiglio Nazionale delle Ricerche and Fondazione Trentina per la Ricerca sui Tumori. Urska Vrhovsek was supported by a fellowship from the Slovenian Ministry for Science and Technology.

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