EPR investigation of direct oxyradical scavenging and metal chelation activities of chitosan as an alternative to sulfites in enology

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INTRODUCTION

Oxidation of wines is a crucial problem to be faced by winemakers. **Electron paramagnetic resonance** (EPR) spectroscopy studies combined with spin trapping on nitrones have revealed the implication of reactive oxygen species, **including hydrogen peroxide (H_2O_2), hydroxyl (HO•) and 1-hydroxyethyl (1-HER)** radicals, in the oxidation mechanism [1-4]. Thus, low levels of H_2O_2 formed by metal catalyzed reduction of dissolved oxygen under a redox cycle established by polyphenols (*eg*, catechols) can undergo a Fenton reaction to yield HO• which ultimately oxidizes ethanol into acetaldehyde through intermediate formation of 1-HER (Fig. 1A). Reacting with H_2O_2, **sulfur dioxide (SO_2)** is extensively used to protect wines. Since sulfites are growingly rejected by consumers because of their chemical nature and known allergenic properties, developing biologically relevant alternatives is an expanding field in enology.

Another antioxidant strategy in wine could be to decrease the catalytic potential of metals, which are endogenous to the grape or present in agrochemical additives. Recently **chitosan** (Fig. 1B), an abundant biopolymer obtained by deacetylation of chitin from animal or vegetal sources, has been proposed as alternative fining agent in wines, showing antimicrobial, biodegradable and non-allergenic properties. Chitin derivatives also demonstrated metal chelating effects (which slower oxidation in wines), interacting with grape polyphenols such as caftaric acid (whose enzymatic oxidation results in excessive browning [5]), and inhibiting (+)-catechin oxidation in model white wines [6].

Here, using EPR/spin trapping and HPLC techniques we have tested the protecting efficacy of chitosans against oxidation of a model and sulfite free white wine under realistic treatment conditions and doses.

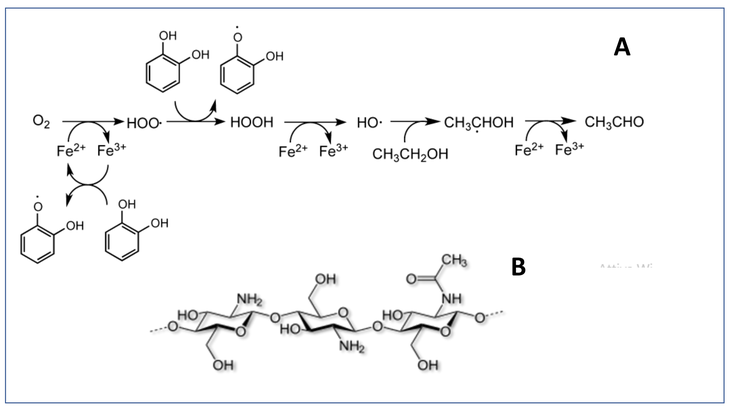


Fig. 1. (A) Free radical mechanism of wine oxidation. (B) General structure of chitosan.

MATERIALS AND METHODS

MATERIALS. Chemicals and reagents were from commercial suppliers, including DNPH, 4-methyl catechol (4-MeC, a representative polyphenol in grapes), the iron chelator ferrozine and the spin traps 5,5-dimethylpyrroline-1-oxide (DMPO; 3.3 mM) and (4-pyridyl-1-oxide)-*N*-tert-butyl nitrone (4-POBN; 15 mM). Studied chitosans were either from Sigma (CHI-1) or supplied by IOC (CHI-2).

EPR. Signals were acquired at 20°C in glass capillaries on a Bruker ESP 300 spectrometer operating at 9.80 GHz equipped with an in situ Oriel UV photolysis system. Standard routines were used throughout, with typical settings: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 0.625 G; gain, 1 x 10^5; sweep time, 41.94 s/scan; accumulated scans, 10. Quantification used double integration of simulated signals (WinSim software).

BASIC PRINCIPLE OF SPIN TRAPPING (see also Fig. 2)

R•	+	SPIN TRAP	→	[SPIN ADDUCT]•	→	*EPR spectrum*	→	*informations on R•*
not EPR detectable (eg, hydroxyl, 1-HER)		*nitrone (eg, DMPO, 4-POBN)*		*EPR detectable (eg, DMPO-OH, 4-POBN-1-HER)*				*structure, concentration, inhibition* if conditions (*eg*, spin trap) are kept constant

In the presence of a competitor having increased scavenging properties (and/or being at higher concentration) than the spin trap, spin adduct concentration (thus, the EPR signal) will be dose dependently decreased.

PROTOCOLS FOR EPR

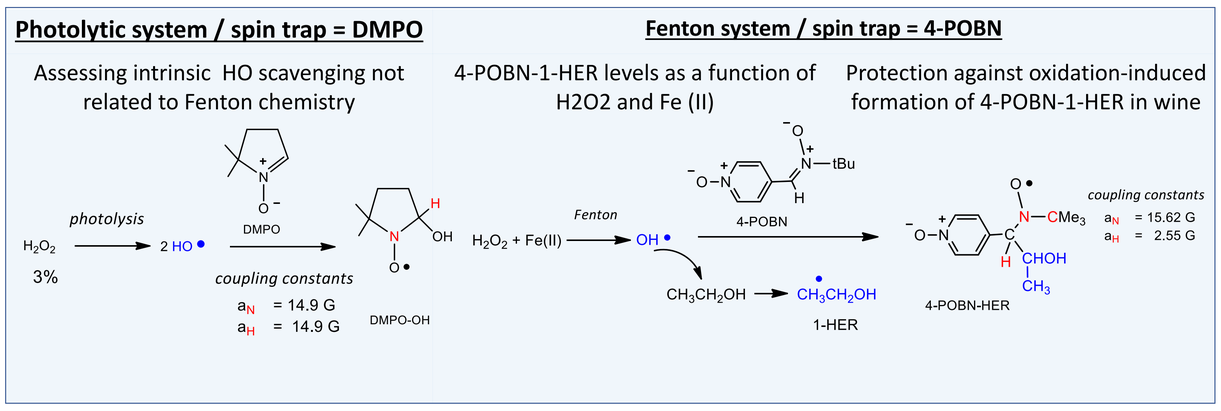


Fig. 2. Principle of spin trapping, spin traps and related and spin adducts detected in the study.

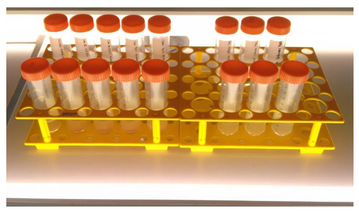
PROTOCOL FOR INCUBATIONS

All reactions, and EPR spectroscopy were performed in the dark to avoid extra photolysis of H_2O_2.
In all incubations, the mixtures were kept under continuous agitation throughout.

- Model wine (consisting of 12% of ethanol + 8 g/L of tartaric acid, pH 3.5) or white wine were pretreated with 0.1 mM Fe(II) ± inhibitor (CHI, ferrozine or SO_2) for 2 days ;
- Supernatant was recovered and were added: 4-POBN (15 mM) in real wine or a mixture of 4-POBN and 4-MeC (1 mM) in model wine (to start oxidation);
- 4-POBN-1-HER relative concentration was monitored sequentially up to 4 days (96 h).

HPLC.

DNPH 10 mM was used to derivatize samples. Separation of adducts a Nucleodur C18 Htec (250 x 4.6 mm; 5µm) with a isocratic flow rate of 0.8 mL·min^{-1}. Solvent A is acetonitrile; solvent B is water containing 0.05% [2] v/v solution of phosphoric acid (pH 2.7). The elution program was the following: 0 min, 40% A, 8 min, 85% A, 9 min, 40% A, 13 min, 40% A. The identification of the observed derivatives was based on their retention time compared with those of standards tested at 360 nm as well as their spectral characteristics.



RESULTS

1. Both chitosans dose dependently and efficiently scavenged **photolytically-generated HO•** at similar rate constants (Fig. 3). At maximum concentrations of 2 g·L-1, both chitosan exhibit an scavenging effect of 90%

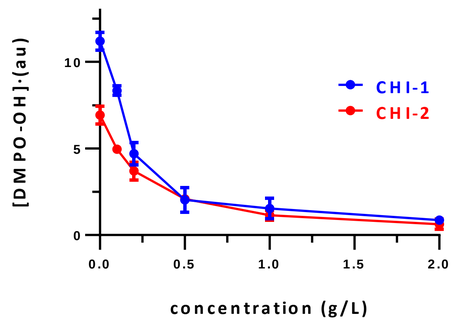


Fig. 3. Direct scavenging of hydroxyl radical by chitosans determined by EPR spin trapping with 3.3 mM DMPO (bars = SEM).

2. In a **Fenton system** performed in model wine oxidation 4-POBN-1-HER concentration increased with increasing H_2O_2 or Fe(II) concentrations, with the effect of varying iron content being dramatically more important. In this system maximal spin adduct levels as obtained in incubations with 0.1 mM iron(II) (see below) are compatible with H_2O_2 concentrations ranging 0.025-2.5 µg/mL (Fig. 4).

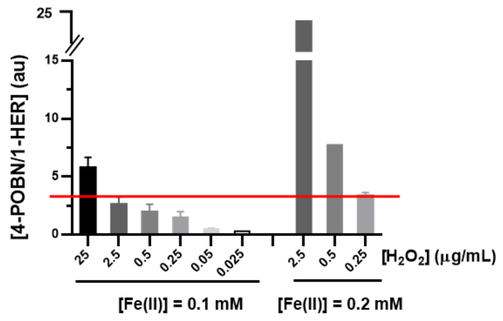


Fig. 4. Fenton-formed 4-POBN-1-HER adduct in model wine is more sensitive to iron(II) than H_2O_2 content. The red line visualizes the maximum spin adduct levels detected in incubations (bars = SEM).

3. In **model matrix** both chitosans efficiently inhibited 4-POBN-1-HER formation as a result of oxidation. CHI-2 was found > CHI-1 and >> high ferrozine or SO_2 treatments under wine conditions. and their profiles are close to that obtained when low ferrozine was present. After peaking at day 2 CHI treated samples showed decreased oxidation while samples containing SO_2 (50 mg/mL) reoxidized as to reach the same level at day 3 (Fig. 5).

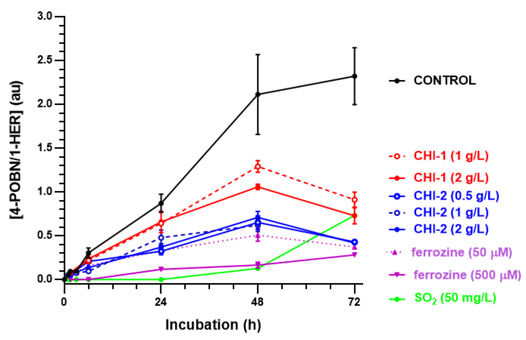


Fig. 5. EPR monitoring of oxidation of model wine at 20°C as a function of treatments. Oxidation was triggered by adding a mixture of 0.1 mM Fe(II) and 1 mM of 4-MeC (bars = SEM).

4. In **SO_2 free white wine** CHI-2 at the highest dose allowed by french wine regulations efficiently inhibited 4-POBN-1-HER formation. Up to day 4 the iron chelator ferrozine remained more efficient than CHI-2 while wine samples supplemented with SO_2 (50 mg/mL) tended to reoxidize as to yield a similar spin adduct content. (Fig. 6).

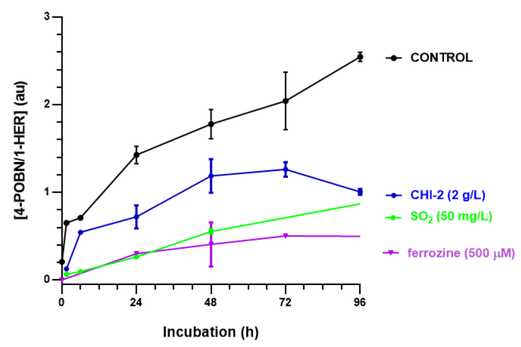


Fig. 6. EPR monitoring of oxidation of SO_2 free white wine (Chardonnay) at 20°C as a function of treatments. Oxidation was triggered by adding 0.1 mM of Fe(II) (bars = SEM).

5. Figure 7. contains the chromatograms of the **aldehyde oxidation intermediates**, obtained after a 48 h exposure of the model wine samples. As can be seen, the content of glyoxylic acid and acetaldehyde was reduced when the samples were treated with chitosan. As previously obtained in the EPR experiments, a dose-depentent effect was shown, with reductions of 70% at higher concentrations of 2 g · L-1 chitosan.

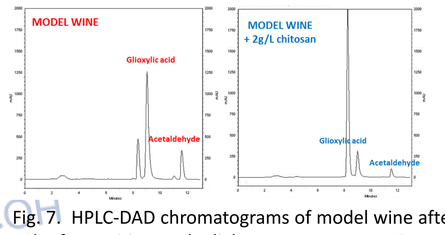


Fig. 7. HPLC-DAD chromatograms of model wine after 48h of exposition to the light.

DISCUSSION AND PERSPECTIVES

- Although the very persistent 4-POBN-1-HER spin adduct (half-life of weeks) is a standard EPR index of stability in beer studies, it has been scarcely used to follow wine oxidation [1-4,7]. Using this endpoint we demonstrated that natural chitosans can be good alternatives to SO_2 in protecting wine. Although tested CHIs demonstrated rather strong HO• scavenging properties in test tube assays, such mechanism of action yet seems unrelevant given the many other targets available for free radical attack among wine constituents.
- More likely, our data suggest iron chelation a major antioxidant action of CHIs, in line with previous findings of the better protective efficacy of decreasing the levels of metal catalysts *vs* H_2O_2 [2-4]. However, since chitosan can also react with H_2O_2, such third mechanism of action may play a role in the overall inhibition of spin adduct formation. In this regard spin trapping investigations using photolytic, H_2O_2-free HO• generators are in progress in our laboratory.
- HPLC experiments revealed a reduction of aldehidic intermediates responsible of cross-linking of flavanols leading to the development of browning. These results agreed with those already obtained with EPR
- Since chitosan has been approved in oenology by the OIV, interest of its use in winemaking process is growing. In our group, different proves are being developed, in order to study the influence of chitosan on the fixed and volatile composition of wine. Different studies have already been carried out by adding chitosan in different stages of winemaking (stabilization of must, alcoholic fermentation [7].

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Acknowledgement The sulfite free white wine used in the study was from pure Chardonnay (côteau champenois, 2016 and 2017) kindly provided by Champagne J. De Telmont, Damery, France.