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Antioxidants in white wine (cv. Riesling): I. Comparison of different testing methods for antioxidant activity

by

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S u m m a r y : This paper provides a study on different testing methods for antioxidant activity. Four commonly used methods (LDL oxidation, TAS measurement, β-carotene bleaching as well as a rapid screening test published by Pryor *et al.* 1993) are compared on the basis of a set of model compounds. The differing results concerning the ranking order of the tested substances are discussed. Furthermore three methods which showed appropriate results were used in order to determine the antioxidant activity of Riesling wine fractions.

K e y w o r d s : antioxidants, testing systems, LDL oxidation, TAS measurement, β-carotene bleaching, Riesling wine.

Introduction

It is now widely accepted that moderate consumption of wine reduces mortality from coronary diseases (Renaud and De Lorgeril 1992; Criqui and Ringel 1994; Gronbaek *et al.* 1995), with one likely explanation being the antioxidative properties of its phenolic constituents (Frankel *et al.* 1993). More recent studies have furthermore demonstrated that wine consumption alters the redox properties of blood plasma (Maxwell *et al.* 1994; Nigdikar *et al.* 1998). The antioxidative properties of the phenolic compounds have been widely studied, but also prooxidative effects of white wine on low-density-lipoprotein (LDL) oxidation have been reported (Fuhrman *et al.* 1995). Other investigators (Vinson and Hontz 1995; Caldú *et al.* 1996) found that white wine polyphenols were more effective than red wine polyphenols in inhibiting *in vitro* LDL oxidation. For white wine polyphenols the average IC50 (*i.e.* concentration which exerts 50 % inhibition on *in vitro* LDL oxidation) was found to be 1.7 µM, whereas for red wines an average value of 2.9 µM has been determined (Vinson and Hontz 1995). This finding was explained by differences in the polyphenolic composition of red and white wine, *i.e.* the predominance of polymeric phenols (tannins) in red wine and low molecular weight polyphenols in white wine.

Although the major polyphenolic constituents of white wine are known (Singleton and Esau 1969; Ritter and Dietrich 1994; Teissedre *et al.* 1996), there has been no systematic search of wine constituents based on antioxidative activity. The aim of our study was to evaluate the antioxidant activity of the components of a commercial Riesling wine. For unknown constituents with antioxidant activity structure elucidation is attempted. A prerequisite for this study was the availability of suitable testing systems for antioxidative activity. Here, different testing methods for antioxidants are evaluated based on their response to different antioxidants and their suitability for screening. Appropriate methods are then used for measuring the antioxidative activity in Riesling wine fractions obtained after separation by solvent extraction and subsequent countercurrent chromatography.

Material and Methods

All commercial chemicals used were of analytical grade quality. Solvents were redistilled before use. Water was purified by a MilliQ system (Waters).

R e f e r e n c e c o m p o u n d s : Ascorbic acid, catechin, quercetin dihydrate, rutin, α-tocopherol and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma or Fluka. Caftaric acid was a standard from the Department of Viticulture and Enology, Davis, California (Meyer *et al.* 1998).

P r e p a r a t i o n a n d f r a c t i o n a t i o n o f a R i e s l i n g w i n e e x t r a c t (cf. Fig. 1): For antioxidant testing, a commercial Riesling wine (20 l QbA, Ilbesheimer Herrlich, Rheinpfalz, 1993 vintage) was used. Prior to work-up, the wine was diluted 1:1 with distilled water. Then the wine was passed through a column of Amberlite XAD-2 resin in portions of 10 l. After rinsing with water, the retained material was eluted with MeOH. The methanolic eluate was concentrated under reduced pressure. The residue (20 g) was dissolved in distilled water and partitioned into a polar and a nonpolar fraction by all-liquid extraction using diethyl ether as solvent. For fractionation of the aqueous residue, multilayer coil countercurrent chromatography (MLCCC) was used (Multilayer Coil Separator-Extractor, P.C.Inc., Potomac, USA; equipped with a 85 m x 2.6 mm i.d. PTFE tubing; solvent system: $CHCl_3$/MeOH/H_2O 7:13:8 (v/v/v), flow rate: 1.5 ml·min^{-1}). Eighty fractions were obtained which were grouped into 6 combined fractions (TLC monitoring).

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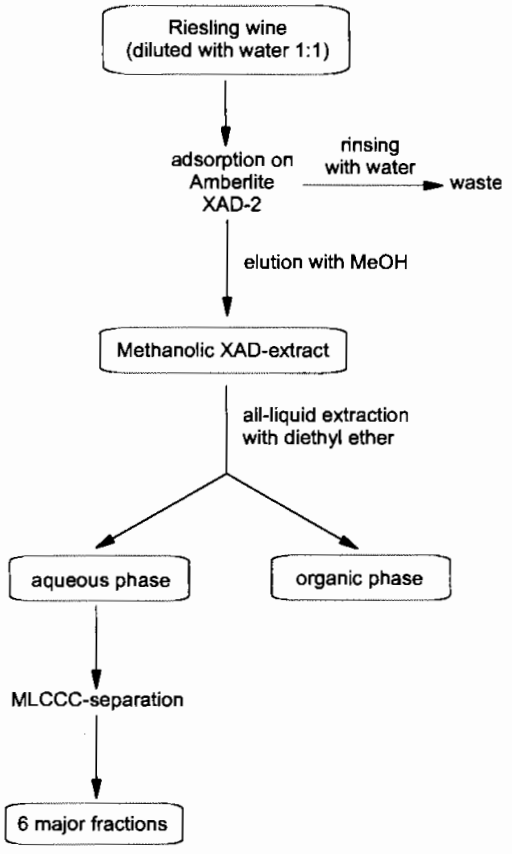

Fig. 1: Preparation of wine fractions for determination of their antioxidative activity.

Testing systems

a) LDL oxidation (FRANKEL *et al.* 1992): Inhibition of human LDL oxidation was investigated using the "Frankel method" (FRANKEL *et al.* 1992) which measures hexanal formation, a secondary lipid oxidation breakdown product. For the preparation of human LDL, blood was collected by venipuncture into EDTA tubes from healthy volunteers and centrifuged at 1500 *g* and 4 °C to obtain the plasma. The plasma LDL was obtained by sequential density ultracentrifugation in the presence of 0.1 $g \cdot l^{-1}$ EDTA. Prior to oxidation, LDL was thoroughly dialysed with de-oxygenated phosphate-buffered (10 mM, pH 7.4) saline (100 mM). The LDL protein concentration was determined using the Lowry protein analysis kit (Sigma) and LDL was diluted to 1000 $\mu g \cdot ml^{-1}$ protein concentration with phosphate-buffered saline. Freshly dialysed LDL solution (250 µl) was placed into GC-headspace vials containing 10 µl of the antioxidant solution either in water or in DMSO. After addition of 5 µl of a 3.88 mM (15.7 $mg \cdot 25\ ml^{-1}$) aqueous copper sulphate solution the vials were sealed, mixed and incubated for exactly 2.0 h in a shaking waterbath at 37 °C. After incubation, hexanal formation was determined by static headspace gas chromatography. Relative inhibition of LDL oxidation obtained by either test compound or wine fraction was calculated as follows:

$$\%\ \text{inhibition} = \frac{C - S}{C} \times 100$$

where C is the hexanal formed in the control experiment and S is the hexanal formed with the sample.

b) Measurement of the total antioxidant status (TAS) (MILLER *et al.* 1993): The total antioxidant status was determined using a test kit purchased from Randox Laboratories (Ireland).

Incubation of the chromogen 2,2'-azobis(ethylbenzothiazolinsulfonate) (ABTS) with metmyoglobin and hydrogen peroxide produces the radical cation $ABTS^{\cdot+}$ which has a relatively stable green color and can be measured at 600 nm. Antioxidants of an added sample inhibit the reaction and cause a suppression of color production to a degree which is proportional to the antioxidant capacity of the sample. The assay is calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and results are expressed in mM Total Antioxidant Status (TAS) units, where one unit is equivalent to the color suppression of 1 mM Trolox.

Solutions of the reference compounds were initially diluted to one mM concentration. For wine fractions, the total phenolic content was determined in gallic acid equivalents (GAE) by the Folin Ciocalteu analysis and then the solutions were diluted to one GAE. Samples which gave a result > 2.5 mM TAS units were diluted with H_2O to 0.5 mM and measured again.

Assays were performed at 37 °C using a HP 8452A diode array spectrophotometer with a thermostated cell-holder. 20 µl H_2O (blank), standard solutions or sample solutions were added to 1 ml of the chromogen solution. After mixing, initial absorbance was read (A1). 200 µl substrate solution were added, the solution mixed and after exactly 3 min the final absorbance was read (A2).

A2 - A1 = ΔA of sample/standard/blank

TAS was obtained by the following calculation:

$$\text{TAS(mM)} = \frac{\Delta A\ \text{blank} - \Delta A\ \text{sample}}{\Delta A\ \text{blank} - \Delta A\ \text{standard}} \times \text{conc. standard}$$

c) β-Carotene bleaching (MARCO 1968): β-Carotene (5.0 mg) was dissolved in 50 ml acetone. An aliquot (4 ml) of this solution was added to a flask containing 40 mg linoleic acid and 400 mg Tween 40. Acetone was evaporated with nitrogen. The residue was mixed with H_2O and the emulsion diluted with water to a total volume of 100 ml. 100 µl of the sample solutions were placed in semimicrocuvettes and 2 ml of the β-carotene/linoleic acid emulsion were added. After mixing, the initial absorbance at 500 nm was measured and the cuvettes were placed in a water bath (50 °C).

After 50 min final absorbance was read. Antioxidant activity was expressed as % inhibition relative to a control (containing water instead of antioxidant solution) using the following equation:

$$\%\ \text{inhibition} = \frac{\Delta\ \text{control} - \Delta\ \text{sample}}{\Delta\ \text{control}} \times 100$$

Δ control = absorbance (t = 0) - absorbance (t = 50);
Δ sample = absorbance (t = 0) - absorbance (t = 50).

d) Rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants (Pryor *et al.* 1993): This method requires the following solutions: 0.1 M SDS (sodium dodecylsulfate) in 0.05 M sodium phosphate buffer (pH 7.4); 2.6 mM linoleic acid in 0.1 M SDS in 0.05 M sodium phosphate buffer (pH 7.4); 0.05 M 2,2'-azobis(2-amidino-propane)dihydrochloride (ABAP) in 0.05 M phosphate buffer (pH 7.4) as well as sample solutions (0.1 mM in H_2O or ethanol).

The linoleic acid solution (2.4 ml) was placed in an UV cuvette and thermostated (40 °C). A reference cuvette contained 2.4 ml of the SDS solution in phosphate buffer. After 10 min 50 µl of the ABAP solution (0.05 M) were added to each cuvette. The conjugated diene formation was monitored at 234 nm until the rate was constant. 50 µl of the antioxidant solution were added to each cuvette and time was measured until the oxidation rate returned to the initial value (lag phase).

Total phenolic content: The concentration of total phenols in the wine fractions was determined by the Folin-Ciocalteu method (Singleton and Rossi 1965) and expressed as mmol gallic acid equivalents (GAE) per g wine extract. Furthermore the model compounds' response to this method was investigated and calculated as mmol GAE per mmol. 20 µl of standard solution, sample solution or water, respectively, were pipetted into semimicrocuvettes, followed by 1.58 ml water. After mixing, the Folin-Ciocalteu reagent (100 µl) was added to each cuvette and the solutions were mixed again. After 30 s and before 8 min 300 µl of an aqueous sodium carbonate solution (20 %) were added. The solutions were left at room temperature for 2 h. Then the absorption of the developed blue color was determined at 765 nm, being proportional to the amount of phenolic (oxidizable) material present in the sample.

Results and Discussion

Assays of antioxidant activity: We selected a set of 7 model compounds (Fig. 2): ascorbic acid (**1**) and α-tocopherol (**2**) are well known nutritional antioxidants; the water soluble tocopherol derivative Trolox (**3**) was included along with 4 wine phenolics with different structural features: caftaric acid (**4**), catechin (**5**), quercetin (**6**) and rutin (**7**).

The results of the different testing procedures are shown in Fig. 3. With the β-carotene bleaching method and the Pryor method, α-tocopherol and its derivate Trolox gave the highest antioxidant activities, whereas the *in vitro* LDL oxidation and the TAS measurement revealed the polar phenolic compounds to be most potent antioxidants. These differences may be caused by the different reaction environments. The LDL oxidation method and the TAS measurement are carried out in aqueous media, but the β-carotene bleaching procedure as well as Pryor's method uses detergents (Tween 40, SDS) to dissolve an oxidizable lipid, linoleic acid. Thus, in the latter cases, emulsions define

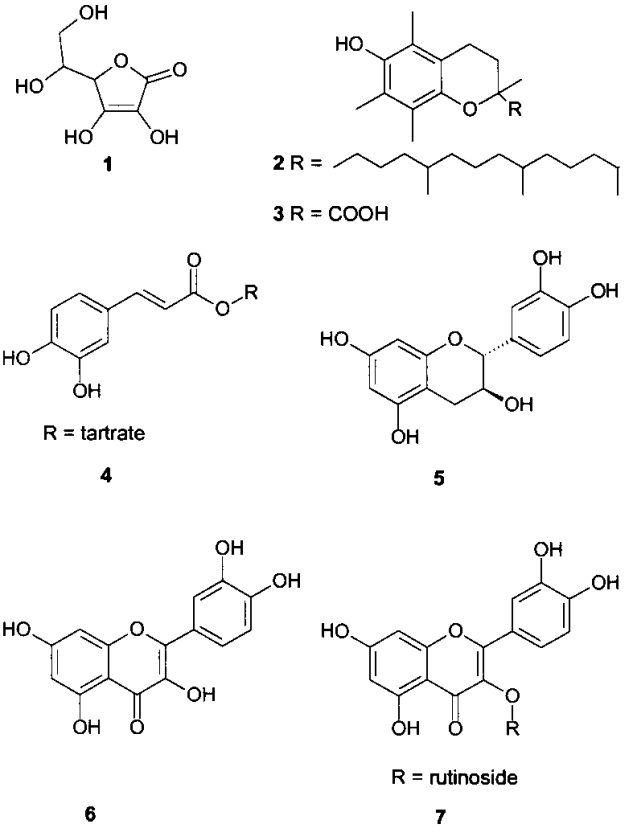

Fig. 2: Structures of model compounds used in antioxidant activity tests (**1** ascorbic acid, **2** α-tocopherol, **3** Trolox, **4** caftaric acid, **5** catechin, **6** quercetin, **7** rutin).

where the important oxidation reactions take place, usually within or at the surface of lipid particles. Oil-soluble antioxidants like α-tocopherol will be more efficient at scavenging lipid peroxyl radicals inside the particles, whereas the polar phenolic compounds and the water-soluble antioxidant ascorbic acid can merely react in the water phase or at the surface of the particles where they cannot interrupt the critical oxidation reactions.

Vice versa, in aqueous media the polar antioxidants are more likely to react with metal ions, important in the LDL oxidation test, and with the tungsten and molybdenum ions in the Folin-Ciocalteu assay. In addition, these polar compounds are more likely to interact with free radicals in an aqueous environment.

If the antioxidant efficiencies of just the phenolics are compared, only small differences are found between the screening methods. However, the β-carotene bleaching method and the TAS measurement gave quercetin as the most potent antioxidant followed by catechin and rutin. The LDL method changed the rank order for antioxidant efficiency to catechin > rutin > quercetin. Caftaric acid, the compound with only two phenolic hydroxyl groups showed the lowest antioxidant activity in each testing system. Pryor's method turned out to be not practicable for determining the antioxidant activity as no clear end point of inhibited reaction could be observed for the phenolic testing compounds.

The Folin-Ciocalteu method is actually not an antioxidant test but instead an assay for the quantity of oxidizable

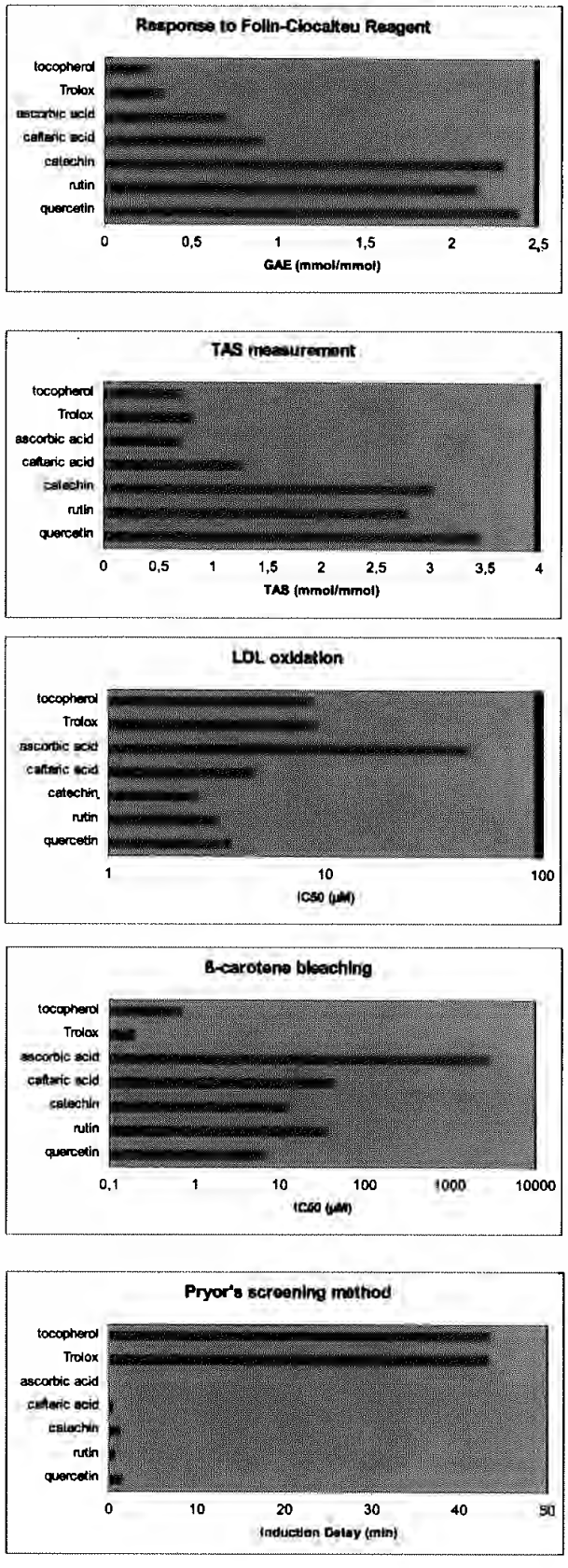

Fig. 3: Comparison of different testing methods for antioxidant activity using model compounds. GAE = gallic acid equivalents; TAS = total antioxidant status; LDL = low-density-lipoprotein. IC50 = concentration which exerts 50 % inhibition on *in vitro* LDL oxidation.

substances, *i.e.* phenolic compounds. The response order of the model compounds was found to be very similar to the TAS measurement, with quercetin having the highest response and α-tocopherol the lowest. Thus, while the Folin assay is not an antioxidant test, it certainly may be useful for detecting antioxidants.

The relationship between structure and antioxidant activity of flavonoids and phenolic acids in the aqueous phase has been investigated by RICE-EVANS *et al.* (1996), stating that the structural features of polyphenols are predictive for their antioxidant activity. In the case of flavonoids, the authors demonstrated the importance of the 2,3-double bond and the 4-oxo function for antioxidant efficiency. Catechin, lacking this structural features, was shown to give only half of the antioxidant activity compared to quercetin. Glycosylation of the 3-hydroxyl group of quercetin (*e.g.* rutin) decreases antioxidant activity as well. Our observations for the β-carotene bleaching method and the TAS measurement are in good agreement with these findings, however, our results for the LDL oxidation are in contrast to this theory. In the latter case the efficiency of inhibiting *in vitro* LDL oxidation is obviously influenced by several additional factors. Likely factors are copper chelating properties of the antioxidants and their ability to protect the α-tocopherol in LDL from oxidation *via* regeneration of α-tocopherol from its oxidized form. It is furthermore important that the antioxidant can get in close contact to the LDL particle. This means that the partition coefficient of the antioxidant greatly influences its efficiency. The latter two properties are also likely to be important for the β-carotene bleaching method. So far, all the influences on the ranking for antioxidant activity in the LDL testing system are still not fully understood.

However, these discrepancies are - relatively speaking - only minor differences in antioxidant potency, and it is possible to make some general statements about the ability of the various testing systems to be used as a screening method for antioxidants. There was a close but not total concordance between the relative rankings of these antioxidants by the LDL oxidation test, the metmyoglobin assay, and the Folin-Ciocalteu method for phenol analysis. For that reason, it is reasonable to suggest that for screening purposes, *i.e.* to find "antioxidant" compounds, any of these provide a similar response. Other, more appropriate tests could be used to compare antioxidant potency with regard to a specific oxidation system. It is clear that Pryor's method is unsuitable for polar compounds due to its poor response to these compounds.

In summary, it can be concluded that assessing antioxidant activity is problematic since the results are strongly dependent on the method used. On the other hand, if the goal is somewhat limited, then most assays will indicate active compounds. Apart from this, one general problem remains. It is always difficult to draw conclusions from results obtained by *in vitro* testing systems on the antioxidant efficiency *in vivo*. Factors like absorption of a compound into blood circulation, metabolism and excretion cannot be simulated by simple oxidation tests, and these additional factors play a major role for an antioxidant's effectiveness *in vivo* – not to mention the particularly contentious point which

in vitro test best reflects the *in vivo* antioxidant event; but our comparison was not intended to address that question. For our Riesling wine study additional factors were taken into consideration, *i.e.* the method's practicability, instrumental requirements and the time, expertise and cost necessary for an analysis.

Antioxidant activity of white wine fractions: In order to investigate how far the above mentioned testing systems produce comparable results, a commercial Riesling wine was worked up as shown in Fig. 1. In the fractions obtained the antioxidant activity was measured. Results were compared with the fractions' total phenolic contents which were determined by using Folin-Ciocalteu reagent according to the procedure of SINGLETON and ROSSI (1965). As shown in Fig. 4, different methods gave comparable results for the antioxidant capacity of the separated fractions. Thus, for further investigations we used the β-carotene bleaching method because of its simple practicability. It is also apparent that the antioxidant efficiency is correlated with the amount of GAE obtained by the Folin method. This is not unexpected because the response to the Folin-Ciocalteu reagent depends on the number of phenolic hydroxyl groups or other potentially oxidizable elements (AMERINE and OUGH 1988).

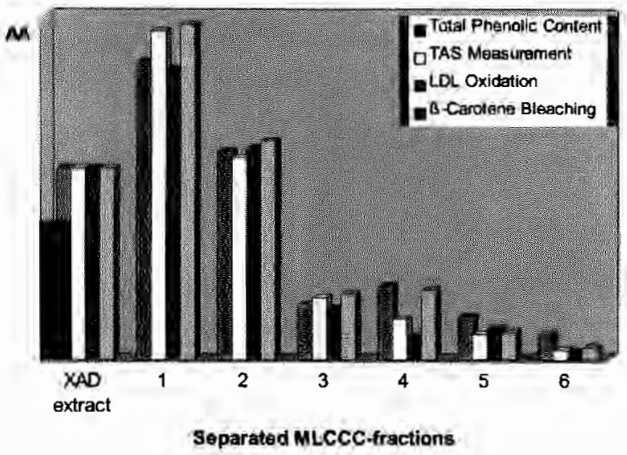

Fig. 4: Antioxidative activity (AA) of separated wine fractions relative to the activity in the aqueous XAD extract. For abbreviations see Fig. 3; MLCCC see Material and Methods.

In the case of Riesling wine, the most polar fractions 1 and 2 were found to possess highest antioxidant activity and therefore contribute considerably to the total activity of the polar wine extract. An almost equal antioxidant capacity was also determined for the nonpolar ether extract. Isolation and structure elucidation of antioxidative compounds and evaluation of their contribution to the antioxidant activity of white wine is subject of our present research.

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