Preamble
This report summarises research work on oxidation processes in white wine and model white wines, placing particular emphasis on the role of copper. The research was carried out at The University of Melbourne. Research staff and students involved in the project were :

- Dr Geoffrey R. Scollary (School of Chemistry, Project Coordinator)
- Dr Victoria A. Vicente-Beckett (RMIT Department of Applied Chemistry, Project Supervisor)
- Ms Minerva Mitri, (PhD student, The University of Melbourne)
- Mr Andrew C. Clark (BSc(Hons) student, The University of Melbourne, 1996).
- Dr Patrick Williams of The Australian Wine Research Institute assisted in the formative stages of this project.

Project Objectives
1. To characterise the chemical forms of copper and iron in wine.
2. To propose a working model for copper- and iron-induced oxidative spoilage.
3. To examine the role of copper and iron in haze formation.

Early in the project, it became apparent that the chemistry of both the oxidative and haze formation systems was extremely complex. The research team leaders made a decision to investigate the role of copper in wine oxidation in the first instance and to address the role of iron subsequently. In the final outcome of the project, it proved possible to investigate only the copper/oxidation question. As a consequence, the objectives were refined by the research team to be :

R1. To characterise the chemical forms of copper in wine.
R2. To propose a working model for copper-induced oxidative spoilage.

This report will be based on these revised objectives only. An indication of the future direction of the project will be given in the conclusion.

Project Funding and Dates
Support for this project has extended over four years of GWRDC funding.

Commencement date	1 January, 1993
Revised completion date	30 June, 1996

The annual funding amounts were

1992/93	$25,854
1993/94	$26,549
1994/95	$26,366
1995/96	$16,558

In addition, a summer scholarship of $1,000 was provided for November and December, 1996.

PROJECT OBJECTIVE R1 :

To characterise the chemical forms of copper in wine

Background

Copper, which finds its way into grape juice from either vineyard sprays or contact with copper-based equipment, tends to precipitate with yeast cells during and after fermentation such that the copper concentration of freshly fermented wine is generally less than 0.1 mg/l. The main source of copper in finished wine is a consequence of the practice of adding copper(II) sulfate for the removal of sulfidic off-odours. The added copper(II) precipitates as copper sulfide which can be separated by settling or filtration. A complication of this vinification procedure is that the added copper(II) is in significant stoichiometric excess of the amount of sulfide present, resulting in high residual copper(II) concentrations in the wine.

High residual copper(II) has been linked with an enhanced rate of oxidative spoilage which ultimately results in the browning of the wine, especially white wine [1]. Further, copper(II) can contribute to the formation of hazes (copper casse) which may not become apparent until after the wine is bottled. To minimise the incidence of these spoilage problems, it is generally recommended that the copper concentration in finished wines should remain below 0.3 to 0.5 mg/l [2]. However, there is sufficient anecdotal evidence that wines with total copper concentrations less than 0.3 mg/l may be subject to copper-induced spoilage, suggesting that knowledge of the chemical forms of copper in wine, rather than its total concentration, may be a better indicator of the potential for spoilage. The discrimination between the different chemical forms of copper, or any metal, is known as **speciation analysis**.

Electrochemical methods have long been established as suitable techniques for speciation determination and electrochemical stripping techniques in particular have been found to have the desired sensitivity for speciation analysis [3]. One simple approach to speciation assessment is to determine the **labile** and **total** metal concentrations, in this case for copper. The labile concentration refers to that fraction of the total concentration which can be deposited at the electrode surface by the application of a suitable electrolysis potential. It is generally assumed, based on speciation studies in water chemistry, that this labile concentration represents the chemically active form of the metal. Stripping potentiometry was the technique selected for this speciation study. The theory of stripping potentiometry is outside the scope of this report, but a detailed review of the principles of the technique was published in 1982 [4]. In addressing this Objective, the aim was to determine the labile concentration and to relate this concentration to the potential for spoilage of the wine.

References

1. Loubser GJ, Sanderson RD (1986) S. Afr. J. Enol. Vitic. 7:47-51
2. Rankine B (1991) Making good wine. Sun, Australia p277.
3. Soares HMVM, Vasconcelos MTSD (1995) Anal. Chim. Acta 314:241-249.
4. Jagner D. (1982) Analyst 107:593-599.

Experimental Approach

The equipment used was the Radiometer Analytical (Lyon, France) TraceLab 20 system, purchased from funding provided by this grant. This operating system allows the ready application of stripping potentiometry to the determination of copper.

Total copper concentrations were determined by stripping potentiometry in a high acid/high chloride matrix. This matrix was optimised to permit the ready determination of the total copper concentration in white wine.

A check method by flame atomic absorption spectrophotometry (Flame AAS), routinely used by the wine industry, was used to verify the accuracy of the stripping potentiometry.

Labile copper concentrations were determined by performing the copper analysis in the wine without any form of sample pre-treatment. The absence of any pre-treatment ensured that there was no perturbation of the copper distribution pattern in the wine and the results are thus a reflection of the labile concentration.

Results and Discussion

Table 1 presents the results of the measurement of the total and labile copper concentration for four white wine samples. The agreement between the total copper concentration by stripping potentiometry and the check value by Flame AAS is excellent. In addition to the four samples listed in Table 1, the stripping potentiometric method was applied to seven additional white wine samples, again with excellent agreement. These results demonstrate that stripping potentiometry is a viable alternative to methods based on atomic spectrometry.

The labile concentrations of copper determined by the procedure developed in this project represent about 70 to 80% of the total copper. As this method for assessing labile copper was only applied to three white wine samples, it is not possible to draw any conclusions between the labile copper concentration and the possible onset of copper-induced spoilage.

It must also be noted that the calibration matrix used for quantifying the labile copper concentrations was tartrate based (12% aqueous ethanol saturated with potassium hydrogen tartrate and adjusted to pH 3.2 with tartaric acid). That is, as all the copper in the tartrate calibration matrix is labile, then it is reasonable to assume that the 70 - 80% labile copper in the three wine samples investigated is in a chemical form similar to that of the calibration matrix. The form of the remaining (inert) copper has not been determined.

Table 1. Total and labile copper in white wine by stripping potentiometry (mean and the 95% confidence limit are quoted). The number of determinations is given in parentheses.

Wine	Flame AAS (mg/l) as check method	Total copper by stripping potentiometry (mg/l)	Labile copper by stripping potentiometry (mg/l)
White 1	0.28 ± 0.01 (5)	0.27 ± 0.01 (7)	0.18 ± 0.01 (3)
White 2	0.27 ±0.01 (8)	0.23 ± 0.01 (9)	0.18 ± 0.01 (2)
White 3	0.69 ± 0.05 (3)	0.65 ± 0.02 (10)	
White 4	0.64 ± 0.08 (6)	0.67 ± 0.04 (5)	0.52 ± 0.07 (3)

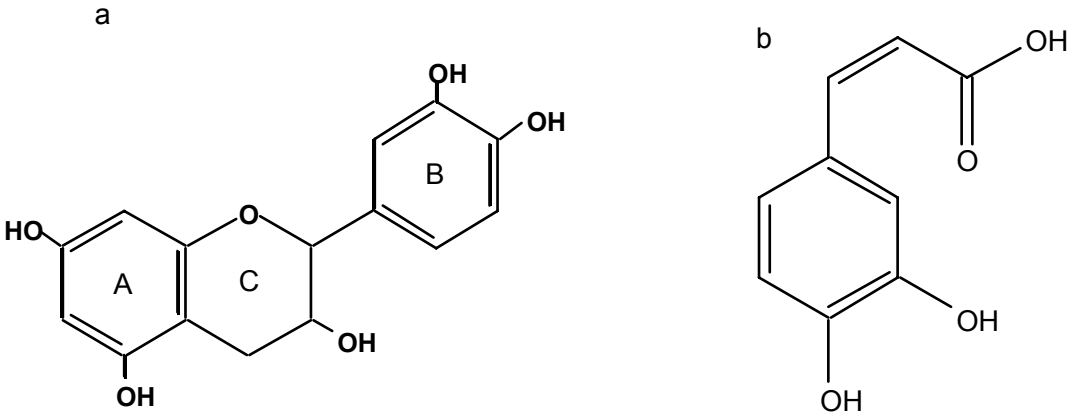
PROJECT OBJECTIVE R2 :

To propose a working model for copper-induced oxidative spoilage

Background and Study Approach

The chemical system used for this component of the project was a model wine developed at The Australian Wine Research Institute by the Chemistry Group. The potentially oxidisable organic substrate in the model wine was either catechin (Fig 1a), as an example of a flavonoid, or caffeic acid (Fig 1b), as an example of a non-flavonoid. The medium was 12% (v/v) aqueous ethanol, saturated with potassium hydrogen tartrate and adjusted to pH 3.2 with tartaric acid. Varying concentrations of copper(II) and ascorbic acid were also added to this model wine, either individually or together.

Figure 1. Structures of catechin (a) and caffeic acid (b).



There is an extensive discussion in the literature on the interaction of copper(II) with organic molecules similar to catechin, particularly those with *o*-dihydroxy substituents on an aromatic ring (as ring B in Fig 1a). However, much of this published work has used either non-aqueous solutions or aqueous media totally unlike wine. In many cases, the pH used was in excess of 7 (and in some cases closer to pH 9). As the reactivity of the dihydroxyaromatic substances is generally dependent on pH, these previous studies do not allow any conclusions to be drawn within respect to copper(II)-induced reactions at wine pH.

Thus, the intention of the study undertaken to address this objective was to examine the behaviour of the copper and to attempt to find changes in either the chemical speciation or the oxidation state of the copper. The electrochemical technique of anodic stripping voltammetry (ASV) was used to examine changes in the copper chemistry. ASV was applied, rather than stripping potentiometry, as it is more flexible at the copper concentrations used in this study.

Experimental Approach

Solutions of the model wine containing either catechin or caffeic acid together with copper(II) and, in some experiments, ascorbic acid, were prepared and stored with screw capped bottles with dioxygen in the headspace. Reactions were allowed to occur at 45^{o}C in the dark. Bottles were opened daily and swirled to refresh the dioxygen concentration in the headspace. Changes in the labile copper concentration were measured by ASV using a hanging mercury drop electrode. A calibration matrix prepared in the model wine (tartrate-based, but omitting

the catechin or caffeic acid) was used to relate peak heights in the reacting systems to concentration. The study for Objective R1 had shown that all copper in this model wine was in the labile form.

Browning was estimated by measuring the absorbance at 440 nm in 10 mm cuvettes. The wavelength of 440 nm was used, rather than the industry standard of 420 nm, as a spectrum of the model wine containing oxidised catechin or caffeic acid showed a peak at 440nm.

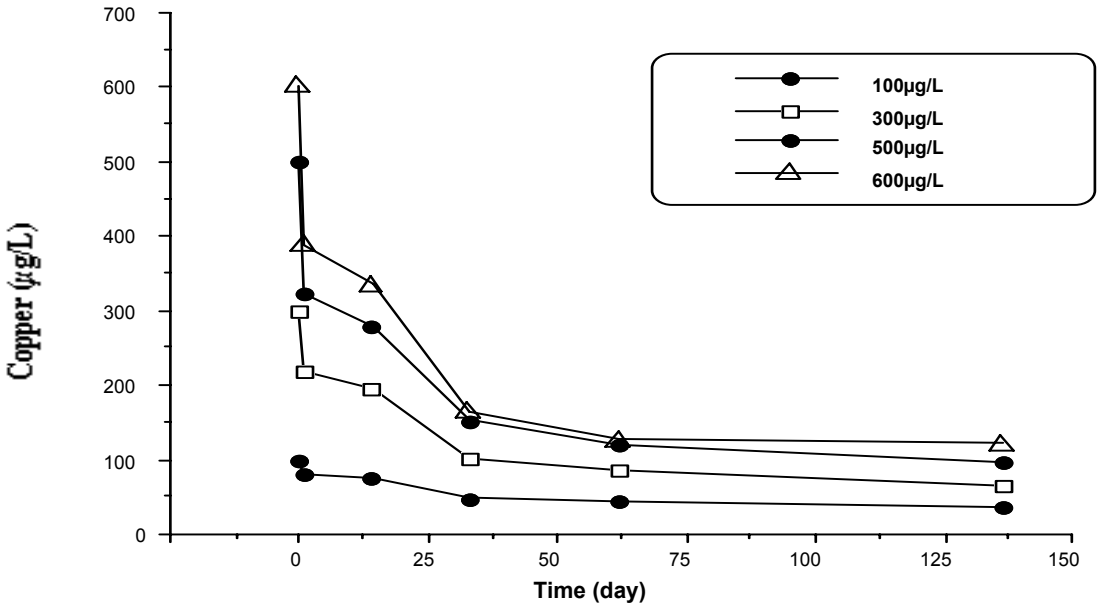
Absorbance measurements were also used to determine the amount of copper(I) present. The colorimetric agent, 4,4'dicarboxy- 2,2'biquinoline, was used as it is specific for copper(I).

Results and Discussion

Studies with catechin

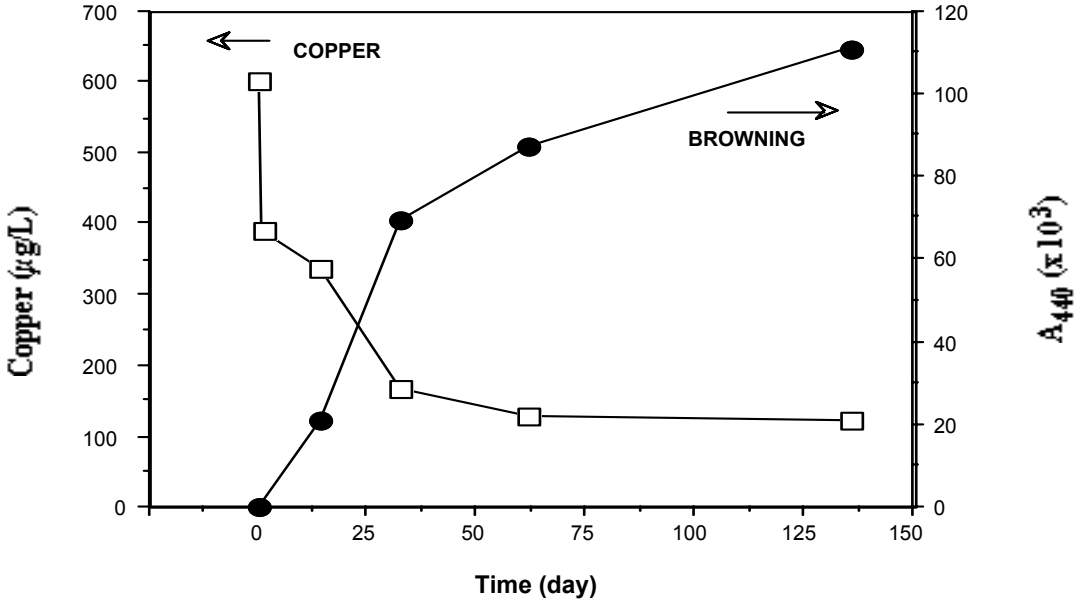
The technique of ASV is capable of determining the labile or electroactive concentration of copper and it was used in this study to monitor changes in the copper concentration during the oxidation of the organic substrate present in the model system. Fig 2 (catechin as reactant) demonstrates clearly that the concentration of ASV labile copper decreases significantly over time. There is a marked decrease within the first 16 hours (after overnight reaction), followed by a slower loss of labile copper up to approximately 30 days after mixing before reaching a plateau with the residual labile copper concentration being about 25 to 30% of the original added concentration. The total copper remained constant, and equal to the original concentration, throughout each experiment. In the absence of catechin, the copper remains electrochemically active over the same period of time.

Figure 2. Change in the ASV labile copper concentration in the presence of catechin.



The loss of ASV labile copper is reflected in the increase in browning (Fig 3; catechin as reactant). It is apparent from a comparison of the relative rates of change in Figs 2 and 3 that the increase in browning is much less dependent on the added copper concentration. Further, the incremental change in the rate of browning is much slower than the rate of loss of ASV labile copper.

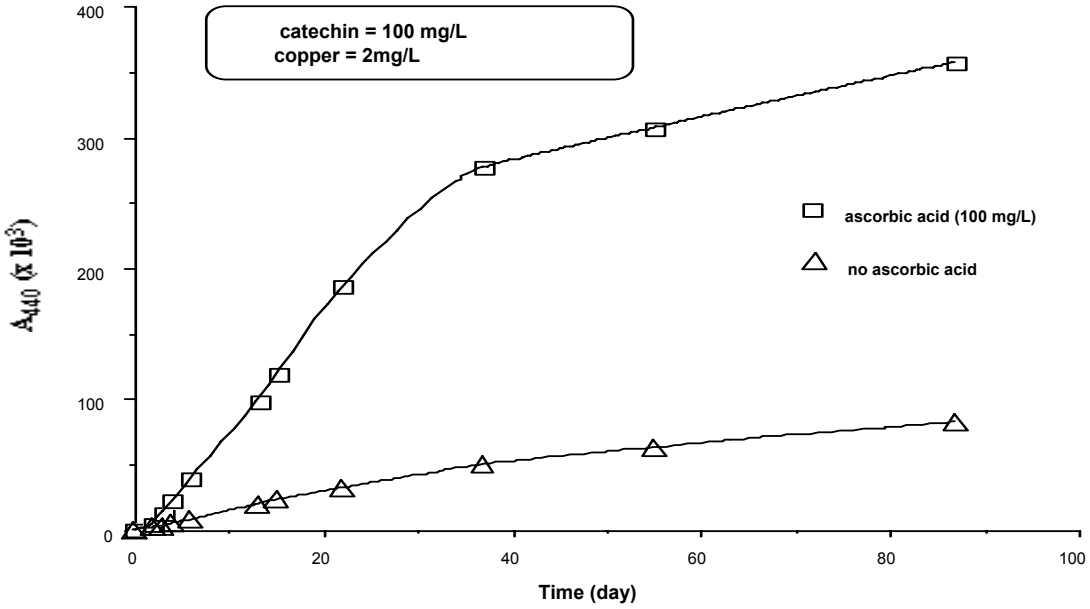
Figure 3. Change in the rate of browning of catechin compared with the rate of loss of ASV labile copper.



Effect of ascorbic acid

The presence of ascorbic acid, especially when added with copper(II), caused a significant enhancement in the rate of browning (Fig 4) in a model wine containing catechin. The loss of labile copper, as measured by ASV, was also enhanced when ascorbic acid was present. The concentration of labile copper was reduced by more than 70% of the original concentration after 10 days in the reaction system containing ascorbic acid, copper and catechin compared with about 50 days in the reaction system containing copper and catechin only.

Figure 4. Effect of ascorbic acid on the rate of browning of catechin



Oxidation state of the copper

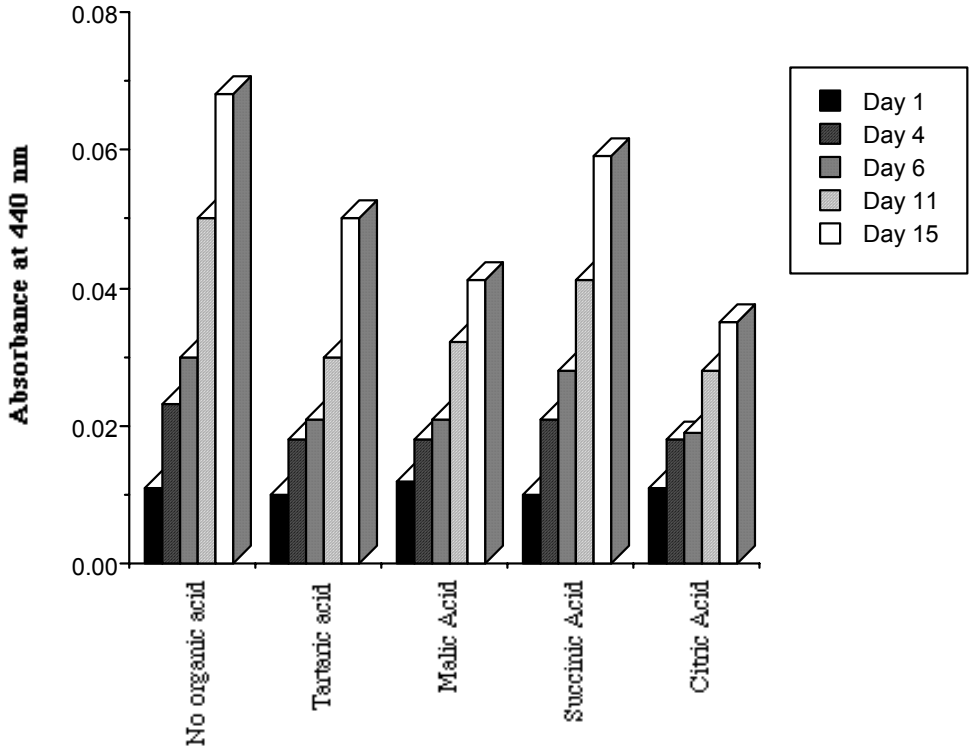
The change in labile copper, as sensed by ASV, gives no indication of the oxidation state of the copper in the reaction system. Rather, ASV indicates only the degree of reactive copper. To obtain further insight into the oxidation state of the copper, a colorimetric assay, specific for copper in the +1 oxidation state, was used. Considerable experimental difficulties were encountered in setting up this colorimetric assay and it was found necessary to remove all catechin (unreacted and oxidised) by extensive PVPP fining prior to the colour development step. This physical/mechanical treatment of the solution (fining plus filtration) may have caused some perturbation of the reaction systems. Irrespective of this potential limitation, the presence of copper(I) was clearly identified by this colorimetric assay throughout the entire reaction period. It must be noted that ascorbic acid has long been used to generate standards in colorimetric assays for copper(I). Thus, it is reasonable to conclude that the enhanced rate of browning observed when ascorbic acid is added to the copper/catechin reaction system in a consequence of a higher concentration of copper(I) being present.

Influence of organic acid

Tartaric acid hinders the rate of browning, as can be seen in Fig 5. The other organic acids used also hinder the rate of browning with citric acid being the most efficient inhibitor and succinic acid being the least effective. The control (labelled “no organic acid”) was an aqueous ethanol system set to pH 3.2 and this showed the fastest rate of browning. The most obvious explanation of these data is that the organic acid binds the copper(II), thereby reducing its availability for involvement in the oxidative reaction.

A separate study by polarography on the complexation, or binding, reaction between copper(II) and tartrate at pH 3.2 have shown that a 1:1 complex between copper(II) and tartrate forms immediately on mixing. This observation supports the argument that the reduced rate of browning in the presence of tartrate and other organic acids is a consequence on the reduced availability of the copper ion for the oxidative reaction of the catechin.

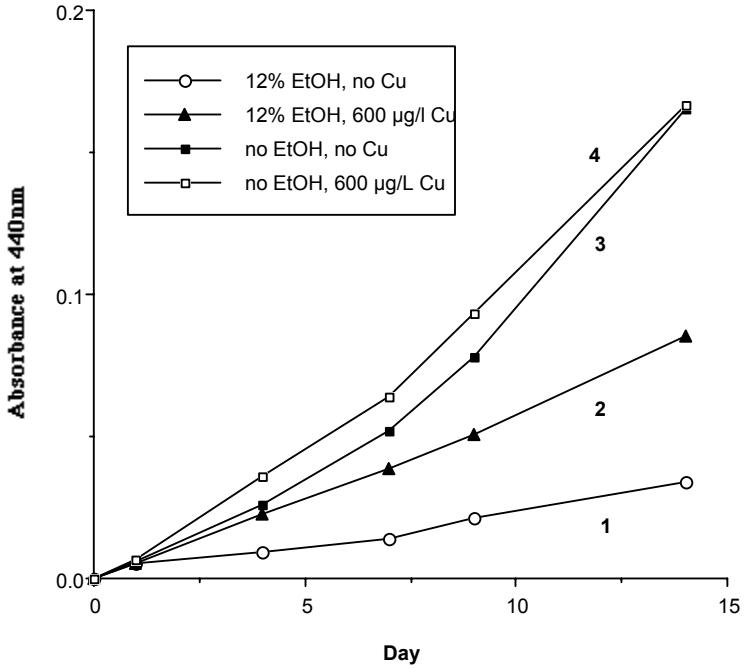
Figure 5. Influence of organic acids on the rate of browning of catechin.



Influence of ethanol

Ethanol at 12 % (v/v) significantly reduces the rate of the oxidative browning reaction involving catechin (Fig 6). A comparison of curves 2 (12% ethanol) and 4 (no ethanol) in Fig 6 clearly demonstrates this point. It is also apparent from a comparison of curve 1 with 2 and curve 3 with 4 in Fig 6 that the rate of browning is much more dependent on copper when ethanol is present. From ASV experiments, it was also observed that the rate of loss of ASV labile copper is considerably faster when ethanol is omitted from the reaction medium. Obviously ethanol is capable of acting as a scavenger of one of the reactive intermediates in the oxidative process. The detailed role of ethanol has not been identified however.

Figure 6. Influence of ethanol on the rate of browning of catechin.



Studies with caffeic acid

Loss of ASV labile copper was also observed when caffeic acid was used in place of catechin as the oxidisable substrate. However, the rate of decrease in the labile copper concentration was not as significant with caffeic acid compared with catechin. Absorbance measurements at 440 nm on the reaction system containing caffeic acid showed that there was only little development of browning after 180 days and that this browning development was not dependent on the copper concentration.

Thus, although the ASV results indicate that there is an interaction between copper and caffeic acid, the caffeic acid is not subject to the same degree of copper-induced browning as is catechin. This, undoubtedly, is related to the different chemical structures of caffeic acid and catechin (Fig 1).

Possibility of free radical processes

Electron paramagnetic resonance (epr) was used to check for the presence of free radicals in this oxidative process. Radical trapping agents were used because of the low concentration and short lifetime or high reactivity of some free radicals, such as superoxide or hydroxyl, which may be present.

No free radicals could be detected in the reaction system involving copper and catechin in the tartrate-based model wine system. Similar observations have been reported for analogous studies (see Balla et al., Inorg Chem, 1992, 31:58-62). The absence of detectable free radicals implies that the copper-mediated oxidative process is a direct chemical process.

Proposed Mechanism

The mechanism proposed in this discussion has been developed only for catechin, as it is the more reactive of the two organic substrates considered in this project. The proposed mechanism is based on observations reported in the literature (Balla et al., Inorg Chem, 1992, 31:58-62) as well as the following interpretation of the results described above :

- ASV studies suggest that there is a strong interaction between catechin and copper.
- The oxidised and presumably polymerised form of catechin generates the brown colour. It is relatively easy to convert the *o*-dihydroxy groups on catechin to the oxidised quinone form.
- Copper(I) has a critical role to play, especially as the rate of oxidation is enhanced by ascorbic acid. The combination of copper(II) and ascorbic acid produces copper(I).
- Free radicals are not involved in the copper-mediated oxidation process.

Balla et al. (Inorg Chem, 1992, 31:58-62) and others before them have postulated the existence of CuO_2^+, that is a copper(I)-dioxygen complex, as the "dioxygen-carrier" in the reaction systems involving the oxidation of dihydroxyaromatic compounds. There is no direct evidence for the existence of CuO_2^+, but its involvement in the oxidation reaction mechanism is essential to the interpretation of the reaction kinetics. Its existence is also assumed in the mechanism described here.

In the following mechanism, H_2A represents catechin and HA^- and A^{2-} are the mono- and di-anions of catechin, respectively, formed by the loss of protons. Q symbolises the oxidised form of catechin and CuA is the copper(II)-catechin complex.

STEP 1

$$Cu^{2+} + H_2A \varnothing CuA + 2H^+$$

Formation of CuA may well be responsible for the loss of ASV labile copper.

STEP 2

$$Cu^{2+} + CuA \varnothing 2Cu(I) + Q$$

This disproportionation reaction produces copper(I) and oxidised catechin, Q.

STEP 3

$$Cu(I) + O_2 \rightleftharpoons CuO_2^+$$

The dioxygen carrying species is thus generated.

STEP 4

$$CuO_2^+ + HA^- \varnothing Cu(I) + HO_2^- + Q$$

This reaction is only one of the several ways by which dioxygen is transferred to produce another mole of the quinone, Q. Copper(I) is regenerated and thus is available for further dioxygen transfer processes. Hydrogen peroxide is also proposed as a product in Step 4 and without being scavenged, it is capable of oxidising the catechin. Superoxide is an alternative product. Ethanol is an effective scavenger of both hydrogen peroxide and superoxide and the observation (Fig 6) that ethanol inhibits the rate of oxidative browning may well be a consequence of this scavenging process. The initial oxidation product of ethanol is acetaldehyde, but its presence could not be detected with the experimental protocol used in this study.

In the absence of ethanol scavenging of hydrogen peroxide or superoxide, the role of the copper ion would be to produce these oxidants (by Step 4). The hydrogen peroxide or superoxide could then oxidise the catechin to the quinone form. That is, a copper dependence on the rate of oxidation would not be expected (Fig 6, curves 3 and 4).

When ethanol is present, so that scavenging of the oxidants produced in Step 4 occurs, the only means of dioxygen transfer (that is, oxidation) involves the CuO_2^+ species. A copper concentration dependence in the rate of oxidation would then be expected (Fig 6, curves 1 and 2).

WINE MAKING IMPLICATIONS

The winemaking implications of these findings can now defined :

1. The extent of copper(II) additions should be minimised.
2. The use of ascorbic acid requires caution.
3. The combination of ascorbic acid and copper(II) enhances probability of rapid oxidation occurring.

SUMMARY OF MAJOR FINDINGS

Project Objective R1 : To characterise the major forms of copper in wine.

R1.1 Stripping potentiometry is a viable alternative to Flame AAS for the determination of total copper concentrations.

R1.2 The determination of labile copper in white wine can be achieved using stripping potentiometry.

R1.3 The use of a tartrate-based calibration medium for the assessment of the labile concentrations suggests that the 70 - 80% labile copper in wine is in a chemical form analogous to that of the calibration matrix.

Project Objective R2 : To propose a working model for copper-induced oxidative spoilage

R2.1 Catechin is more reactive than caffeic acid with respect to copper mediated oxidation.

R2.2 There is a rapid interaction between copper(II) and catechin, leading to a significant loss of electrochemically active (labile) copper.

R2.3 A measurable, and significant proportion, of the copper exists in the +1 oxidation state for a protracted period of time.

R2.4 Ascorbic acid enhances the rate of copper mediated oxidation, presumably because it converts all initial copper to the +1 oxidation state.

R2.5 Tartaric acid hinders the rate of copper mediated oxidation, presumably be decreasing the availability of free copper(II) through complexation.

R2.6 Ethanol significantly reduces the rate of the browning reaction involving catechin, suggesting that ethanol acts as a scavenger of the reaction intermediates.

R2.7 There is no evidence for free radicals species being involved in the copper mediated oxidation process.

DISSEMINATION OF INFORMATION

One paper describing the analytical aspects of he measurement of the labile copper concentration has been submitted to *Fresenius Journal of Analytical Chemistry*.

A manuscript on the results of the oxidative studies is in preparation for the *Australian Journal of Grape and Wine Research*.

A technical paper summarising the implications of the project for winemaking will be prepared.

FUTURE WORK

It is proposed to address the more fundamental issues of the mechanism of copper-induced oxidation in a PhD program funded by DEETYA. This new program will commence in March, 1997.

Under an ARC funded project in 1997, a more suitable assay for the direct detection of copper in the +1 oxidation state will be carried out.