NATIONAL Wine&Grape Industry CENTRE

Australian Government
Grape and Wine Research and Development Corporation

Mechanism of oxidative browning of white wine by copper(II) and ascorbic acid



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CSU01/02**

Principal Investigator: **Professor Geoff Scollary**

Research Organisation:
National Wine and Grape Industry Centre

Date: **17 September, 2004**

EXECUTIVE SUMMARY

1. Project CSU 01/02 addressed the issues of the role of copper(II) in mediating the oxidation of (+)-catechin and related polyphenolic compounds, the factors that influence the degradation of tartaric acid, and the chemistry of ascorbic acid as a pro-oxidant.

2. An examination of the role of copper(II) in enhancing the development of colour in a model white wine containing (+)-catechin as the oxidisable substrate, as measured at 440 nm, showed that the increase in absorbance was not directly proportional to the copper(II) concentration.

3. The species that gave rise to the increase in colour was identified as a xanthylium pigment for which the basic building block is two (+)-catechin molecules bridged by glyoxylic acid. There is no copper present in the coloured species.

4. Copper(II) ions were found to enhance the bridging of the (+)-catechin molecules by glyoxylic acid. For this reason, the term "copper mediated oxidation" is used to describe the process.

5. Glyoxylic acid can be produced by exposure of tartaric acid solutions to sunlight.

6. Hydrogen peroxide is also formed in the sunlight exposed solutions of tartaric acid.

7. Copper(II) increases the amount of glyoxylic acid formed in light exposed tartaric acid solutions by reducing the amount of hydrogen peroxide. If copper(II) ions are absent from the model system, hydrogen peroxide oxidises glyoxylic acid to formic acid.

8. The hydroxyl radical was identified as the species responsible for initiating the chemical reaction sequence that leads to the formation of glyoxylic acid from the cleavage of tartaric acid.

9. Ethanol in inhibits the cleavage of tartaric acid as it is an effective scavenger of the hydroxyl radical

10. An analytical procedure based on ion exchange chromatography (IEC) was developed and verified for the quantification of hydrogen peroxide in the model systems. The IEC method allowed simultaneous determination of hydrogen peroxide and the organic acids formed from the breakdown of hydrogen peroxide.

11. A chemiluminescence method for hydrogen peroxide quantification was investigated. A rapid method using flow injection analysis was developed, but the method did not allow simultaneous quantification of the organic acids.

12. A comparison of the oxidation the related flavanols, (+)-catechin and (-)-epicatechin, showed that similar concentrations of the xanthylium pigments were produced, but that the absorbance of the (-)-epicatechin based xanthylium pigment was almost double that of the (+)-catechin based pigment. This result suggests that knowledge of the flavanol profile is necessary in order to predict the propensity of a wine towards oxidative coloration.

13. The colour of the glass in wine storage bottles was found to be important in determining the extent of oxidative colour development of a white wine. A Sauvignon Blanc wine stored in Classic Green and Antique Green bottles developed a higher colour, as measured at 440 nm, than the same wine stored in Flint and French Green bottles.

14. Significant colour development was found to occur only when the concentration of (+)-catechin type phenolic compounds was increased through additions. This underpins the important role played by this class of phenolic compounds in being responsible for colour development resulting from oxidative processes.

15. The main driver for the higher colour development of the darker bottles appeared to be temperature, with the wine in the Classic Green and Antique Green being 5^{o}C to 12^{o}C warmer than the wine stored in Flint and French Green bottles.

16. A semi-preparative chromatographic method for separating the various fractions arising from the breakdown of ascorbic acid was established. However, the species within each fraction were far too reactive to allow their identification. New technologies that allow on-line identification of the chemical structures during a chromatographic run will be employed post this project.

17. A comparison of the capacity of ascorbic acid and erythorbic acid to induce oxidative coloration in catechin-type flavanols showed that ascorbic acid induced higher absorbance values at 440 nm.

18. The highest oxidative coloration was achieved with the combination of (-)-epicatechin and ascorbic acid while the lowest oxidative coloration resulted from the combination of (+)-catechin and erythorbic acid.

SIGNIFICANT OUTCOMES

1. Tartaric acid solutions undergo oxidative degradation under reasonably mild storage conditions.

2. Glyoxylic acid, a precursor to xanthylium pigment formation, is produced from the cleavage of tartaric acid

3. Ethanol inhibits, but does not prevent, the degradation of tartaric acid.

4. Copper(II) is capable of mediating the bridging of (+)-catechin by glyoxylic acid and of providing increased stability for glyoxylic acid by hindering its further oxidation.

5. Hydrogen peroxide is produced in tartaric acid solutions that are stored in light.

6. The mechanism of the tartaric acid cleavage process is consistent with the production of hydroxyl radicals.

7. The role of the hydroxyl radical was confirmed by gamma-radiation experiments.

8. (-)-Epicatechin produced xanthylium pigments that had a higher absorbance at 440 nm for the same concentration of (+)-catechin derived xanthylium pigments.

9. Ascorbic acid is capable of inducing greater oxidative colour development in catechin-type flavanols than is erythorbic acid.

10. White wine contained in dark green bottles and stored in light showed greater colour development than wine stored in light green or colourless bottles.

COMMUNICATION OUTCOMES

1. Three scientific publications published in refereed journals.

2. Five scientific publications are in preparation.

3. One workshop and two posters at the 12th Australian Wine Industry Technical Conference in 2004.

4. One oral (invited plenary) lecture at the 3rd *In Vino Analytica Scientia* conference in 2003.

5. Three oral and three poster presentations at wine industry technical symposia and scientific meetings

6. Discussion of the major outcomes of this project in CSU's Wine Chemistry subject in 2002, 2003 and 2004 (approximately 80 students each year).

PROJECT OVERVIEW

Preamble

This report summarises research activities into the mechanism of oxidative browning of white wine by copper(II) and ascorbic acid. The emphasis in the project has been on elucidating the fundamental chemistry of the phenolic oxidative process as mediated by copper(II) and induced by ascorbic acid. The majority of the research work was performed using model systems, although some preliminary experiments with a white wine of relatively high phenolic composition were carried out. The outcomes of the project show how the degradation of tartaric acid and ascorbic acid establish conditions that can lead to enhanced rates of phenolic oxidation. Further insights into the factors influencing the white wine quality defect of random oxidation can be obtained from the results of this research project.

Research venues

- National Wine and Grape Industry Centre, Wagga Wagga
- School of Biological and Chemical Sciences, Deakin University, Geelong
- ANSTO, Lucas Heights

Research staff

- Professor Geoffrey R Scollary, NWGIC, Wagga Wagga
- Dr Paul Prenzler, NWGIC, Wagga Wagga
- Dr Andrew Clark, NWGIC, Wagga Wagga (from 1 July 2003 to 30 June 2004)

Post-doctoral Fellows appointed on GWRDC funds

- Dr Andrew Clark, from 1 August 2001 to 30 June, 2003
- Dr Chantal Maury, from 1 July 2003 to 30 June 2004

Dr Clark was appointed to the academic staff of Charles Sturt University on 1 July 2003 and he was replaced for the final year of the project by Dr Chantal Maury.

Visiting student

Mr Florian LaBrouche, from the Institut National Agronomique, Paris-Grignon, from 16 June 2003 to 12 September 2003.

Mr Labrouche's visit was funded by the Institut National Agronomique, Paris-Grignon.

Project dates and funding

Project commencement date	1 July 2001
Project completion date	30 June 2004

Funds (GST exclusive figures) provided by the GWRDC for the project were

2001/02	$80,677
2002/03	$83,991
2003/04	$88,478

Project Objectives

1. To identify the free radicals generated in oxidative browning of (+)-catechin by copper(II) and ascorbic acid
2. To determine the reaction sequence involving free radicals in the formation of brown products from (+)-catechin
3. To apply the results from model studies to commercial wines
4. To determine the efficiency of wine components other than polyphenols to scavenge free radicals

OUTPUTS

Scientific publications

Published

Andrew C Clark and Geoffrey R Scollary (2002). Copper(II)-mediated oxidation of (+)-catechin in a model white wine system. Australian Journal of Grape and Wine Research, **8**, 186-195.

Andrew C Clark and Geoffrey R Scollary, (2003). Influence of light exposure, ethanol and copper(II) on the formation of a precursor for xanthylium cations from tartaric acid. Australian Journal of Grape and Wine Research, **9**, 64-71.

Andrew C Clark, Paul D Prenzler and Geoffrey R Scollary (2003). The role of copper(II) in the bridging reaction of (+)-catechin by glyoxylic acid in a model white wine. Journal of Agricultural and Food Chemistry, **51**, 6204-6210.

In preparation

Florian Labrouche, Andrew Clark, Paul Prenzler and Geoffrey Scollary. Isomeric influence on the oxidative coloration of phenolic compounds in a model white wine: comparison of (+)-catechin and (-)-epicatechin.

Andrew C Clark, Paul D Prenzler and Geoffrey R Scollary. Time-dependent production and stability of glyoxylic acid in tartaric acid solutions stored outdoors.

Andrew C Clark, Chantal Maury, Paul D Prenzler and Geoffrey R Scollary. Mechanism of hydroxyl radical degradation of tartaric acid solutions.

Chantal Maury, Andrew C Clark, Paul D Prenzler and Geoffrey R Scollary. Influence of wine bottle colour on the oxidative coloration of a white wine.

Chantal Maury, Andrew C Clark, Paul D Prenzler and Geoffrey R Scollary. Comparison of ascorbic acid and erythorbic acid in the oxidative coloration of (+)-catechin and (-)-epicatechin.

Technical publications

Andrew C Clark and Geoffrey R Scollary. Is it brown? Is it yellow?...No it's oxidation. In preparation for Australian and New Zealand Grapegrower and Winemaker

Conference and symposium presentations

Oral presentation – Opening plenary lecture

Geoffrey R Scollary. Analytical techniques to examine random oxidation in white wine systems, ***In Vino Analytica Scientia***, Aveiro, Portugal, 10-12 July, 2003.

Oral presentation

Andrew C Clark. Understanding troubled wines, 2003 National Wine and Grape Industry Centre Industry Symposium, Wagga Wagga, 19 June 2003.

Oral presentation
Andrew C Clark. Phenolic oxidation: implications for white wine quality, 2004 National Wine and Grape Industry Centre Industry Symposium, Wagga Wagga, 24 June 2004

Oral presentation
Andrew C Clark. White wine oxidation. 2003 CSU Research Symposium, Wagga Wagga, 10 October, 2003.

Poster presentations
12th Australian Wine Industry Technical Conference, Melbourne, 24 - 28 July, 2004
Chantal Maury, Andrew Clark, Paul Prenzler and Geoffrey Scollary. Oxidation of flavanols in the presence of ascorbic and erythorbic acids.

Andrew Clark, Paul Prenzler and Geoffrey Scollary. The time-dependent production of a pigment precursor from tartaric acid.

2003 National Wine and Grape Industry Symposium, Wagga Wagga, 19 June, 2003
Andrew Clark, Mark Bradshaw and Geoffrey Scollary. Effect of copper(II) and ascorbic acid on the coloration of a model white wine

Workshop presentations
W22, Oxidation of wine. 12th Australian Wine Industry Technical Conference, Melbourne, 24 - 28 July 2004.

Geoffrey R Scollary. White wine oxidation: introduction

Andrew C Clark. Tartaric acid degradation

Andrew C Clark. Phenolic (catechin type) and oxidant (ascorbic acid type) chemistry

ABBREVIATIONS

IEC	ion exchange chromatography
LC/DAD	liquid chromatography/photodiode detection
LC/MS	liquid chromatography/mass spectrometry
PPO	polyphenoloxidase
rsd	relative standard deviation
SWV	square wave voltammetry
UV/Visible	Ultraviolet/Visible spectrophotometry

DETAILED PROJECT REPORT

BACKGROUND

This project is in three parts

1. An examination of the role of copper(II) in mediating the oxidation of (+)-catechin
2. Factors influencing the degradation of tartaric acid
3. An examination of the chemistry of ascorbic acid.

The work on copper (II) and tartaric acid degradation in this project developed from studies carried out by Dr Andrew Clark in his PhD thesis research (Studies on the copper-mediated oxidation of white wine; The University of Melbourne, 2001).

The outcomes of GWRDC project UM96/1 provided the basis for the work on ascorbic acid in this project.

Based on a study published by Simpson in 1982 (Factors affecting oxidative browning of white wine (1982), Vitis, 21, 233-239), the oxidisable phenolic substrate chosen for the majority of this work was (+)-catechin (Figure 1). (+)-Catechin is characterised by possessing one aromatic ring with phloroglucinol functionality (Ring A; Figure 1) and a second aromatic ring (B) with catechol functionality. Caffeic acid (Figure 2) possesses only one aromatic ring, equivalent to the B ring of (+)-catechin showing catechol functionality. Based on Simpson's observations, there is no significant correlation between caffeic acid concentrations in white wine and the potential of the wine to undergo oxidative coloration.

Figure 1. The structure of (+)-catechin

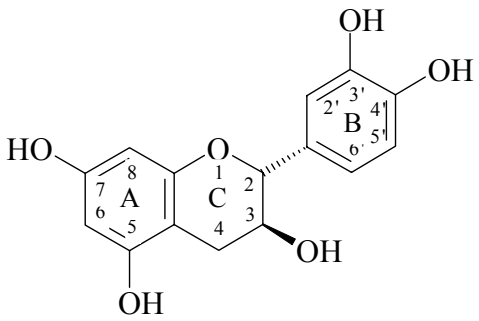
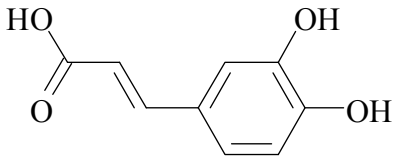


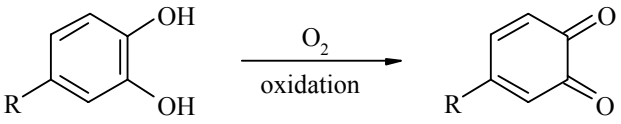
Figure 2. The structure of caffeic acid



Non-enzymic oxidation, the type of oxidation of phenolic compounds that is being examined in copper(II)-mediated oxidation and ascorbic acid induced oxidation, is generally assumed to involve the oxidation of the *ortho*-hydroxy groups in the ring showing catechol-type functionality to give the *ortho*-quinone compound. The *ortho*-quinone compound is then capable of reacting with other wine components to form coloured polymers. Figure 3 summarises the oxidation process. Note that the direct reaction of molecular oxygen with the

phenolic compound requires a catalyst to allow the triplet energy state oxygen molecule to react with the singlet state phenolic compound. Metal ions, particularly copper, iron and manganese, are thought to be important catalysts.

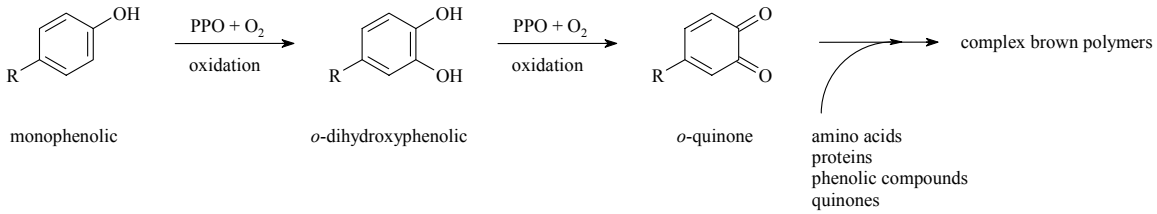
Figure 3. The commonly reported reaction for the conversion of an *ortho*-dihydroxyphenolic compound to the corresponding *ortho*-quinone



o-dihydroxyphenolic o-quinone

This proposed reaction for non-enzymic oxidation is based on the well-established mechanism for enzymic oxidation (Figure 4). However, much of the published work on non-enzymic oxidation has been carried out at pH 6 or higher (see, for example, Singleton (1987) Oxygen with phenols and related reactions in musts, wines and model systems: observations and practical implications. American Journal of Enology and Viticulture **38**, 69-77) and it is questionable whether studies at such high pH values are relevant to wine pH. Clearly, at pH 6 and higher, the *ortho*-dihydroxyphenolic groups will be almost fully ionised and this will make conversion to the quinone through a semi-quinone intermediate reasonably easy to achieve. At typical white wine pH values (around pH 3.2), the *ortho*-dihydroxyphenolic groups will be essentially fully protonated, limiting the quinone production reaction.

Figure 4. Mechanism for the enzymic oxidation of phenolic compounds where PPO is polyphenoloxidase, the oxidative enzyme



The results of this project will in fact show that the oxidative processes that occur involve the A ring of the (+)-catechin type phenolic compounds and not the B ring. In addition to phenolic oxidation, it will be shown that it is possible to achieve the oxidative cleavage of tartaric acid. That is, it will be argued in this report that there are two oxidative processes: tartaric acid oxidation and (+)-catechin type phenolic oxidation.

Brown, yellow or oxidation?

The term 'browning' has become a commonly used wine industry term to describe phenolic oxidative processes. Traditionally, browning has been measured by the value of the absorbance at 420 nm and, in enzymic oxidation, the solution colour is clearly brown to the observer. In cases of extensive enzymic oxidation, a brown solid may separate from the wine or juice.

Non-enzymic oxidation has also been assessed by absorbance measurements at 420 nm and, by analogy with enzymic processes, 'browning' has been used to describe the process.

However, the extent of colour development in non-enzymic oxidation is considerably less than in enzymic oxidation and the development of a yellow colour is a more appropriate descriptor. Use of a single colour descriptor is not particularly helpful in non-enzymic oxidation as a range of coloured products can be formed that absorb in the 400 – 500 nm region.

In this report, 'oxidation' without a colour descriptor will be used to describe the phenolic reaction process.

THE MODEL SYSTEM USED

Model wine base

The model wine base was prepared by adding 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid to aqueous ethanol (12 % v/v, 2L) and stirring overnight at room temperature. The pH of the model wine base was 3.2 ± 0.1

Model wine

(+)-Catechin (generally at 150mg/litre) was used as the oxidisable substrate in the model wine system and wad added to the wine base just prior to the commencement of the induced browning process.

Induced oxidation process

Phenolic oxidation reactions were performed by adding a sample of the model wine system in a Schott bottle and placing this Schott bottle in the dark in a water bath held at 45^{o}C. Flasks were exposed to air on a daily basis to replenish the molecular oxygen content. In this way the system remained saturated with respect to the molecular oxygen content.

Absorbance measurements

The increase in colour was assessed by measuring the absorbance of the sample at 440 nm. The model wine base (unheated) was used as the blank in these absorbance measurements. The wavelength of 440 nm was used as separate experiments had indicated that it is the wavelength corresponding to the absorbance maximum in this model system that has undergone extensive colouration.

COPPER(II)-MEDIATED OXIDATION OF (+)-CATECHIN

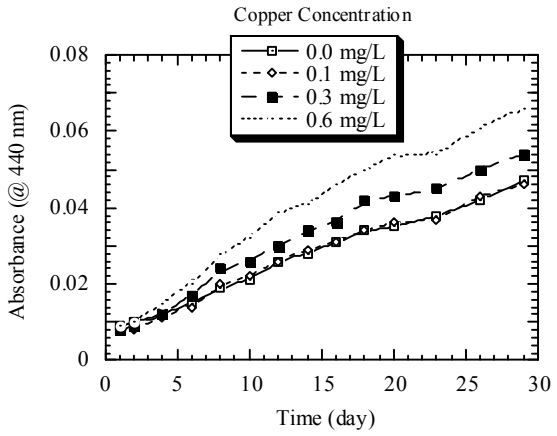
The non-enzymic oxidation of phenolic compounds (Figure 3) requires a catalyst for the activation of molecular oxygen. Metal ions, including copper, iron and possibly manganese, have been assumed to be appropriate species for the catalyst.

In this project, copper(II) was used as the metal ion in the phenolic oxidation studies. Copper(II) was chosen as one of the reasons for this project was industry concern regarding 'copper spoilage' of white wine. It should be noted here that a parallel study at the INRA in Montpellier, France, used iron(II) as the metal ion.

The concentrations of the components of the model wine system were chosen to reflect typical concentrations in white wine as well as to allow reactions to be studied in a reasonable period of time. The (+)-catechin concentration used (150 mg/litre) is at the high end of the concentration normally found in white wines. (+)-Catechin tends to be located in the seeds of the grape and high concentrations in wine are usually a reflection of extensive maceration of the grape marc. Copper(II) concentrations up to 0.6 mg/litre reflect the upper value common in white wines.

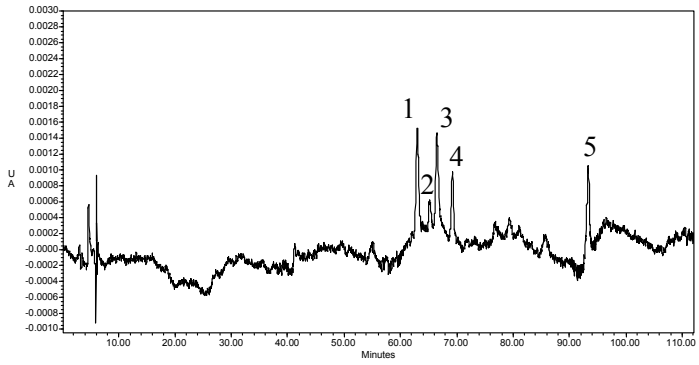
When the model white wine, with differing concentrations of added copper(II), underwent the induced oxidation process, the relationship between the increase in absorbance at 440 nm was not directly proportional to the copper(II) concentration (Figure 5). Intriguingly, an increase in absorbance at 440 nm was observed in the absence of copper(II), the same as that found for 0.1 mg/litre coper(II). An accelerated increase was only observed when the copper(II) concentration was at 0.3 mg/litre or higher (Figure 5). At the end of the induced oxidation process, the solutions were coloured but not cloudy, indicating that no precipitation had occurred.

Figure 5. Changes in absorbance at 440 nm with time for the model white wine at varying copper(II) concentrations during the induced oxidation process



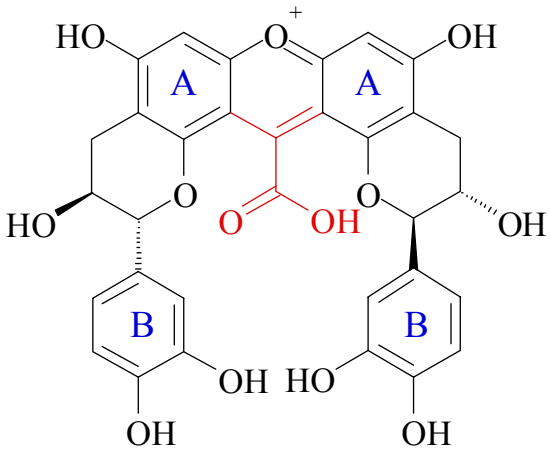
The LC/DAD chromatogram (440 nm) of a sample taken after 29 days of induced oxidation of the solution containing 0.6 mg/litre copper(II) is presented in Figure 6. The most striking characteristic is the set of four peaks at retention times between 65 and 72 minutes together with a single peak at 92 minutes. The UV/Visible spectrum of peaks 1 to 4 in Figure 6 (not shown) had a major absorbance at 440 nm as well as a shoulder at 310 nm. All 5 peaks in the 440 nm chromatogram had corresponding peaks in the 278 nm chromatogram, indicating that the catechin moiety was part of the species giving rise to these peaks.

Figure 6. LC/DAD chromatogram at 440 and 278 nm for the model white wine containing 0.6 mg/litre copper(II) after 30 days of induced oxidation



LC/MS combined with LC/DAD showed that peaks 1 to 4 with a mass of 617 (positive ion mode) were xanthylium pigments (see Figure 7) while peak 5 (mass 617 in positive ion mode) was due to the ethyl ester of the xanthylium pigment. Peaks 1 to 4 in the 440 nm chromatogram are in fact isomers resulting from various combinations of linkages between the 6- and 8-carbon positions on the A-ring of (+)-catechin (Figure 1). The linkage group is glyoxylic acid that result from the cleavage of tartaric acid. The xanthylium pigments identified here are identical to those reported by Es-Safi et al. (Es-Safi et al. (1999) Journal of Agricultural and Food Chemistry, **47**, 5211-5217) when iron(II) was used as the 'catalytic' metal ion.

Figure 7. The xanthylium pigment formed from the oxidative coupling of two catechin molecules bridged by glyoxylic acid (the fragment shown in red) via the A rings of the two catechin molecules



Fascinatingly, there is no copper in the product of the (+)-catechin oxidation. It is apparent from Figure 5, however, that at copper(II) concentrations higher than 0.3 mg/litre in the experimental conditions used in this study, enhanced colouration of the reaction system occurs resulting from higher concentrations of xanthylium pigment formation. The mechanism for the formation of the xanthylium pigments has been shown to be a four-step process (Es-Safi et al. (2000), Journal of Agricultural and Food Chemistry, **47**, 5211-5217):

STEP 1 Cleavage of tartaric acid to give glyoxylic acid (an oxidative process)

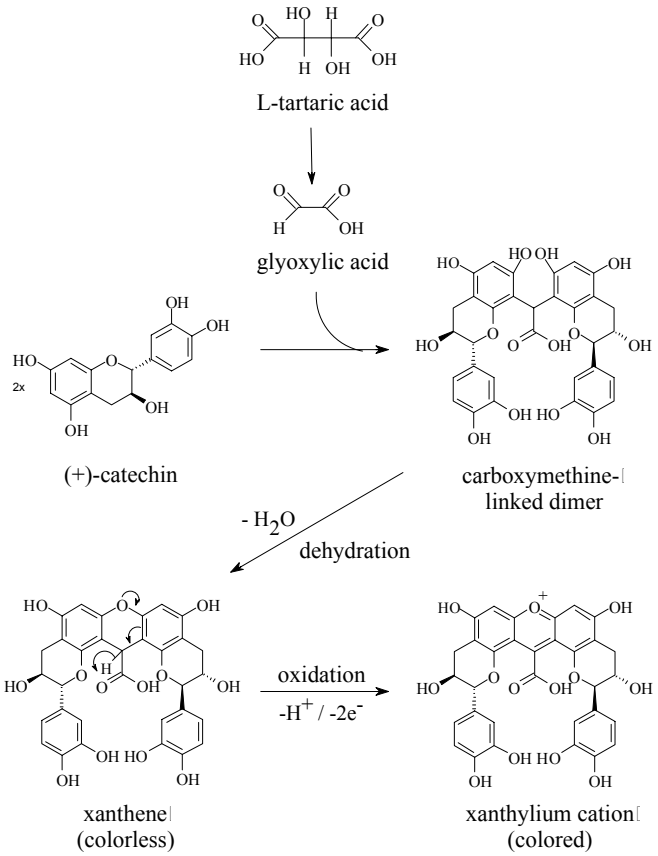
STEP 2 Bridging of two (+)-catechin molecules to give a carboxymethine linked dimer

STEP 3 A dehydration step to generate a xanthene

STEP 4 Oxidation of the xanthene to give a xanthylium pigment.

This mechanism is shown schematically in Figure 8 after Es-Safi et al. (Es-Safi et al. (2000), Journal of Agricultural and Food Chemistry, **47**, 5211-5217).

Figure 8. Schematic showing the four step reaction sequence leading to the formation of a xanthylium pigment from (+)-catechin



The next phase of the study involved a determination of the step or steps in the mechanism that copper(II) influences.

More detail on this study is set out in Appendix 1.

Role of copper(II) in the bridging reaction of (+)-catechin by glyoxylic acid

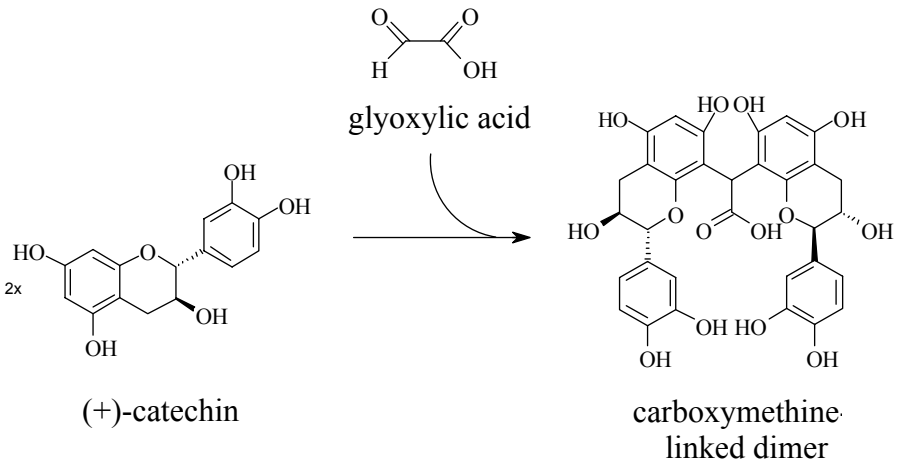
The oxidative cleavage of tartaric acid to give glyoxylic acid is discussed in detail in the next main section of this report. The amount of glyoxylic acid formed from the spontaneous cleavage of tartaric acid is small and highly dependent on the storage conditions of the tartaric acid solutions. To ensure that there was sufficient glyoxylic acid present to allow the bridging of (+)-catechin to occur without limitation of glyoxylic acid, experiments in this section of the

project were performed with added glyoxylic acid in a mole ratio of 2:1 for (+)-catechin to glyoxylic acid.

A full description of this study is set out in Appendix 2.

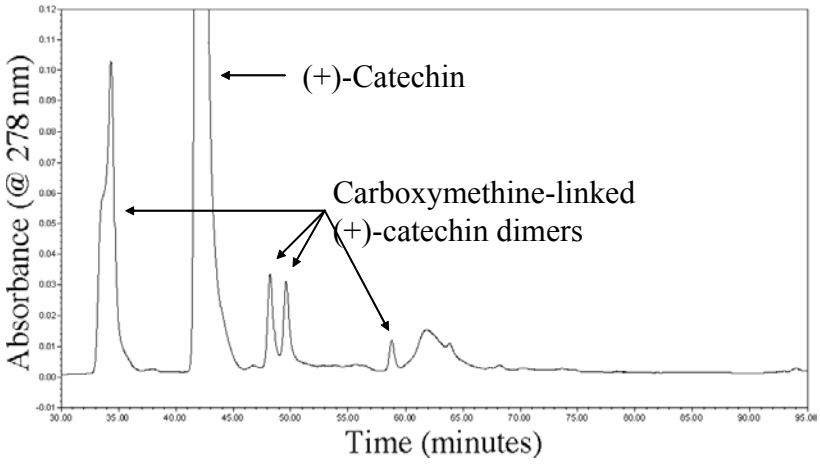
At 45°C, copper(II) increased the maximum levels of the carboxymethine-linked dimer as well as the xanthylium pigment. However, the increase in the amount of the xanthylium pigment was found to be a consequence of the higher levels of the carboxymethine-linked dimer. That is, Step 2 of the xanthylium pigment production (Figure 9) is enhanced by copper(II).

Figure 9. The formation of the carboxymethine linked dimer from the reaction between (+)-catechin and glyoxylic acid



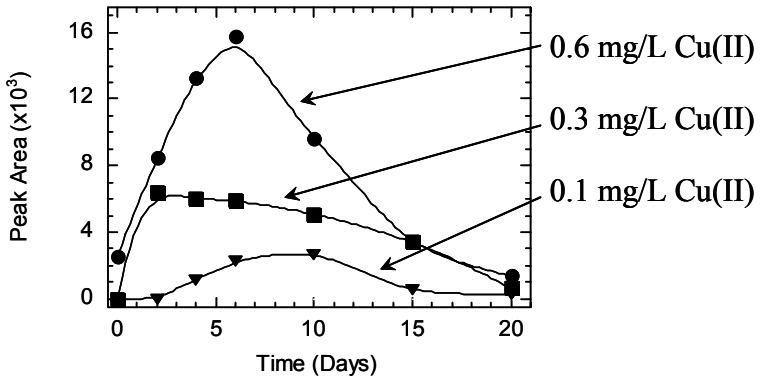
The formation of this dimer was monitored by LC/DAD at 278 nm (Figure 10). Four peaks were identified by LC/MS as corresponding to the dimer: these correspond to the different bridge links between carbon-6 and carbon-8 of the A-ring on (+)-catechin. As the reaction progressed, peaks due to the xanthylium pigment isomers appeared (data not shown).

Figure 10. LC/DAD chromatogram (278 nm) showing the four peaks for the various isomers of the carboxymethine-linked dimer of (+)-catechin



If the change in total peak area (corresponding to concentration) for the four carboxymethine-linked dimer peaks are plotted against reaction time at various concentrations of added copper(II), the impact of the copper(II) ions on the bridging reaction is evident (Figure 11).

Figure 11. The change in peak area for the four carboxymethine-linked dimer peaks (Figure 10) at various copper(II) concentrations as a function of reaction time



The process of bridging catechin-type phenolic compounds by aldehydes is well-known. The classic reaction that has been extensively studied used acetaldehyde as the bridging species. When the bridging reactions using glyoxylic acid and acetaldehyde were compared (at 10^{o}C to minimise losses of acetaldehyde), no copper(II) enhancement was observed for the reaction involving acetaldehyde (see Appendix 2 for data). This implies that the acid group of glyoxylic acid allowed the copper(II) to influence the bridging reaction.

To assist in clarifying terminology, the term 'copper(II)-mediated oxidation' is used as the role of the copper ions is to accelerate the reaction process.

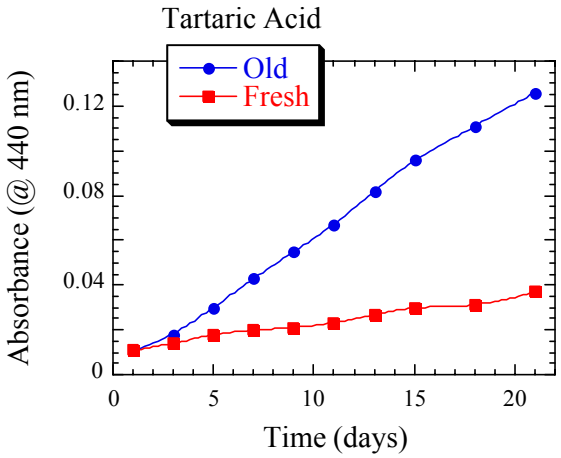
Environmental factors influencing the formation of glyoxylic acid

One of the intriguing issues faced in the early part of this project related to the reproducibility of the absorbance values when accelerated oxidation studies were performed in the model wine. For each run, final absorbance values at 440 nm were highly repeatable with rsd values always less than 5%. When absorbance values were compared on different days, sometimes with different tartaric acid solutions, final absorbance values could be highly variable.

The answer to this problem was found to be related to the age of the tartaric acid solution used to prepare the model wine system. Figure 12 demonstrates the issue very effectively. When an aged tartaric acid solution was used, that is a solution which had been on the bench for several months, the final absorbance at 440 nm was markedly higher than that obtained for a freshly prepared tartaric acid solution.

The data in Figure 12 suggested the formation of a precursor to the xanthylium pigments was occurring during the ageing of the tartaric solutions.

Figure 12. Influence of the age of tartaric acid solutions used to prepare the wine base for the accelerated oxidation reaction studies.



A detailed examination of tartaric acid solutions revealed the following:

- Exposure of tartaric acid solutions to sunlight resulted in the production of glyoxylic acid
- Tartaric acid solutions stored in the dark, even at 45°C, did not produce glyoxylic acid
- Hydrogen peroxide was also observed in solutions of tartaric acid exposed to sunlight
- Copper(II), when added to the tartaric acids solutions, appeared to increase the amount of glyoxylic acid formed, but reduced the amount of hydrogen peroxide detected
- The presence or absence of ethanol appeared to have some influence on the amount of glyoxylic acid and hydrogen peroxide produced.

Appendix 3 describes these experiments in detail.

The formation of glyoxylic acid from tartaric acid and the production of hydrogen peroxide in solutions of tartaric acid exposed to light and heat have implications for the link between random oxidation and enhanced coloration of white wines.

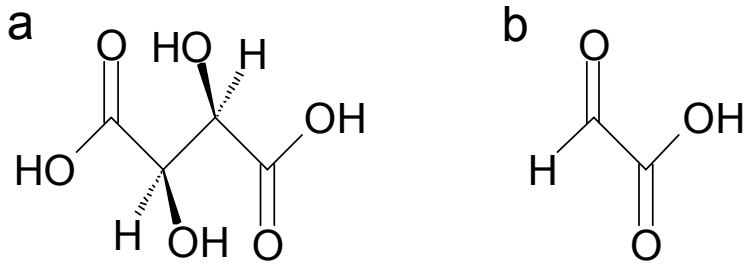
The next phase of the project therefore investigated the chemistry of tartaric acid degradation.

TARTARIC ACID DEGRADATION

Tartaric acid (Figure 13) is a stable organic acid and its degradation in wine is not normally considered to be a basis for inducing quality defects such as random oxidation and consequent coloration of white wine.

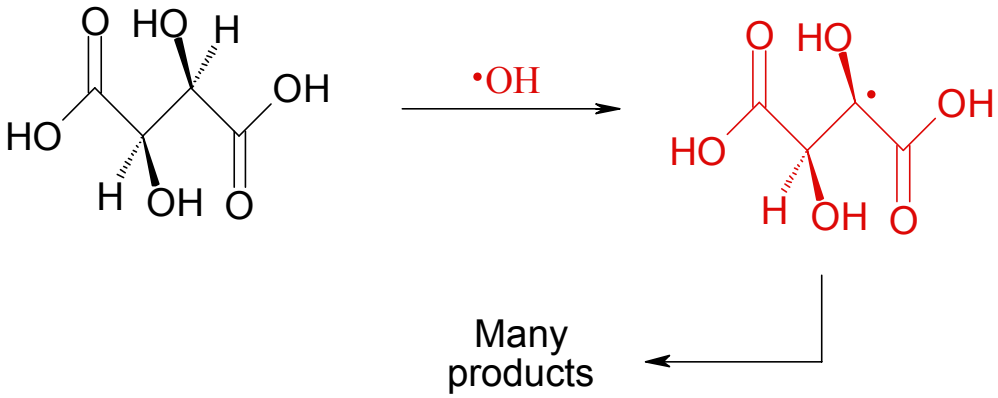
It is clear from the above discussion that cleavage of tartaric acid must occur in order to produce glyoxylic acid (Figure 13), a molecule containing both carboxylic acid and aldehyde functionalities. The cleavage of the central carbon-carbon bond in tartaric acid is required to produce glyoxylic acid. Carbon-carbon bonds are extremely strong and considerable energy is required to achieve the cleavage.

Figure 13. Structure of (a) tartaric acid and (b) glyoxylic acid



The source of energy for the bond cleavage (an oxidative process) can come from free radicals, especially the hydroxyl radical ($^{\bullet}$OH). The hydroxyl radical is an extremely powerful oxidant and can react with tartaric acid to produce a 'tartaric acid' radical as shown in Figure 14.

Figure 14. Reaction between tartaric acid and the hydroxyl radical.



The hydroxyl radical can be produced in several ways, including:

- Fenton chemistry; that is, the reaction of iron(II) with hydrogen peroxide
- gamma-radiation
- the combination of hydrogen peroxide and UV radiation.

These three methods of hydroxyl radical production were investigated to determine whether hydroxyl radicals, hydrogen peroxide (from the decay of two hydroxyl radicals) or glyoxylic acid could be detected in solutions containing tartaric acid.

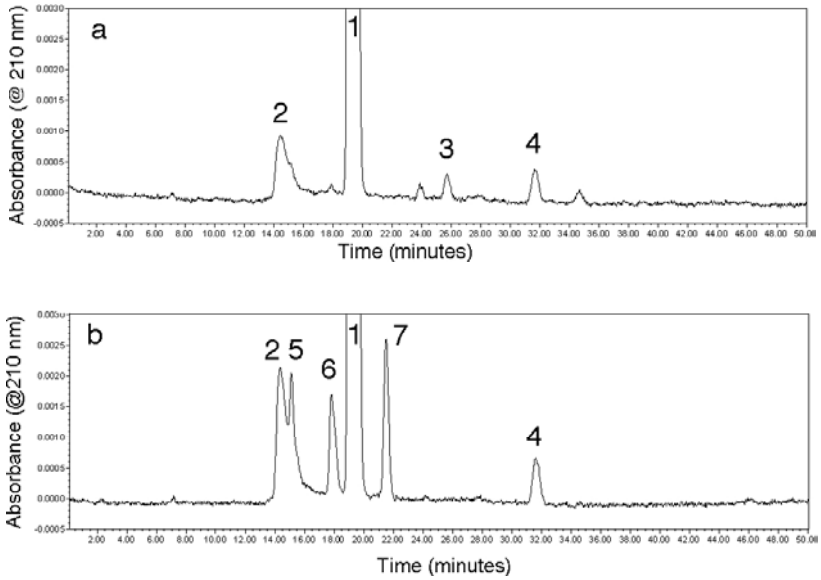
Factors influencing the formation of glyoxylic acid from tartaric acid

Solutions of tartaric acid in water at pH 3.2 or in 12% (v/v) aqueous ethanol at pH 3.2 were stored outdoors (sunlight) with daytime temperature reaching approximately 40°C, indoors in darkness at ambient temperature and in a enclosed water bath (essentially no light exposure) at 45°C.

Figure 15 shows LC-DAD chromatograms for 12% (v/v) aqueous ethanol and aqueous solutions of tartaric acid stored outdoors for 10 days. It is clear that glyoxylic acid, oxalic acid and formic acid are generated from the tartaric acid and that hydrogen peroxide is also formed. The organic acids were identified by comparing retention times with standards and confirmed by LC-MS. Characterisation of hydrogen peroxide is discussed below.

Figure 15. LC-DAD chromatograms at 210 nm for tartaric acid in 12% (v/v) aqueous ethanol solution at pH 3.2 (a) and in water at pH 3.2 (b) after 10 days exposure outdoors. Peak assignments are:

1 tartaric acid **2 hydrogen peroxide**
3 mono-ethyl tartrate **4 formic acid**
5 oxalic acid **6 unidentified acid**
7. glyoxylic acid

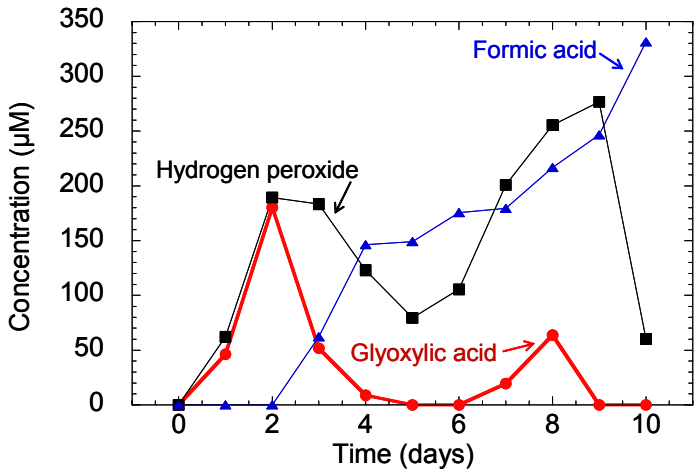


There are some significant differences between the two chromatograms in Figure 15:

a) the peak for glyoxylic acid is quite apparent for the pH 3.2 aqueous sample, but is missing from the pH 3.2 aqueous ethanol solution; this, as discussed below, is a reflection of the time at which the samples were taken
b) the hydrogen peroxide peak is more obvious in the pH 3.2 aqueous solution; again, this is a reflection of the time at which the samples were taken
c) the unidentified acid (peak 6) is observed in the pH 3.2 aqueous solution and only a trace is apparent in the pH 3.2 aqueous ethanol solution; this peak has a mass of 120 and is most probably a three-carbon dicarboxylic organic acid. Further work is being undertaken to elucidate the structure of this molecule, as its characterisation will assist in defining the mechanism for the oxidative cleavage of tartaric acid
d) more oxalic acid is produced in the pH 3.2 aqueous solution
e) the concentration of formic acid is approximately the same in both solutions

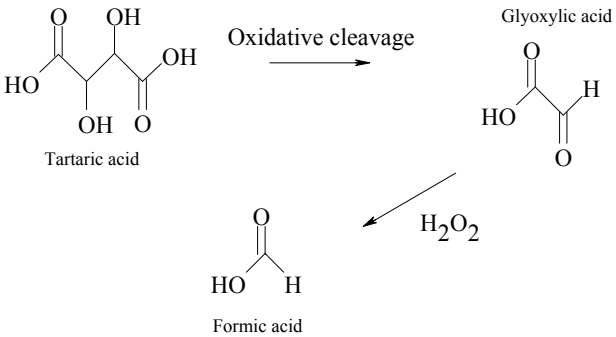
When chromatograms were recorded on a daily basis, it became apparent that the concentrations of glyoxylic acid and hydrogen peroxide in the pH 3.2 aqueous ethanol solution shows some form of cyclic behaviour. As can be seen in Figure 16, the concentrations of glyoxylic acid and hydrogen peroxide increase to Day 2 and then decrease before rising again. However, the concentration of glyoxylic acid does not rise in its second production cycle to the concentration observed in the first production cycle. In fact, by Day 10, the concentration of glyoxylic acid is again at zero, indicating why its peak was absent in the chromatogram shown in Figure 15a.

Figure 16. The concentration profiles for glyoxylic acid, hydrogen peroxide and formic acid during the 10 day outdoor exposure of the pH 3.2 aqueous ethanol solution of tartaric acid



The peak for formic acid increases continually over the 10 day reaction period, although there is a lag period of about 2 to 3 days before its concentration can be detected. This lag period corresponds to the time required for both glyoxylic acid and hydrogen peroxide to reach their maximum concentrations in the first production cycle (Figure 16). These observations imply that hydrogen peroxide is capable of oxidising glyoxylic acid to produce formic acid, as shown in Figure 17.

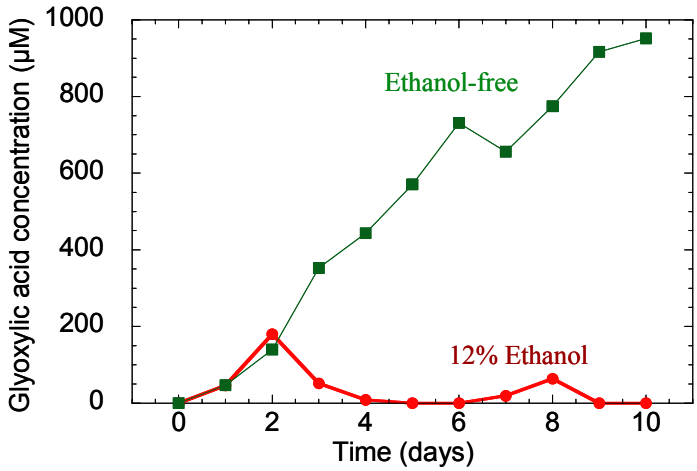
Figure 17. The reaction sequence for the production of formic acid from glyoxylic acid, the latter being formed from tartaric acid



Ethanol has a pronounced inhibitory effect on the production of glyoxylic acid. Figure 18 compares the concentration profiles for glyoxylic acid in the 12% aqueous ethanol solution of

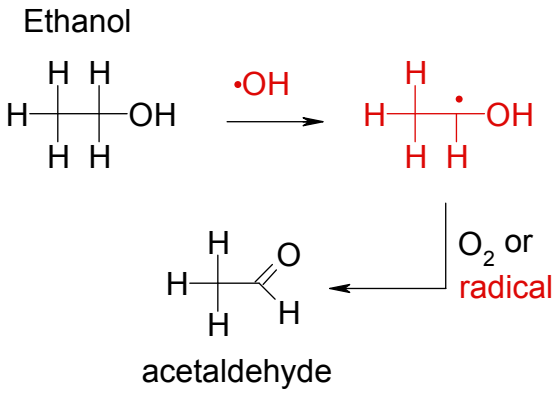
tartaric acid with the pH 3.2 aqueous solution (Ethanol-free in Figure 18). Clearly, considerably more glyoxylic acid is produced when ethanol is absent.

Figure 18. The concentration profiles for the production of glyoxylic acid over the 10 day outdoor exposure of tartaric acid in pH 3.2 12% aqueous ethanol and pH 3.2 aqueous (ethanol free) solutions



These observations provide tentative evidence for the role of the hydroxyl radical in the reaction process. Ethanol is a highly effective scavenger of the hydroxyl radical ($k = 1.9 – 2.2 \times 10^9$ L $mol^{-1}s^{-1}$) and at 12% (v/v) concentration, it is the species with the highest molar concentration in the reaction medium (2.1 M compared to 0.02 M tartaric acid). In the absence of ethanol, the tartaric acid would not be scavenged and more extensive cleavage of tartaric acid to give glyoxylic acid would be expected.

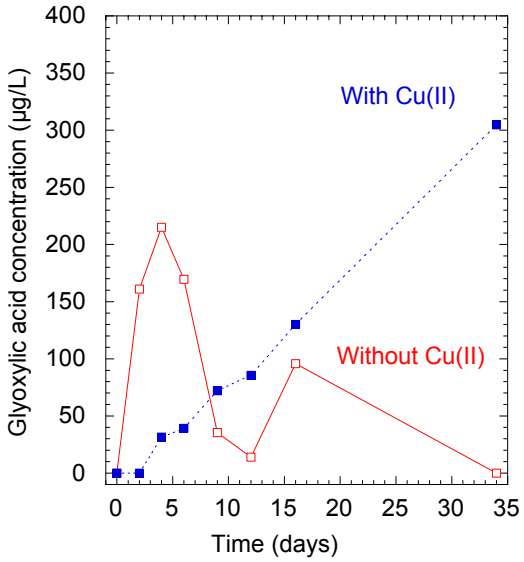
If the hydroxyl radical reacts with ethanol, it is most likely that acetaldehyde would be produced as follows:



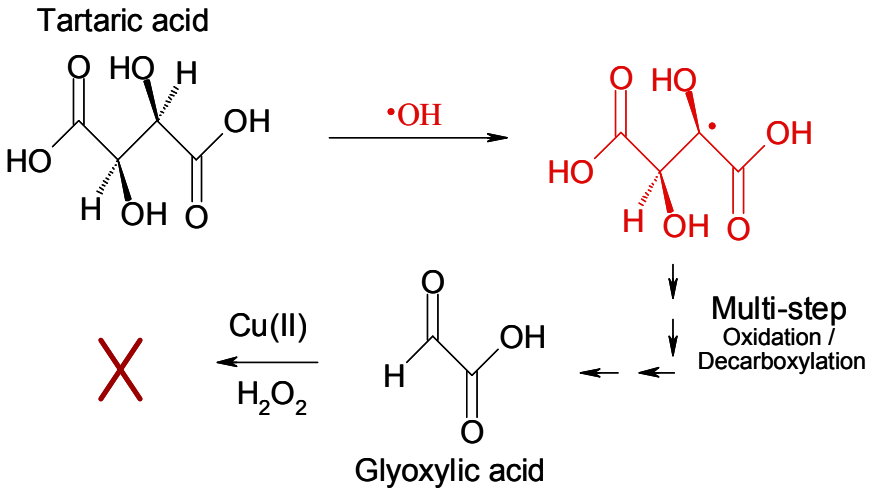
Detection of the highly volatile acetaldehyde under the conditions of these experiments is not a simple task, as it would be readily lost from the reaction medium. A peak was observed at 275 nm in the LC-DAD chromatogram. The retention time and spectrum of this peak was identical to an authentic sample of acetaldehyde, providing *prima facie* evidence for the generation of acetaldehyde. Further experiments are presently under way using aldehyde-specific chromatographic conditions to verify the presence of acetaldehyde in the aqueous ethanol reaction mechanism.

Copper(II) ions play an important role in the production of glyoxylic acid. The copper(II) will prevent the accumulation of hydrogen peroxide by causing its rapid breakdown. This in turn will limit the extent to which glyoxylic acid can be oxidised by hydrogen peroxide. Figure 19 demonstrates that copper(II) is in fact highly effective in increasing the concentration of glyoxylic acid.

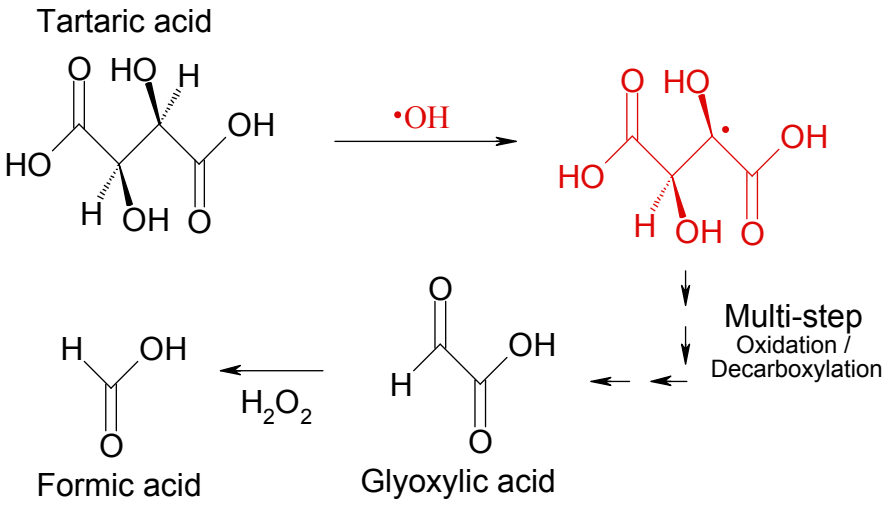
Figure 19. Concentration profiles for the formation of glyoxylic acid in the presence (6.0 mg/litre) and absence of copper(II) in 12% aqueous ethanol tartaric acid solutions at pH 3.2 stored outdoors for 35 days



The proposed mechanism for the generation of glyoxylic acid from tartaric acid in the presence of copper(II) can be represented by:



That is, the copper(II) causes the breakdown of the generated hydrogen peroxide and allows accumulation of glyoxylic acid. In the absence of copper(II), the generated hydrogen peroxide can react with the glyoxylic acid to produce formic acid:



In summary,

a) sunlight exposure enhances the production of glyoxylic acid
b) ethanol inhibits the production of glyoxylic acid, possibly by scavenging the hydroxyl radical
c) in tartaric acids solutions containing ethanol, there is a time dependence to the production of both glyoxylic acid and hydrogen peroxide
d) copper(II) ions enhance the concentration of glyoxylic acid generated from tartaric acid by causing the breakdown of hydrogen peroxide

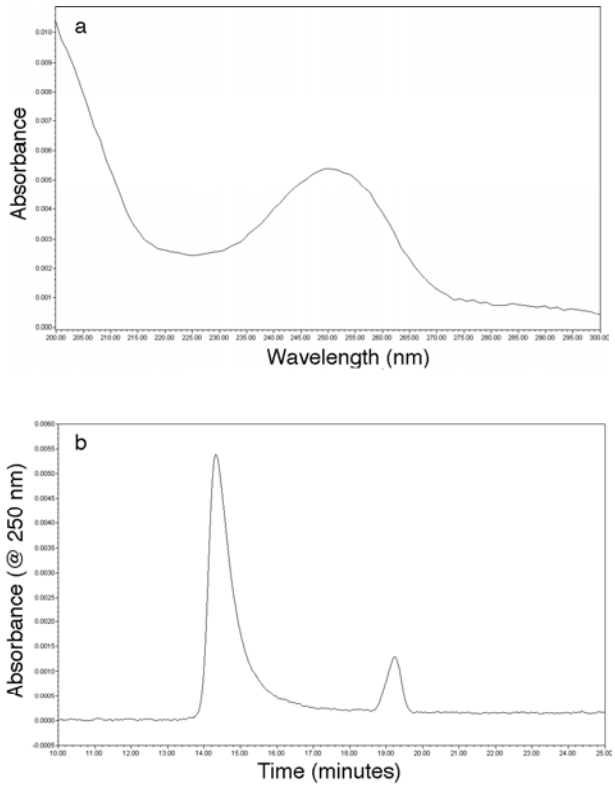
The specific effect of temperature and the combination of light and temperature requires further examination.

Analytical approaches to the measurement of hydrogen peroxide generation

The identification and quantification of hydrogen peroxide was one of the analytical challenges faced in this project. In project UM96/1, a square wave voltammetric method for hydrogen peroxide determination was evaluated and published (Bradshaw et al., Electroanalysis **14**, 546-550). While this method is simple and straightforward, it does not allow the simultaneous detection of hydrogen peroxide with the organic acids that are formed from the oxidative cleavage of tartaric acid.

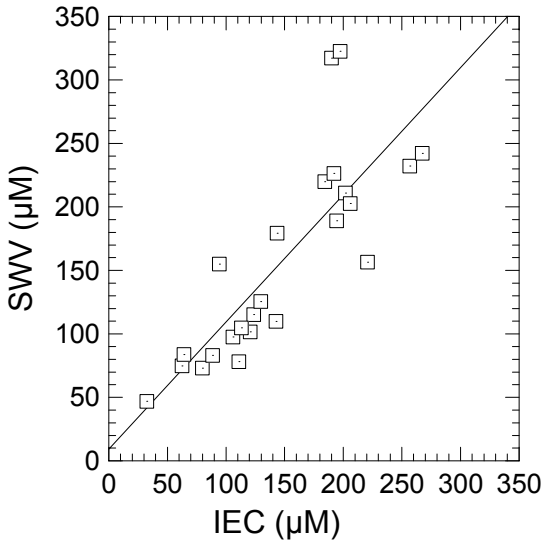
When the ion exchange chromatograms for the measurement of organic acids were examined closely, it became apparent that a peak close to the injection front was in fact hydrogen peroxide. While the organic acids are normally detected at 210 nm, the diode array detector used afforded much better detection of hydrogen peroxide at 250 nm. Figure 20 presents the spectrum of hydrogen peroxide and a chromatogram of a sunlight exposed tartaric acid solution. A well-defined peak for hydrogen peroxide occurs at 14.5 min, well separated from the tartaric acid peak at 19.3 min.

Figure 20. The spectrum (a) and chromatogram at 250 nm (b) generated from the analysis of hydrogen peroxide by ion exchange chromatography with diode array detection. The peaks at 14.5 min and 19.3 min are due to hydrogen peroxide and tartaric acid respectively



To validate the quantification of hydrogen peroxide using the peak at 250 nm, a correlation between the concentrations determined by ion exchange chromatography with those obtained by the square wave voltametric method was prepared. The correlation graph for hydrogen peroxide standards was excellent: $y = 6.69 + 1.02x$; $R^2 = 0.9964$. Figure 21 presents the correlation plot for the hydrogen peroxide concentrations found in solutions of tartaric acid exposed to sunlight. The correlation is satisfactory ($y = 3.38 + 1.0092x$. $R^2 = 0.6977$). If the two outliers are deleted, the correlation coefficient (R^2) becomes 0.8330 and the gradient improves to 0.8624 (a perfect correlation would have a gradient of 1.0000). Instability of the hydrogen peroxide in these sunlight exposed samples is probably the cause of the correlation being lower than that obtained for standards. None-the-less, the correlation is sufficient to confirm that the IEC method is acceptable for quantification of the hydrogen peroxide concentration.

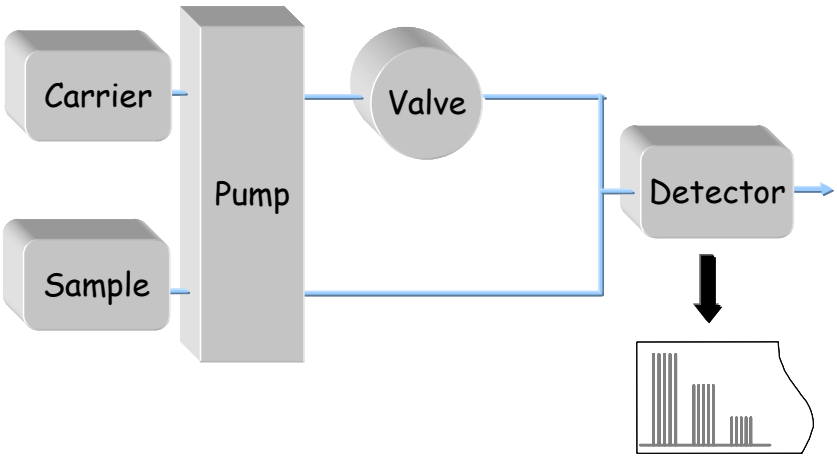
Figure 21. The correlation between ion exchange chromatography (IEC) and square wave voltammetry (SWV) for the quantification of hydrogen peroxide in tartaric acid samples exposed to sunlight over 10 days



Chemiluminescence is an effective method for detecting hydrogen peroxide. Experiments on chemiluminescence were carried out in the laboratories of Professor Neil Barnett of Deakin University to determine if a rapid and effective method for the quantifying hydrogen peroxide could be established.

Luminol is the classic reagent used for hydrogen peroxide chemiluminescence. When luminol is used as the detecting reagent in flow injection (Figure 22), analyses for hydrogen peroxide can be performed approximately every 30 seconds.

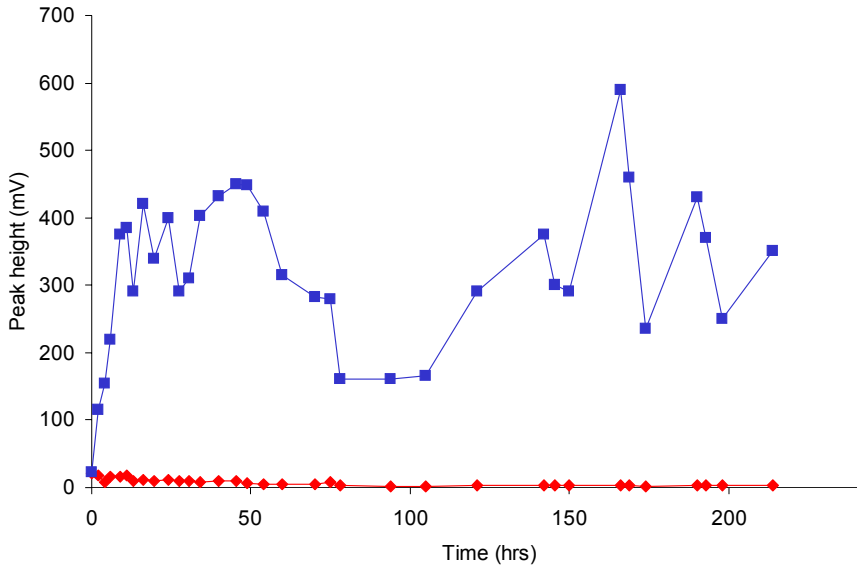
Figure 22. Schematic of the flow injection system used for the determination of hydrogen peroxide



An effective procedure for hydrogen peroxide quantification by flow injection/luminol chemiluminescence was established. Figure 23 plots the hydrogen peroxide concentration found in light-exposed solutions of tartaric acid. Sampling and analyses could be performed every 15 minutes. However, as with the square wave voltammetric method, the flow

injection/chemiluminescence method did not allow the simultaneous detection of hydrogen peroxide and the organic acid degradation products of tartaric acid. Consequently, the ion exchange chromatography validated method was used in all experiments where hydrogen peroxide was to be quantified.

Figure23. Chemiluminescence detection of hydrogen peroxide using luminol in the flow injection mode. The upper curve represents the hydrogen peroxide concentration found in light exposed solutions of tartaric acid. The lower curve is for solutions stored in the dark.



<u>Investigations into the involvement of the hydroxyl radical</u>

The above mechanisms are predicated on the assumption that the hydroxyl radical is present in the reaction system. To confirm the role of the hydroxyl radical, two visits were made to ANSTO at Lucas Heights to use the Gamma-irradiator (Figure 24). Gamma irradiation of water produces the hydroxyl radical and a hydrogen atom:

$$\gamma + \text{H-O-H} \longrightarrow \text{H-O}\cdot + \cdot\text{H}$$

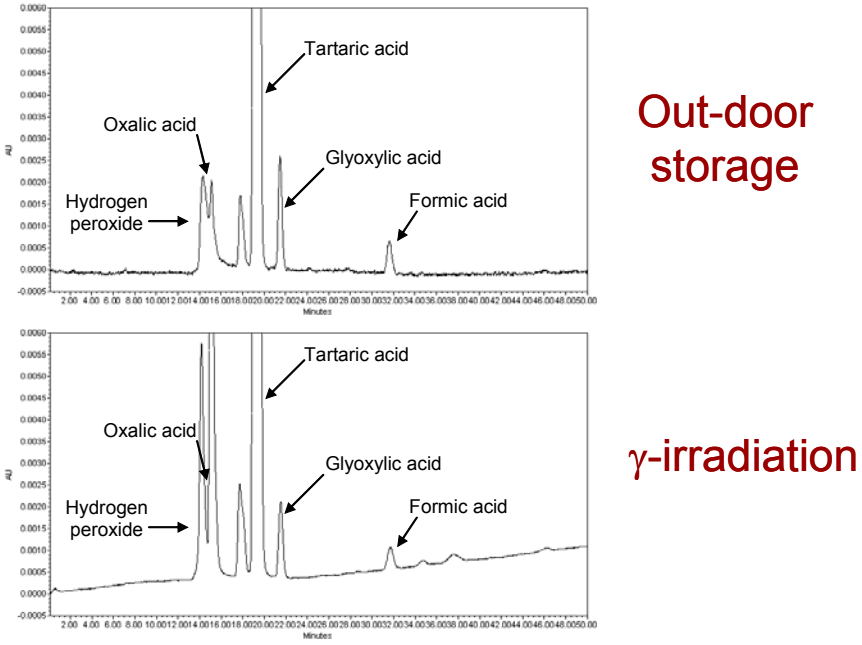
The released hydrogen atoms can then be converted to hydroxyl radicals in the presence of nitrous oxide. This technology for hydroxyl radical production is a standard procedure used at ANSTO and was applied with considerable success in these experiments.

Figure 24. The Gamma-irradiator used at ANSTO



Figure 25 compares the chromatograms for the gamma-irradiated sample with a sample of tartaric acid that had been stored out-doors. The organic acids that are produced in the gamma-irradiated sample are the same as those obtained from the out-doors treatments, confirming that the hydroxyl radical is critical to the cleavage of tartaric acid.

Figure 25. Chromatograms comparing the organic acids produced from gamma-irradiation and out-door storage of tartaric acid solutions in water at pH 3.2

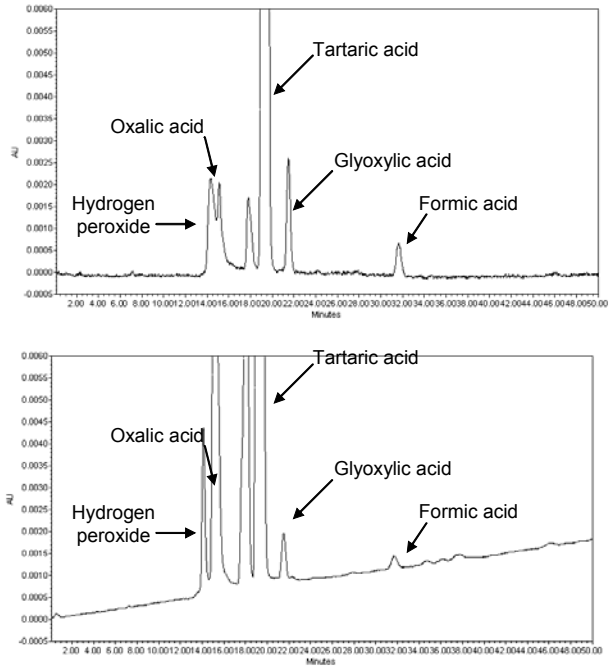


The classic chemical process for generating free radicals is the so-called Fenton reaction in which iron(II) reacts with hydrogen peroxide:

$$Fe(II) + H\text{-}O\text{-}O\text{-}H \longrightarrow H\text{-}O^{\cdot} + {}^{\cdot}O\text{-}H + Fe(III)$$

Again the organic acids produced from the Fenton process are the same as those observed in solutions of tartaric acid that have been stored out-doors (Figure 26). Intriguingly, the unidentified organic acid with a retention time of 18.2 minutes is formed in much higher amounts in tartaric acid solutions exposed to Fenton chemistry. The reason for this is not clear, but may become obvious once the identity of this organic acid has been determined.

Figure 26. Chromatograms comparing the organic acids produced from Fenton chemistry treated and out-door storage of tartaric acid solutions in water at pH 3.2



Summary of studies on the oxidative cleavage of tartaric acid

The main conclusions that can be drawn from these studies of the oxidative cleavage of tartaric acid are:

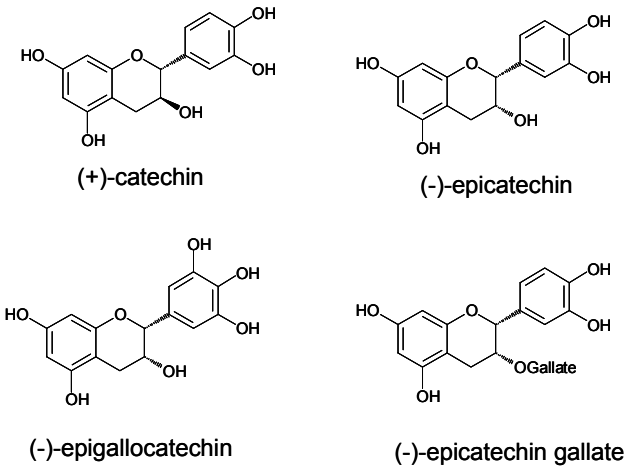
- Tartaric acid solutions can undergo oxidative degradation under reasonably mild storage conditions
- Glyoxylic acid, a precursor to the xanthylium pigment, is produced from the cleavage of tartaric acid
- Oxalic and formic acids are produced from the oxidative cleavage of tartaric acid. An additional unidentified organic acid is also formed.
- Formic acid can be produced by the further oxidation of glyoxylic acid
- Ethanol inhibits, but does not prevent, the degradation of tartaric acid
- Copper(II) provides increased stability for glyoxylic acid, by hindering its further oxidation to formic acid
- Hydrogen peroxide is produced in tartaric acid solutions that are stored in light
- The mechanism of the cleavage process is consistent with the production of hydroxyl radicals.

The mechanism by which hydrogen peroxide and the hydroxyl radical are produced has not yet been fully clarified. Certainly, exposure to light and molecular oxygen is a pre-requisite for hydrogen peroxide formation – no hydrogen peroxide is observed in solutions that are kept in the dark and heated at 45^{o}C. Further work is required to provide a complete understanding of the chemical processes that lead to the generation of hydrogen peroxide and the hydroxyl radical.

PHENOLIC CHEMISTRY AND THE OXIDATIVE COLORATION PROCESS

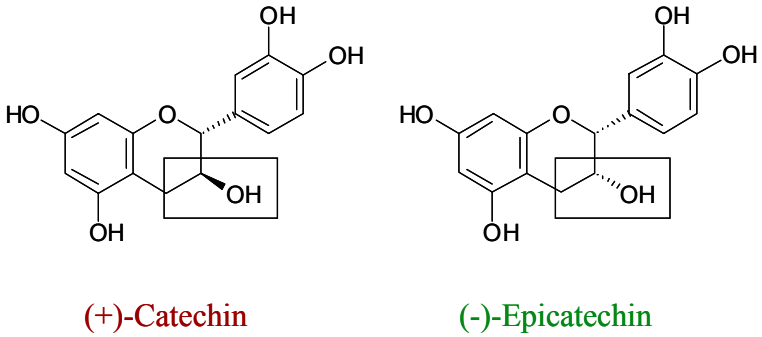
The majority of the work in this project used (+)-catechin as the oxidisable substrate. There is however considerable flavanol diversity in wine and Figure 27 shows the structures of some of the catechin-type flavanols that can be found in white wine. Each of these structures has the required A-ring needed for the formation of xanthylium pigments through the bridging reaction with glyoxylic acid.

Figure 27. Structures of some catechin-like flavanols found in wine



(+)-Catechin and (-)-epicatechin are diastereoisomers, differing only in the geometry at carbon-3 (Figure 28). Experiments to compare the extent of xanthylium pigment production by these related flavanol compounds in the presence of glyoxylic acid and the presence and absence of copper(II) were performed..

Figure 28. Structures of (+)-catechin and (-)-epicatechin. The highlighted carbon is the one giving rise to the isomerism.



In this work, glyoxylic acid was added to model wine bases of (+)-catechin and (-)-epicatechin at a mole ratio of 1 glyoxylic acid to 2 phenolic compound. This was to ensure that sufficient glyoxylic acid was present in the reaction system as only low concentrations are formed spontaneously in freshly prepared model wine bases.

Figures 29 and 30 show the evolution of the absorbance at 440 nm for model wine systems containing either (+)-catechin or (-)-epicatechin together with glyoxylic acid, in the absence (Figure 29) and presence (Figure 30) of 0.6 mg/Litre copper(II) ions. It is obvious that the reaction system containing (-)-epicatechin results in a much higher absorbance at 440 nm in

both the absence and presence of added copper(II) ions. After the 10 day reaction period (Figure 29), the ratio of absorbance values for the (-)-epicatechin to (+)-catechin reaction systems is approximately 2.3.

Figure 29. Comparison of the evolution of the absorbance at 440 nm for model wine systems containing (+)-catechin and (-)-epicatechin in the presence of glyoxylic acid

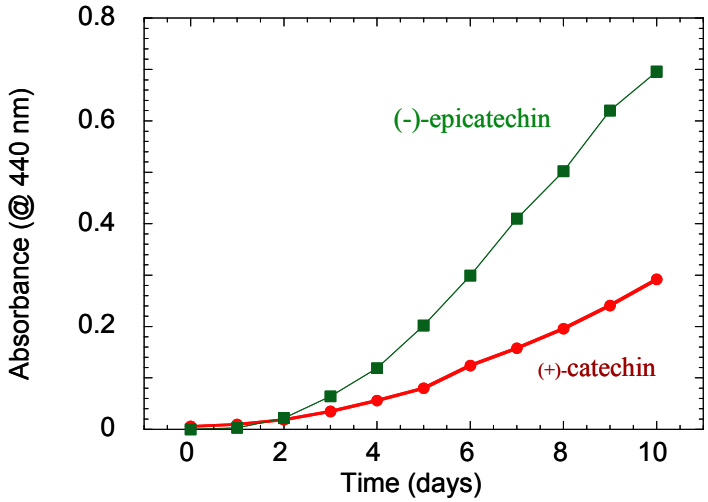
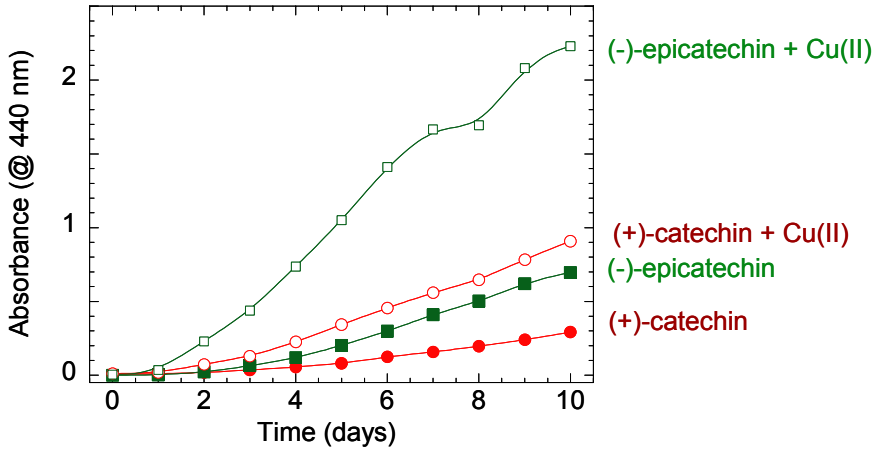


Figure 30. Comparison of the evolution of the absorbance at 440 nm for model wine systems containing (+)-catechin and (-)-epicatechin in the presence of glyoxylic acid and copper(II) ion (0.6 mg/litre)

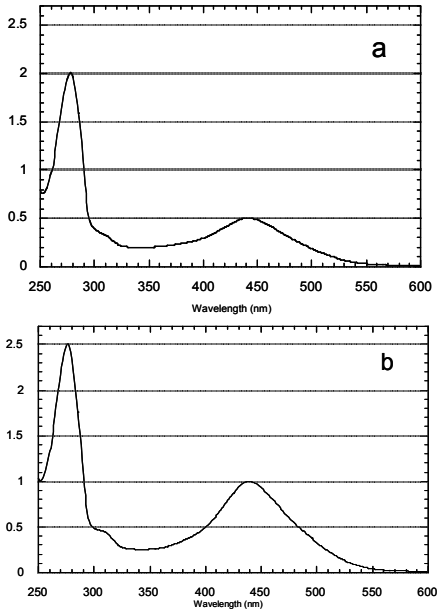


Further, copper(II) ions are capable of enhancing the A_{440} value for the (-)-epicatechin/glyoxylic acid system as the absorbance at 440 nm after 4 days is essentially the same as that obtained after 10 days in the absence of copper(II) (Figures 29 and 30). This enhancement of the A_{440} values in the presence of copper(II) is similar to that discussed above for (+)-catechin. As observed in the absence of copper(II) ions, the absorbance ratio for the (-)-epicatechin to (+)-catechin reaction systems is close to 2.4.

It has been established that the products of the reaction between (+)-catechin and glyoxylic acid in a model wine base system are xanthylium pigments and that the same pigments are formed in the presence of copper(II) ions, except in higher amounts. The UV-visible

spectrum of xanthylium pigments derived from (+)-catechin is characterised by a maximum absorbance at 440 nm and a shoulder at 310 nm together with a peak at 278 nm, corresponding to the catechin component of the xanthylium cation. Figure 31 presents the UV-visible spectra for the (-)-epicatechin/glyoxylic acid and (+)-catechin/glyoxylic acid systems. It is obvious that the spectra are essentially identical, except that the absorbance values at 310 nm and 440 nm for the (-)-epicatechin/glyoxylic acid system are essentially double those for the (+)-catechin/glyoxylic acid system (compare Figure 29). The same spectral characteristics were observed for the reaction systems containing added copper(II). These observations present *prima facie* evidence for the formation of xanthylium pigments in the (-)-epicatechin/glyoxylic acid system.

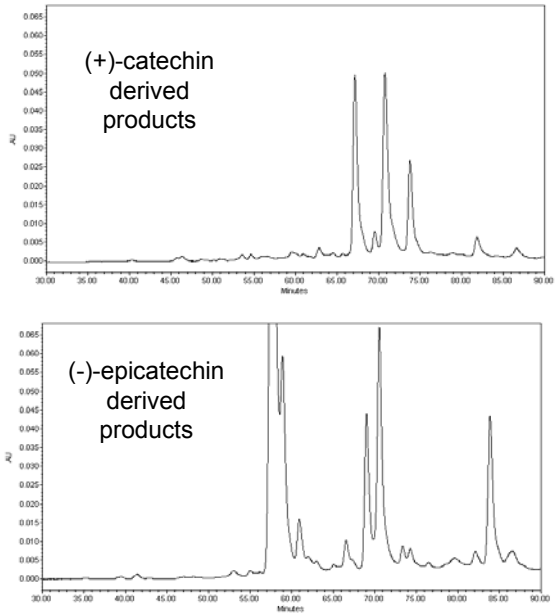
Figure 31. Comparison of the UV/Visible spectra for (a) (+)-catechin/glyoxylic acid and (b) (-)-epicatechin/glyoxylic acid systems



The LC-DAD (440 nm) chromatograms of the (-)-epicatechin/glyoxylic acid and (+)-catechin/glyoxylic acid systems at Day 14 reaction period are compared in Figure 32. The peaks of the (+)-catechin/glyoxylic acid system represent different isomers of the xanthylium pigments as discussed above. The peak at 82 minutes is the ethyl ester of the xanthylium pigment. With the (-)-epicatechin/glyoxylic acid system, the LC-DAD (440 nm) chromatogram shows three distinct peaks with different intensity ratios to those observed for the catechin derived xanthylium pigments. The peak at 84 minutes in the (-)-epicatechin/glyoxylic acid system chromatogram is close to that for the esterified xanthylium pigment, but clearly of different intensity to the peak for the (+)-catechin derived xanthylium pigment.

To confirm that the peaks are xanthylium pigments formed from (-)-epicatechin, the mass chromatogram for each peak was recorded: each peak had m/z 617 (positive ion mode) and m/z 615 (negative ion mode), m/z values corresponding to the xanthylium pigments. When the positive ion mass chromatogram was searched for m/z 617 values, the pattern of peaks found is identical to that observed in the LC-DAD (440 nm) chromatogram shown in Figure 32.

Figure 32. LC/DAD chromatograms for the (+)-catechin/glyoxylic acid and the (-)-epicatechin/glyoxylic acid systems



The (-)-epicatechin/glyoxylic acid system produces xanthylium pigments analogous to those formed from the (+)-catechin/glyoxylic acid system. The different retention times between the two reaction systems may well be a reflection of the diastereoisomeric relationship of the reactants and products as the different spatial geometries of diastereoisomers can produce differences in physical and spectral properties. The observation of 3, rather than 4, peaks and the differing peak intensities in the (-)-epicatechin/glyoxylic acid system compared to the (+)-catechin/glyoxylic acid may well be the consequence of steric effects leading to preferential formation of the different xanthylium pigment isomers.

It is clear from the UV/Visible, LC-DAD and LC-MS data that (-)-epicatechin in the presence of glyoxylic acid polymerises to form xanthylium pigments analogous to those obtained when (+)-catechin is the reactive phenolic compound, although the diastereoisomeric relationship between the two monomeric phenolic compounds is retained in the bridged dimers formed in the reaction process. This diastereoisomeric relationship, however, is unlikely to be sufficient to explain the considerably higher absorbance at 440 nm found at Day 10 for the (-)-epicatechin/glyoxylic acid system compared with the (+)-catechin/glyoxylic acid system (Figure 29).

As the xanthylium pigments are already positively charged, ionisation efficiency does not influence the ion current and thus ion currents can, in this instance, be taken as a measure of the concentration of the two xanthylium pigments. In an experiment using LC/DAD/MS, the ion currents for the (-)-epicatechin based xanthylium was essentially the same as that for the (+)-catechin based xanthylium pigment. On the other hand, the DAD signals at 440 nm were of different magnitude, with a ratio of (-)-epicatechin to (+)-catechin being approximately 1.8. This approximates the absorbance data ratio in Figure 29. The reason why the absorbance values differ has not been resolved but presumably is related to the spatial arrangements of the different functional units in the (-)-epicatechin based xanthylium pigment.

From an oenology perspective, the results of this study suggest that the establishment of an index of oxidation, based on the total phenolic content of the wine alone, is unlikely to succeed. The phenolic composition of white wines depends on a range of factors including seasonal growing conditions and grape and wine processing technologies. For example, in a study of the phenolic composition of Champagnes, the (-)-epicatechin concentration was 1.15 mg/litre in the 2000 vintage and 0.5 mg/litre in the 2001 vintage compared with (+)-catechin concentrations of 0.71 mg/Litre and 2.2 mg/Litre in the respective vintages. These data show that not only the relative amount but also the ratio of (-)-epicatechin to (+)-catechin varies from vintage to vintage. Given the difference in 440 nm absorbances observed for the oxidation products of these two diastereoisomeric phenolic compounds used in this work, an oxidation index will require knowledge of the distribution of the various phenolic compounds that can lead to coloration as a result of oxidation.

INFLUENCE OF GLASS COLOUR ON THE OXIDATIVE COLORATION PROCESS

Light and the type of flavanol clearly influence the oxidation coloration of model systems. This experiment was designed to examine the influence of wine bottle colour on the oxidative coloration process. Both model systems and a white wine were used in this study.

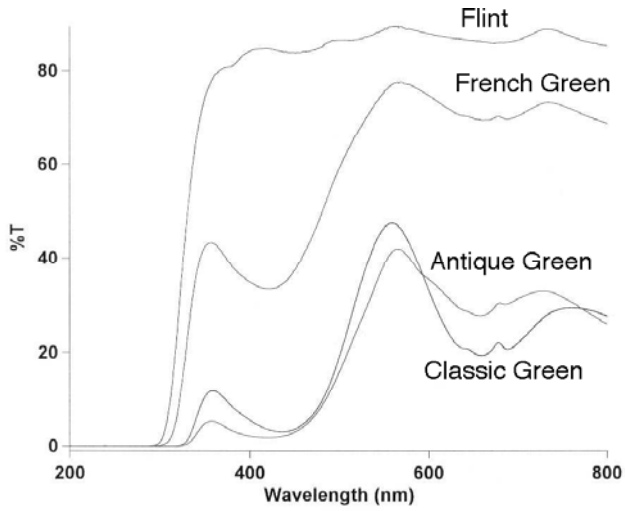
The bottles used were

- Flint
- French Green
- Antique Green
- Classic Green

The 96 bottles were donated to this study by J McCathy and Co Pty Ltd. This donation is acknowledged.

To assess the ability of each of these bottles to limit or stop light reaching the sampling contained with the bottle, transmission spectra were recorded on a section of the glass container carefully removed by a glass cutter. Figure 33 compares the transmission spectra for the four samples.

Figure 33. Transmission spectra of the four wine bottles used in this study



Flint is capable of transmitting all visible and some UV light and French Green allows most light energy above 400 nm to be transmitted. Both Antique Green and Classic Green cut off significant amounts of visible light, although both colours do allow a small amount of UV light to be transmitted.

Studies using model wine systems

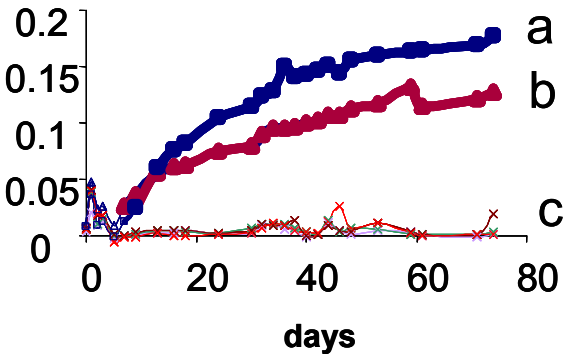
All previous work in laboratory situations had used Schott bottles as the container. Initial experiments for this outdoor exposure study commenced with model systems that were prepared by adding either (+)-catechin or (-)-epicatechin to the model wine base. Flint bottles were used initially as these had the greatest transmission of light in the visible and low UV region. The model systems were stoppered with 'port bottle' corks to allow easy access for daily sampling and aeration. The experimental system is shown in Figure 34. Absorbance values are 440 nm were recorded daily.

Figure 34. Change in colour of a model wine base containing 100mg/litre of (-)-epicatechin (left hand bottle), 100 mg/litre (+)-catechin (centre bottle) and model wine base (right hand bottle) stored in Flint bottles out-doors with daily exposure to air



Figure 35 plots the change in absorbance at 440 nm for the three samples. As was found with the laboratory experiments using Schott bottles, the model system containing (-)-epicatechin had the highest absorbance during the period of the experiment. Intriguingly, the LC/DAD chromatogram of the solutions at the end of the experiment did not show the presence of xanthylium pigments as was found in the laboratory experiments. The reasons for this are not clear, although it should be noted that xanthylium pigments fluoresce and it is possible that during the much longer time course of this experiment compared to the laboratory experiments, the xanthylium pigments may have degraded from excess light exposure. The species that is or are responsible for the increase in absorbance at 440 nm have not yet been identified. This species identification and the impact of prolonged light exposure on xanthylium pigments are the subject of present studies.

Figure 35. Comparison of absorbance data at 440 nm for a model wine base in Flint bottles containing (a) (-)-epicatechin, (b) (+)-catechin, (c) model wine base after 80 days outside exposure.



<u>Studies using a white wine</u>

A Sauvignon Blanc wine was used in a study to examine the impact of oxygen and light exposure on the development of coloration in different coloured bottles. This wine was provided by a wine company supporting the NWGIC research program. The wine details are:

Chemical parameters	pH: 3.13; TA: 7.4 g/litre, free sulfur dioxide: 5 mg/litre
Phenolic composition	(+)-catechin: 8 mg/litre; (-)-epicatechin: 4 mg/litre

The experimental design for this experiment was:

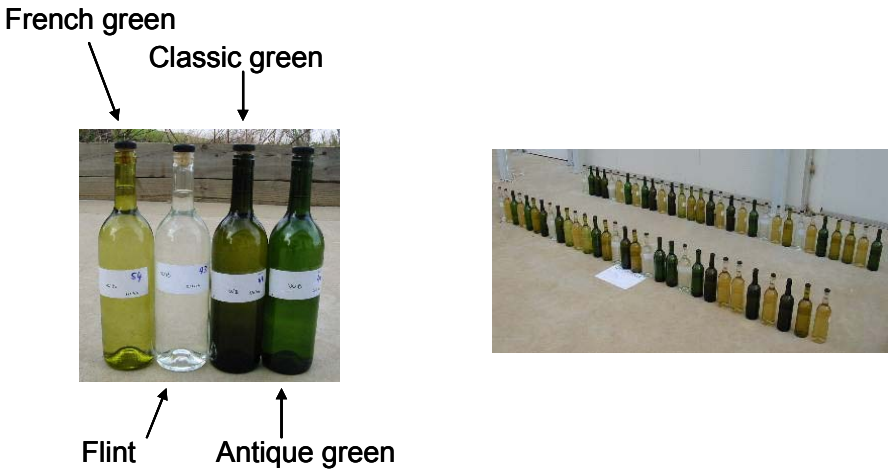
- All samples were prepared in triplicate
- Wine was placed in each of the four bottle types (see above)
- Wine + 100 mg/litre (+)-catechin was placed in each of the four bottle types
- Wine + 100 mg/litre (-)-epicatechin was placed in each of the four bottle types
- The bottles were sealed with a 'port type' cork
- The bottles were placed on a concrete slab with northern exposure
- The position of the bottles in the layout was varied daily to minimise any local environment effects on the outcomes of the experiment
- The bottles were opened daily for sample collection and also to allow aeration.

Absorbance values at 440 nm were recorded daily over a 80-day period.

Figure 36 shows the experimental layout of the bottles.

It is recognised that this is an extreme experiment with respect to normal wine storage, but was designed as a preliminary experiment to assess the impact of light exposure and flavanol content on the development of colour of the wine.

Figure 36. Experimental layout of Sauvignon Blanc in different coloured wine bottles stored outdoors with northern exposure.



The absorbance values at 440 nm at the end of the 80-day reaction for the wine without added flavanol are given in Figure 37. The wine stored in the two darker bottles, Classic Green and Antique Green, had a slightly higher absorbance than the wine stored in Flint and French Green. However, in all cases, while the increase in absorbance was noticeable to the eye, the overall increase in absorbance was minimal.

Figure 37. Absorbance values at 440 nm for the Sauvignon Blanc wine stored in different coloured bottles. The plotted values are the average of three values recorded after 80 days outdoor exposure.

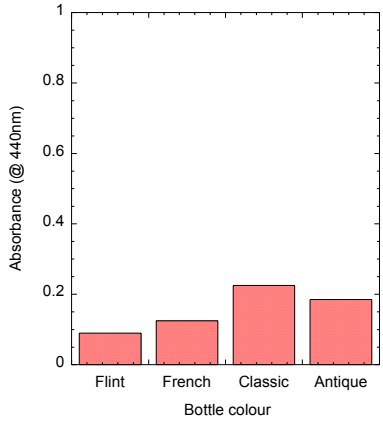
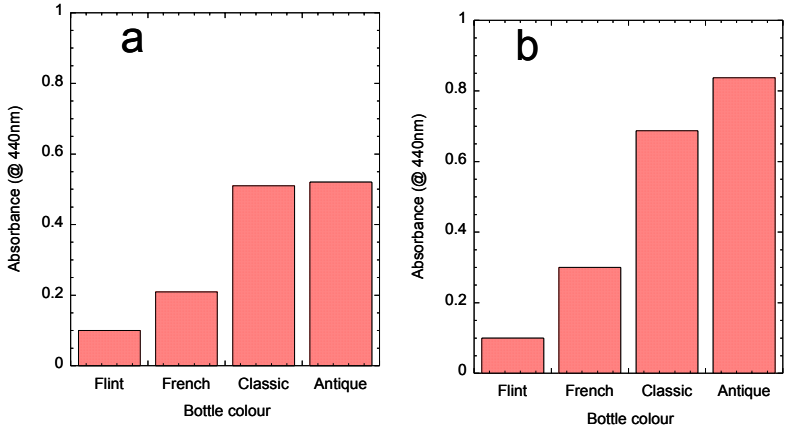


Figure 38 shows the results for the samples containing added (+)-catechin and (-)-epicatechin. A much higher absorbance was found in these samples after the 80-day exposure period than was found for the wine without added flavanol (Figure 37). Similar to what was found with model systems (Figure 35), (-)-epicatechin leads to a significantly higher absorbance than (+)-catechin in the same colour bottle (Figure 38).

Figure 38. Absorbance values at 440 nm for the Sauvignon Blanc wine containing added (a) (+)-catechin at 100 mg/litre and (b) (-)-epicatechin at 100 mg/litre stored in different coloured bottles. The plotted values are the average of three values recorded after 80 days outdoor exposure



The presence of high concentrations of a catechin-type flavanol is obviously essential for the development of colour in these white wine samples. As mentioned above for the model wines, the species that give rise to the increase in absorbance at 440 nm have not yet identified.

The second outcome from this experiment is that increased coloration is found in the darker bottles with lower transmission of light (see Figure 33). With the sample containing (-)-epicatechin in particular, the absorbance at 440 nm increases with the 'darkness' of the bottle. The initial premise for this experiment was that light was necessary to drive the reactions leading to enhanced coloration. While light is necessary to initiate the reaction process, the main driver of enhanced coloration would appear to be temperature. In the latter part of this experiment, the temperature of the wine in the bottles was recorded. In the Classic Green and Antique Green bottles, the temperature of the wine was between 5^{o}C and 12^{o}C higher, depending on the time of day for the measurement, than that for wine in the Flint and French Green bottles.

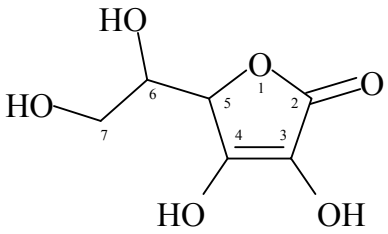
The implications of these results for the safe storage of wine are significant. White wine in warehouses or shipping containers may be subject to elevated temperatures and, based on the preliminary results from this experiment, wine stored in the darker bottles may be more at risk than that stored in darker bottles. That is, there is a potential conflict between the use of darker bottles to minimise light impacting on the wine and the effect of the darker bottles in keeping the wine at elevated temperatures.

A new project in this area is being devised.

STUDIES ON ASCORBIC ACID DEGRADATION

In project UM96/1, the ability of ascorbic acid (Figure 39) to act as a pro-oxidant towards flavanol compounds was demonstrated. It was shown in project UM96/1 that complete oxidation of ascorbic acid was required before the pro-oxidant effect was observed. That is, a degradation product of ascorbic acid is responsible for the pro-oxidant effect.

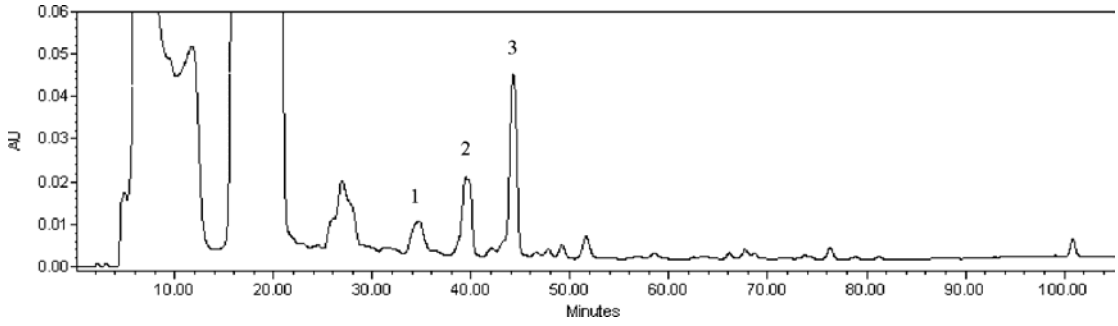
Figure 39. Structure of ascorbic acid



In UM96/1, a detailed chromatographic study of the breakdown of ascorbic acid during its oxidation in the model wine base at pH 3.2 was carried out. A representative chromatogram for 500 mg/litre ascorbic acid is presented in Figure 40. The peaks labelled 1, 2 and 3 varied in height during the course of the experiment, reaching a maximum at the same time as the ascorbic acid was fully oxidised.

The relationship of these peaks with the pro-oxidant capacity of ascorbic acid was considered to be significant in explaining the observed pro-oxidant character.

Figure 40. LC/DAD chromatogram (278 nm) for a solution of ascorbic acid (500 mg/litre) prepared in a model wine base after 4 days reaction period.



A semi-preparative chromatographic procedure with fraction collection was investigated to allow the isolation and identification of these peaks. After a considerable number of trials, a suitable procedure was found that allowed the collection of fractions of reasonable volumes. The intention was to analyse the separated fraction by LC/MS and possibly nmr.

The normal procedure in this type of analysis is to collect and freeze-dry the fractions to remove the eluting solvent and also to allow concentration of the separated species. When freeze-drying was used in this situation, the active species in the isolated fraction was lost. That is, when the freeze-dried fraction was re-dissolved and injected in to the HPLC, no peaks could be detected, implying that the freeze-drying process had caused total breakdown of the species in the original fraction.

The alternate to freeze-drying is further analysis of the fraction as it is collected from the semi-preparative column in the chromatographic procedure. The disadvantage of this approach is simply the potential lack of sensitivity in any subsequent measurement step due to the dilution that occurs in the semi-preparative separation process. As freeze-drying was not an option, the isolated fractions were collected and immediately analysed by LC/MS.

The results of the LC/MS analyses were not successful, as it soon became apparent that the component within the each fraction quickly degraded when isolated. It was found that within 1 hour after fraction collection, the samples had degraded to the extent that no signal could be obtained by either LC/MS or LC/DAD. Further, when a mass spectrum was obtained, it could not be replicated. That is, repeat runs on different samples gave different masses, suggesting that the fractions were degrading as the analysis was being performed.

Unfortunately, a satisfactory resolution to this problem was not obtained before the termination of funding for the project. The results, while frustrating, do add weight to the argument that the breakdown products of ascorbic acid are highly reactive. When masses could be extracted from LC/MS chromatograms, the values were always less than that of ascorbic acid, at least confirming that the peaks in the chromatogram (Figure 40) are breakdown products of ascorbic acid.

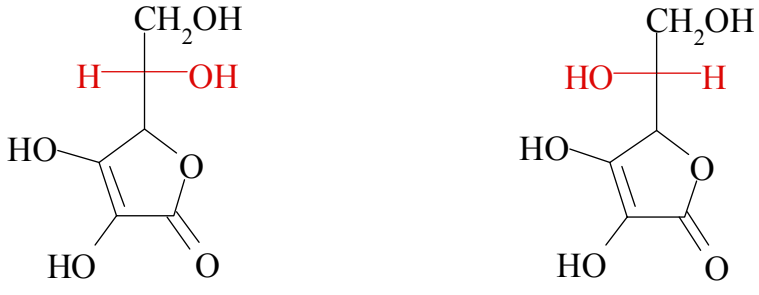
A new approach to identifying these breakdown products will be taken post this project. A new technology, known as LC/NMR, will become available in the near future. Instead of the now traditional DAD or MS detectors, the new technology uses NMR as the detecting device. This will give information on the proton and carbon status of the compounds within each of the isolated fractions which will allow chemical structures to be determined. The instrumentation for LC/NMR will become available in a collaborating laboratory in November this year and it is expected that the work will be complete by the end of the year.

Comparison of ascorbic acid and erythorbic acid

Ascorbic acid and erythorbic acid are diastereoisomers, differing only in the arrangement of atoms on carbon-6 (Figure 41; the numbering system is shown in Figure 39). There is anecdotal evidence that erythorbic acid does not induce the same degree of browning as does ascorbic acid and some preliminary experiments in project UM96/1 appeared to confirm this.

Erythorbic acid itself does not find much application in winemaking, due to its low solubility in wine. The more soluble sodium erythorbate has been used in place of ascorbic acid, but it is no longer a legal additive in Australia.

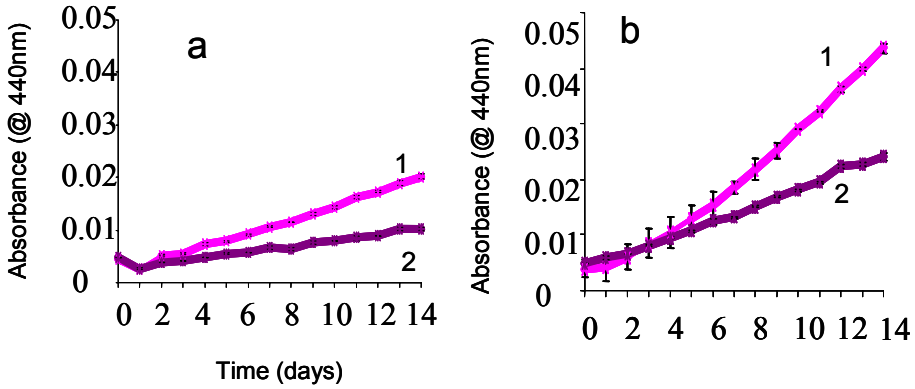
Figure 41. Comparison of the structures of ascorbic acid (left) and erythorbic acid (right)



An experiment was preformed in which 200 mg/litre of either ascorbic acid or erythorbic acid was added to solutions of (+)-catechin or (-)-epicatechin in model wine base at pH 3.2. The increase in colour was monitored at 440 nm.

The results are plotted in Figure 42. Clearly, ascorbic acid brings about an greater increase in colour, as measured at 440 nm, than is achieved with erythorbic acid. It is also obvious from Figure 42 that (-)-epicatechin results in a higher absorbance, compared to (+)-catechin, with either ascorbic acid or erythorbic acid. This is in agreement with the data presented above for the comparison of (+)-catechin and (-)-epicatechin (Figure 30).

Figure 42. Absorbance values at 440 nm for model systems containing (a) (+)-catechin and (b) (-)-epicatechin in the presence of (1) ascorbic acid and (2) erythorbic acid



The results in Figure 42 show:

- the highest oxidative coloration is achieved with the combination of (-)-epicatechin and ascorbic acid.

- the lowest oxidative coloration is achieved with (+)-catechin and erythorbic acid

In summary, these results show that subtle differences in both the phenolic compound structure and oxidant type structure can influence the extent of oxidative coloration. Obviously, any attempt to predict oxidative coloration of a particular could not be achieved without complete knowledge of the distribution of the flavanol and oxidant compounds.

SUMMARY

This project has resulted in several significant conceptual advances in unravelling the complex chemistry that is associated with the white wine quality defect of random oxidation.

Using model systems, it has been demonstrated that tartaric acid can undergo oxidative cleavage when stored outdoors and exposed to sunlight. In chemical terms, for a stable molecule, the environmental conditions required to induce the cleavage are reasonably mild. The breakdown of tartaric acid produces several 2-carbon organic acids and a 1-carbon organic acid. Hydrogen peroxide is also formed. The most important acid formed is glyoxylic acid as this can bridge two catechin-type flavanol compounds that subsequently lead to the formation of xanthylium pigments. These xanthylium pigments absorb at 440 nm, close to the traditional wavelength of 420 nm at which 'browning' of white wine is measured.

Copper(II) ions were found to mediate the bridging of (+)-catechin by glyoxylic acid as well as providing increased stability for glyoxylic acid by hindering its further oxidation.

Using key experiments, some involving irradiation at ANSTO (Lucas Heights), it was demonstrated that the hydroxyl radical is the key species in the cleavage of tartaric acid. The mechanism for the formation of hydroxyl radical remains a challenge to be solved.

Small changes in the geometric arrangement of the substituents on the rings of flavanol compounds result in markedly different values for the absorbance at 440 nm, the wavelength used to measure the induced oxidation of the phenolic compound. For the diastereometric pair of (+)-catechin and (-)-epicatechin, the latter leads to the formation of xanthylium pigments that have approximately double the absorbing power of the (+)-catechin equivalent pigments. This result implies that any model that attempts to predict the propensity of a white wine to undergo oxidation requires prior knowledge of the flavanol profile.

A Sauvignon Blanc wine stored in dark green bottles in light under oxidative conditions showed greater colour development than the same wine stored in light green or colourless bottles. It was observed that the darker bottles were capable of retaining more heat and maintained a higher temperature during daylight hours. Storage of wine must therefore balance the need to use darker bottles to minimise light exposure with the ability of the darker bottles to develop and maintain a higher temperature that enhances colour development.

Detailed knowledge of the chemistry of ascorbic acid and the ability of this molecule to act as a pro-oxidant still remains a challenge. An effective chromatographic method for separating fractions containing degradation products of ascorbic acid was developed. However, the species within the fractions were too reactive to allow identification.

The isomers, ascorbic acid and erythorbic acid, both induce oxidative coloration of (+)-catechin and (-)-epicatechin, but ascorbic acid results in higher levels of colour development, as assessed by the absorbance at 440 nm.

APPENDIX 1

Andrew C Clark and Geoffrey R Scollary

Copper(II)-mediated oxidation of (+)-catechin in a model white wine system

Australian Journal of Grape and Wine Research, **8**, 186-195, 2002

APPENDIX 2

Andrew C Clark, Paul D Prenzler and Geoffrey R Scollary

The role of copper(II) in the bridging reactions of (+)-catechin by glyoxylic acid in a model white wine

Journal of Agricultural and Food Chemistry, **51**, 6204-6210, 2003

APPENDIX 3

Andrew C Clark and Geoffrey R Scollary

Influence of light exposure, ethanol and copper(II) on the formation of a precursor for xanthylium cations from tartaric acid.

Australian Journal of Grape and Wine Research, **9**, 64-71, 2003

Copper(II)-mediated oxidation of (+)-catechin in a model white wine system

ANDREW C. CLARK[1] **and GEOFFREY R. SCOLLARY**[1,2]

[1] National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia

[2] Corresponding author: Professor Geoffrey Scollary, facsimile +61 2 69334068, email gscollary@csu.edu.au

Abstract

This study was undertaken to establish the role played by copper(II) in enhancing the rate of oxidation of flavanols. A model white wine system consisting of 12% (v/v) aqueous ethanol saturated with potassium hydrogen tartrate and adjusted to pH 3.2 was used to allow experimentation under well-defined conditions. (+)-Catechin was the oxidisable substrate and copper(II) concentrations up to 0.6 mg/L were employed. The model white wines were maintained at 45°C to induce the browning process. Under these conditions an increase in absorbance at 440 nm occurred provided the copper(II) concentration was 0.3 mg/L or greater. The coloured species responsible for the increase in absorbance were identified as xanthylium cations, formed by linkage of two (+)-catechin molecules. Glyoxylic acid acted as the bridge between the phloroglucinol-type moiety of the (+)-catechin molecules. The production of the xanthylium cations was inhibited by ethanol and also by mannitol and the implications of these observations for a free-radical induced mechanism are discussed.

Abbreviations

HPLC/DAD high performance liquid chromatography/photodiode array detector; **LC/MS** liquid chromatography/mass spectrometry; **PPO** polyphenol oxidase; **UV** ultra violet; **VIS** visible

Keywords: *browning, (+)-catechin, copper(II), model wine, oxidation, xanthylium cation*

Introduction

Enzymic oxidation and autoxidation

Enzymic oxidation of grape juice occurs just after the grape cells are ruptured in the crushing process. The enzymes responsible for this oxidation, often termed polyphenoloxidases (PPO), include tyrosinase and, in mouldy grapes, laccase. Oxidation of flavanoids, as represented by (+)-catechin (Figure 1), in the presence of PPO type enzymes at pH 3 and 6 yielded both coloured and colourless products (Guyot et al. 1996). The first step in the production of these products was the conversion of the B ring of (+)-catechin (Figure 1), that is, the catechol-like moiety of (+)-catechin, to an *ortho*-quinone. The final coloured products contained multiple interflavan linkages whereas the colourless products contained only a single interflavan link. At the wine-relevant pH 3, the colourless compounds were favoured, possibly formed via semi-quinone intermediates as the corresponding *ortho*-quinone is less stable at pH 3 (Kalyanaraman et al. 1984). This work by Guyot et al. (1996) highlights the importance of the correct experimental conditions being used when modelling processes in white wines.

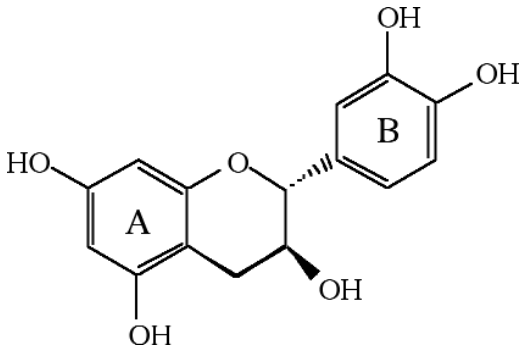


Figure 1. The structure of (+)-catechin. Ring A is of phloroglucinol-type functionality and ring B is of catechol functionality.

In wine, the PPO enzymes are largely absent due to their deactivation or removal with solids in the wine-making process. For this reason, the oxidation process in wine is dominated by indiscriminate chemical reactions rather than the more ordered enzymic oxidation process. In the absence of any catalyst, the non-enzymic oxidation of phenolic compounds is termed autoxidation. Similar to enzymic oxidation, the catechol functionality of phenolic compounds is involved in autoxidation (Figure 2). However, although the overall autoxidation reaction results in an *ortho*-quinone (A in Figure 2), the autoxidation may involve a semiquinone intermediate (B in Figure 2) and under certain conditions the presence of this intermediate species may increase the complexity of the final oxidation products (Singleton 1987).

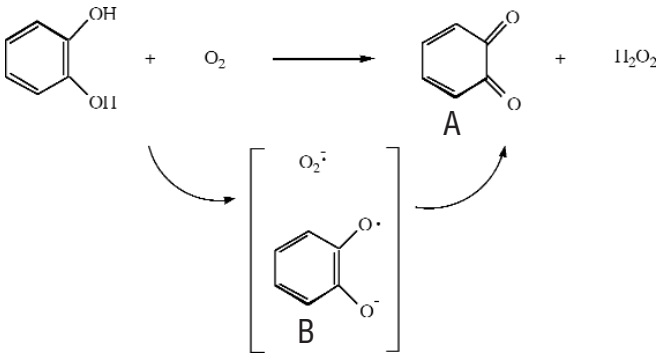


Figure 2. A representation of the autoxidation reaction for catechols to give an *ortho*-quinone (A) and the proposed semiquinone intermediate (B) (Singleton 1987).

Another difference between enzymic oxidation and autoxidation mechanisms is that during the oxidation of catechol-like functional groups a strong oxidant is generated as a byproduct in the latter mechanism (Singleton 1987, Wildenradt and Singleton 1974). Wildenradt and Singleton (1974) assumed that hydrogen peroxide was formed during the autoxidation of (+)-catechin and caffeic acid as they found the presence of acetaldehyde in their reaction systems. That is, they proposed that the generated hydrogen peroxide reacted with ethanol to form acetaldehyde on the grounds that, if the ethanol was replaced by propanol, propanal (the corresponding aldehyde) was formed. The presence of acetaldehyde in wine is known to form methyl-methine interflavan linkages between wine flavanols (Timberlake and Bridle 1976). Later studies proposed that both the superoxide ion and semi-quinone radical were intermediates in the production of hydrogen peroxide and the *ortho*-quinone during the autoxidation of catechol (Figure 2) (Cilliers and Singleton 1992, Singleton 1987). In one study (Singleton 1987), the presence of hydrogen peroxide was confirmed during the autoxidation of gallic acid in water at alkaline pH.

Catechol-like functional groups and metal ions

It has long been assumed that metal ions accelerate autoxidation reactions by increasing the rate of reaction between molecular oxygen and the catecholate functionality of respective phenolic compounds. This is based on the well known ability of catecholate groups to form chelating complexes with metal ions (Avdeef et al. 1978, Carrano and Raymond 1979, Laks et al. 1988, Takeda et al. 1985, van den Berg 1984) and the ability of metal ions to interact directly with molecular oxygen. For example, the copper(II) and copper(I) redox system, in aprotic media, is able to effect a two electron oxidation of catechol-like compounds to the corresponding *ortho*-quinone and finally to the muconic acid ester (Demmin et al. 1981, Rogic and Demmin 1978). The intermediate in both steps was proposed to be a dicopper(II)-catecholate complex. However, the medium, the concentration of copper(II) and copper(I) and the pH in this classical chemistry study were far removed from conditions relevant to wine.

A study more relevant to conditions found in wine was performed on the copper(II) catalysed oxidation of catechol (Balla et al. 1992). This involved an aqueous medium at pH 4.6 to 5.5 with a copper(II) concentration of one to two orders of magnitude higher than those typical of white wine. After 35% completion of the reaction, the overall mechanism was identical to that shown in the top mechanism of Figure 2. Based on kinetic data, the proposed reaction mechanism involved several intermediate copper(II)-catecholate-molecular oxygen complexes and a copper(I)-molecular oxygen species. These intermediates were never identified, however. At the completion of the reaction, the products were mainly unidentified acidic and polymeric species.

It remains to be determined whether similar metal catalysed oxidation reactions will occur in the environment of white wine. Apart from the increased acidity, the matrix of wine is complicated by the number of potential metal ion chelators, such as proteins and organic acids, that will compete with catecholate functional groups. These chelators also may inhibit the redox cycling of copper(II) to copper(I). To date, there has been no published evidence that the presence of metal ions leads to an increase in *ortho*-quinone derived oxidation products in actual or model white wines.

In a study (Oszmianski et al. 1996) conducted on the iron-catalysed oxidation of (+)-catechin in a tartrate buffered model red wine system, the main oxidation products were brown pigments. However, the mechanism for their production was not consistent with either *ortho*-quinone or semi-quinone derived products (Es-Safi et al. 1999a, b). Rather, concentrations of iron(II) ranging from 1–20 mg/L were found to be responsible for the production of a number of coloured xanthylium cations (Figure 3). From the identified mechanism (Es-Safi et al. 1999a, b), it became evident that the reactive site of (+)-catechin was not the catechol functionality but rather the phloroglucinol-type moiety of (+)-catechin (the A ring in Figure 1).

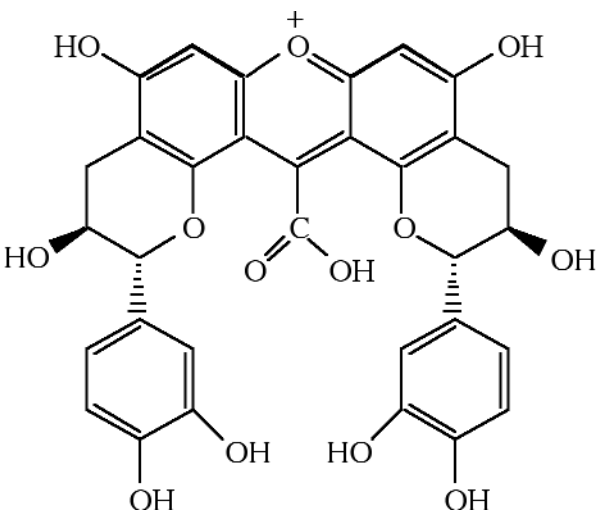


Figure 3. The structure of a xanthylium cation.

The work reported here was conducted to investigate the coloured oxidation products generated in the copper(II)-mediated oxidation of a model white wine system. That is, it was intended to ascertain whether the coloured oxidation products were either *ortho*-quinone or semiquinone derived products, or alternatively, if a mechanism similar to that observed for the iron(II)-mediated oxidation of the model red wine system (Es-Safi et al. 1999a) was dominant. The modelling of white wine was adopted as it is in white wine that oxidative exposure has been attributed with the production of brown pigments (Zoecklein et al. 1995). This process is often referred to as the oxidative browning of a white wine and has been correlated with the concentration of flavanols, such as (+)-catechin (Simpson 1982). Due to the modelling of a white wine, the levels of (+)-catechin adopted were to be lower and the pH more acidic than in the model red wine used previously (Oszmianski et al. 1996). As the average copper(II) concentration found in white wines is generally around or below 0.6 mg/L (Clark and Scollary 2000, Ough and Amerine 1988), depending on the country of origin for the wine, this was the maximum level to be added to the model white wine system. At these concentrations the copper(II) content was to be much lower than the levels of iron(II) used in the previous study (Oszmianski et al. 1996). The effect of some experimental and sample matrix parameters on the production of the coloured pigments was also to be investigated.

Materials and methods

Instrumentation and software

All pH measurements were performed with a TPS pH meter (model 1825 mV) and an Ionode combination pH electrode (IJ40). Buffer solutions (APS Finechem) of pH 7.00 ± 0.05 and pH 4.01 ± 0.05 (at 25°C) were used to calibrate the pH meter before use.

The UV/VIS spectra of samples were recorded on a µQuant Universal Microplate Spectromphotometer that was run by KC4 Software. The single wavelength (440 nm) absorbance of samples was recorded on a Unicam 8625 UV-VIS spectrophotometer.

HPLC/DAD was conducted on a Waters 2690 Separation Module run by Millenium^{32} software and connected to a Waters 996 photodiode array detector. The column used was a reverse phase Wakosil C18RS column of particle size 5 µm and 250 × 2 mm with a guard column of the same type.

LC/MS work was conducted on a SpectraSYSTEM LC run by Xcalibar software with a P4000 sample pump, UV6000LP UV detector and a Finnigan AQA quadrapole MS with an electrospray source. The same column was used as in HPLC/DAD experiments.

Reagents

All glassware and plastic ware were soaked for at least 16 hours in 5% Decon90 E-15 and then rinsed with copious amount of Grade 1 water (ISO 3696). Solutions and dilutions were prepared using Grade 1 water. Copper(II) was added as copper(II) sulfate pentahydrate (BDH, AR) and iron(II) as iron(II) sulfate heptahydrate (Pronalys, AR). (+)-Catechin monohydrate (Sigma, 98%) was used without further purification.

Model white wine system

The model white wine was prepared by adding approximately 10 g of potassium hydrogen tartrate to aqueous ethanol (12% v/v, 2 L) and stirring overnight at room temperature. Excess potassium hydrogen tartrate was removed from the saturated solution by filtration through a 0.45 µm Sartorius cellulose acetate filter fitted to an all glass filter unit. The pH of the solution was adjusted to pH 3.20 with aqueous tartaric acid (300 g/L). The addition of 150 mg/L (+)-catechin was made immediately prior to the commencement of the induced browning process.

Induced browning process

Samples of 150 mL total volume were prepared in 200 mL Schott bottles with screw top lids. These were placed in a water bath at 45°C which was covered with a lid to minimise ingress of light. Unless mentioned otherwise the samples were opened to the atmosphere at least every second day during the measurement process.

Absorbance measurements

The model white wine without added (+)-catechin was used as the blank solution. To obtain a test portion for absorbance measurement, the Schott bottle was removed from the water bath and the solution stirred while approximately 2.5 mL of sample was taken using a disposable pasteur pipette. This test portion was transferred to a disposable plastic cuvette (Sarstedt) of 10 mm path length and the absorbance measured at 440 nm. The solution in the cuvette was then discarded, the lid replaced on the Schott bottle and the remaining sample returned to the water bath.

Analytical HPLC/DAD analyses

The HPLC/DAD work used a flow rate of 0.15 mL/min and sample injection of 100 µL. A gradient elution was adopted which consisted of solvent A, Grade 1 water, and solvent B, methanol, both containing 0.5% acetic acid. The composition of the mobile phase during the analysis was taken from Saucier et al. (1997):

Time(min)	0	1	59	74	75	88	112	120
A (vol%)	100	95	62	56	48	45	0	100

The UV-VIS spectra were recorded from 250–500 nm. The chromatography column was left at room temperature during the analysis.

Analytical LC/MS analyses

LC/MS work was conducted both in the positive ion mode, with an ion spray voltage of +4 kV and orifice voltage of +30 V, and in the negative ion mode, with an ion spray voltage of –4 kV and orifice voltage of –30 V. Simultaneous wavelength detection at 278 nm and 440 nm was performed. The same column, solvent conditions and flow rate were used as for HPLC/DAD experiments. The sample injection was 20 µL.

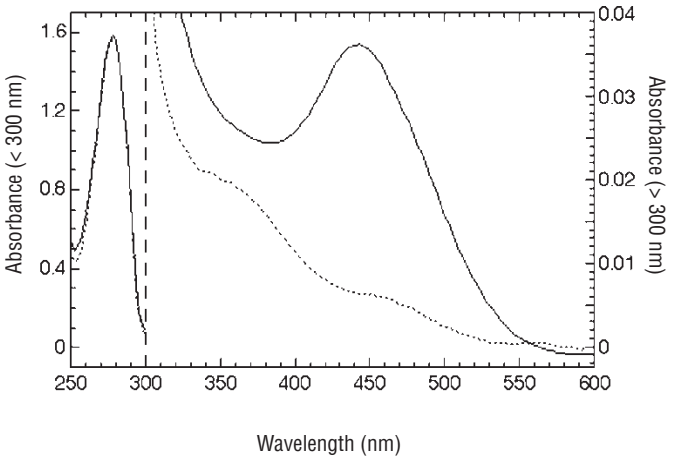


Figure 4. The UV/VIS spectrum of the model white wine system both before (·····) and after (——) 20 days of the induced browning process. The concentration of copper(II) is 0.6 mg/L.

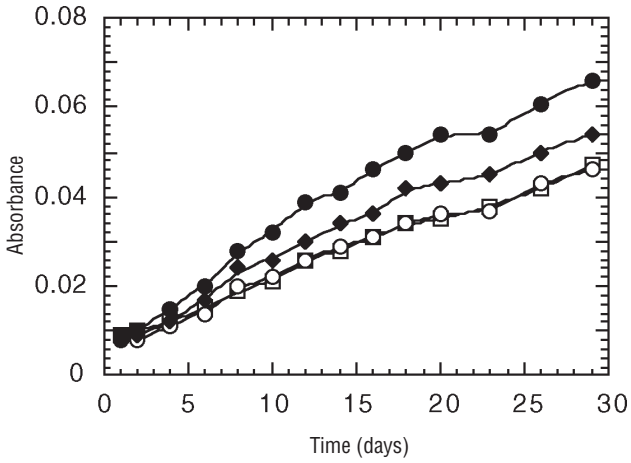


Figure 5. Change in absorbance at 440 nm with time for the model white wine at 0 (□), 0.1 (○), 0.3 (◆) and 0.6 (●) mg/L copper(II) during the induced browning process.

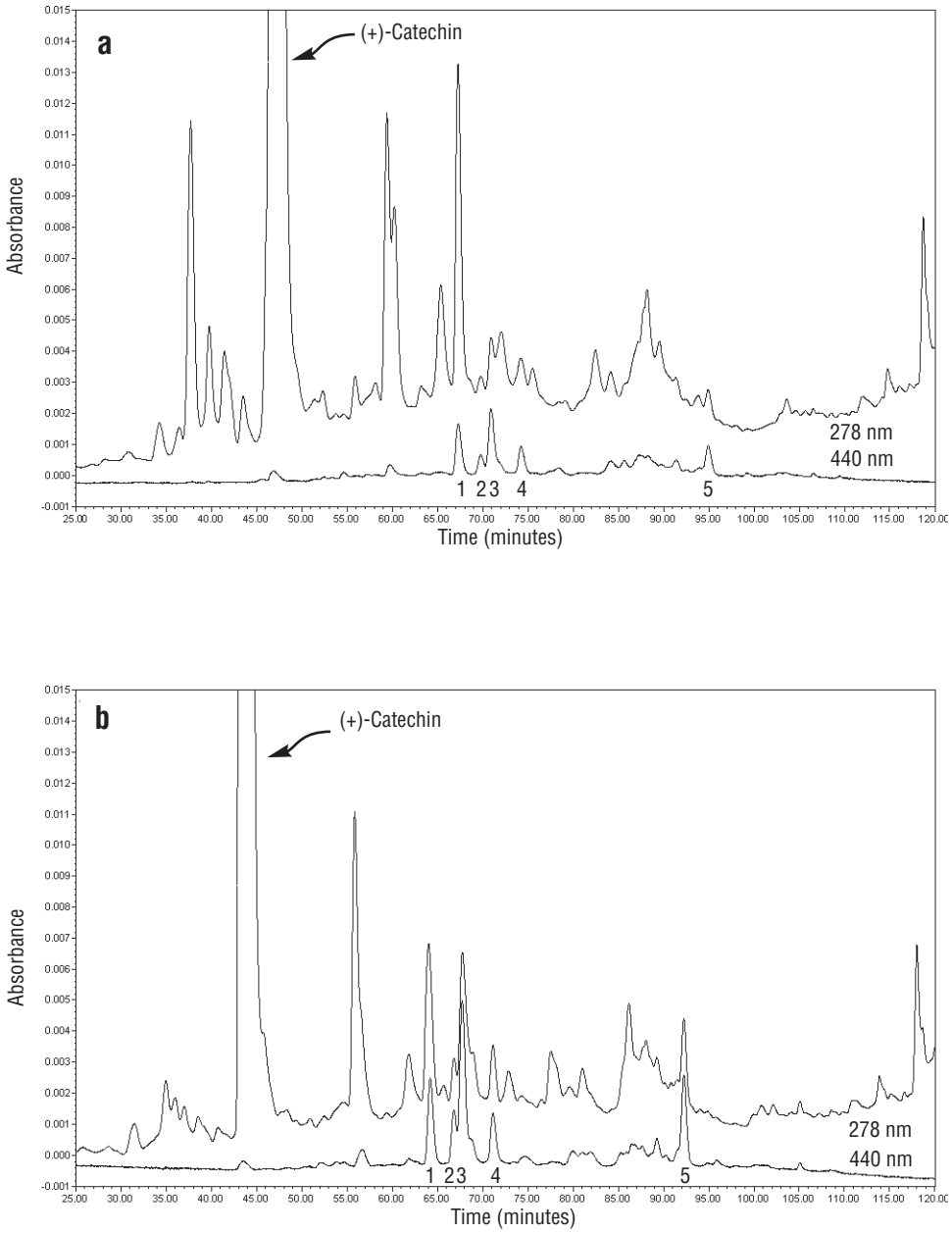


Figure 6. HPLC/DAD chromatograms at 440 and 278 nm of the model white wine with 0 (**a**) and 0.6 (**b**) mg/L copper(II) after 29 days of the induced browning process.

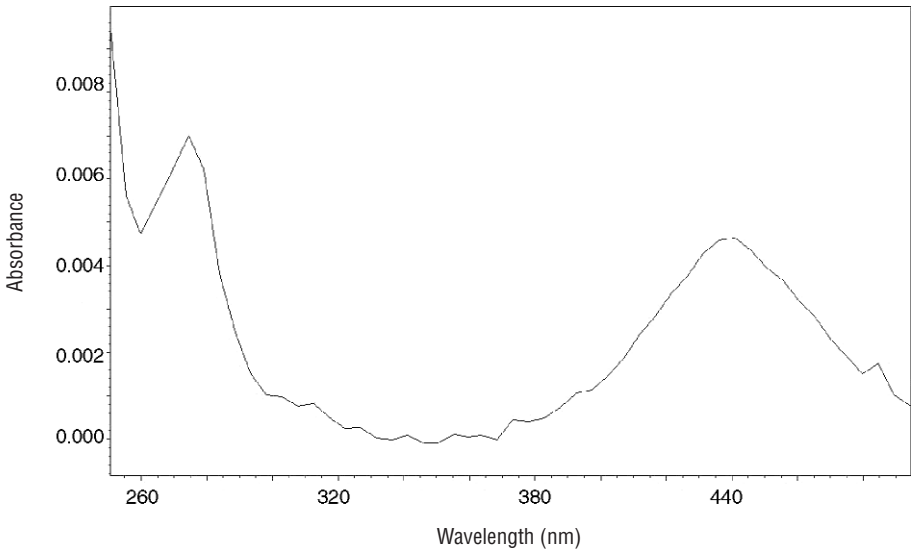


Figure 7. The UV-VIS spectrum of peak 3 in Figure 6b.

Iron(II)-mediated browning of (+)-catechin

The conditions used for the iron(II)-mediated browning of (+)-catechin were identical to those of Osmianski et al. (1996) with 20 mg/L iron(II), added as iron(II) sulfate, except that the sample was incubated at 39°C as performed in the work of Fulcrand et al. (1997).

Reaction between (+)-catechin and glyoxylic acid

Glyoxylic acid (Aldrich, 98%) was added to the model white wine system (150 mL) giving a (+)-catechin to glyoxylic acid ratio of 2:1 (0.50 : 0.25 mM). The sample was stored in darkness at ~ 10°C.

Results and discussion

Influence of Cu(II) concentration on the induced browning process

When the model white wine system underwent the induced browning process, that is, heating at 45°C in darkness, there was an increase in absorbance at wavelengths within the range of 400 to 500 nm (Figure 4). As the absorbance maximum in this region was around 440 nm, all model white wines were measured at this wavelength.

When the model white wine system, with differing concentrations of added copper(II), underwent the induced browning process, the relationship between the rate of browning and the copper(II) concentration was not directly proportional to the copper(II) concentration (Figure 5). Following a lag period, the browning accelerated in all samples and after 29 days it reached an appreciable absorbance of 0.047 to 0.066, depending on the added copper(II) concentration. Negligible difference in the rate of browning occurred in the absence of added copper(II) and with 0.1 mg/L added copper(II), but on the addition of 0.3 mg/L copper(II) (and above) the rate of browning increased considerably. After 29 days of the induced browning process the model white wine solution was coloured but not cloudy, implying that no precipitation had occurred.

All samples were analysed by HPLC/DAD after 29 days of the induced browning process (Figure 6). The photodiode array detector on the HPLC/DAD enabled the chromatograms to be generated simultaneously at 278 nm, a wavelength at which (+)-catechin absorbs, and 440 nm, the wavelength at which the coloured compounds absorb. In the 278 nm chromatograms, the large peak at a retention time of approximately 45 minutes was assigned to (+)-catechin with verification by mass spectral data and from the retention time of a (+)-catechin standard. Throughout the browning process of the model white wine system, both in the presence and absence of copper(II), the peak area of (+)-catechin decreased by less than 5.5%, suggesting that only a minimal fraction of (+)-catechin had been involved in the browning process. It was also apparent that there was some variability in the retention time of peaks between the different sample chromatograms. However, by comparing the retention time of (+)-catechin in different chromatograms and observing the general pattern of eluting peaks, the identification of equivalent peaks in two different chromatograms was easily achieved. This variability was most likely caused by the complex multiple steps in the HPLC/DAD solvent gradient and the lack of temperature control over the chromatography column.

Xanthylium cation formation during the induced browning process

Several peaks were present in the chromatograms at 440 nm with longer retention times than (+)-catechin. Given the nature of the column used in the separation, the elution pattern of these products suggested that they were less polar than (+)-catechin. The profiles of the chromatograms at 440 nm in the absence and presence of added copper(II), and at all different copper(II) concentrations, were identical with only the absorbance intensity of peaks varying, reflecting the differences recorded in Figure 5. The peaks in the 440 nm chromatograms had a corresponding peak in the 278 nm chromatogram.

The UV/VIS spectrum of the compound responsible for the most intense peak in the chromatogram at 440 nm (Figure 6b, peak 3) is shown in Figure 7. The com-

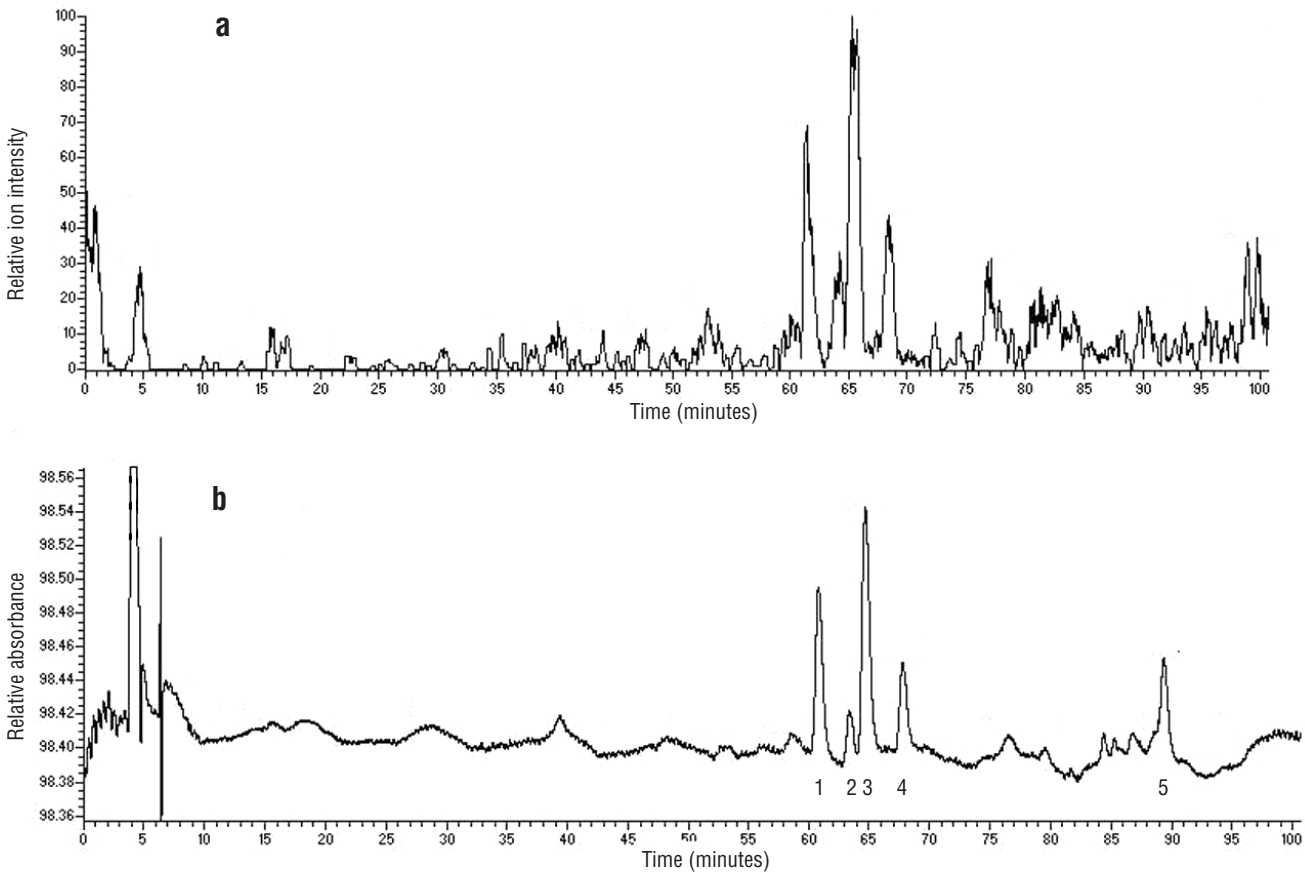


Figure 8. The LC/MS chromatograms generated in the analysis of the model white wine system containing 0.6 mg/L copper(II). The plot of the ion at 617 m/z as monitored by the electrospray mass spectrophotometer detector is shown in (**a**) and the simultaneous plot of absorbance at 440 nm as monitored by the UV detector is shown in (**b**).

pound has absorbance maxima at 280, 310 and 440 nm. The absorbance maximum at 280 nm suggested that this coloured compound had retained some character of (+)-catechin. In fact, peaks 1–4 in the 440 nm chromatogram (Figure 6b) gave identical UV/VIS spectra, while peak 5 (Figure 6b) had a 10 nm bathochromic shift of the maximum in the visible region.

Peak 3 (Figure 6b) was found by LC/MS to have a mass spectrum with a significant ion at 617 m/z. The assignment of this ion as either a fragment or pseudomolecular ion, such as $M+H^+$ or $M+K^+$, was not possible due to the lack of significant ions in the negative ion mass spectrum of peak 3. However, the ion at 617 m/z in the positive ion mode was consistent with the presence of xanthylium cations (Figure 3), analogous to those observed in work published by Cheynier and co-workers (Es-Safi et al. 1999a). The xanthylium cations generated in the iron(II)-mediated oxidation of (+)-catechin (Es-Safi et al. 1999a, Oszmianski et al. 1996) had identical UV/VIS spectra to peaks 1–4 (Figure 7) and were detected by LC/MS (Es-Safi et al. 2000) at 617 (M^+) and 615 ($M^+–2H^+$) m/z in the positive and negative ion modes respectively. Under the LC/MS conditions utilised for the analysis of the model white wine system, the charged xanthylium cations were expected to be more easily detected in the positive rather than negative ion mode.

It had also been shown by Es-Safi et al. (2000) that six different isomeric forms of this specific xanthylium cation are possible. When the mass chromatogram was searched for ions with a m/z value of 617 (positive ion mode) the result in Figure 8a was generated. From the matching profiles it was evident that peaks 1–4 (Figure 8b) had mass spectra with a significant ion at m/z 617 (Figure 8a). Consequently, it was likely that peaks 1–4 were due to the different isomers of the xanthylium cation that had been observed previously (Es-Safi et al. 2000). As only four possible xanthylium cation peaks were found in this study, it was likely that the separation between all isomers had not been achieved and/or not all of the isomers were formed at sufficiently high concentrations to be detected.

To confirm that peaks 1–4 (Figure 6b) were due to xanthylium cations, two samples known to contain xanthylium cations were analysed using the identical chromatographic method as in Figure 6. The first of these samples, containing 20 mg/L iron(II) and 1.16 g/L (+)-catechin, was prepared in a manner identical to that described by Osmianski et al. (1996) with heating at 39°C (Fulcrand et al. 1997) for 12 days. The second sample consisted of glyoxylic acid and (+)-catechin (Es-Safi et al. 1999a) prepared in the model white wine system at a ratio of 1:2 (0.25:0.50 mM). The comparison of 440 nm chromatograms (Figure 9) between these samples and the model white wine system with 0.6 mg/L copper(II)

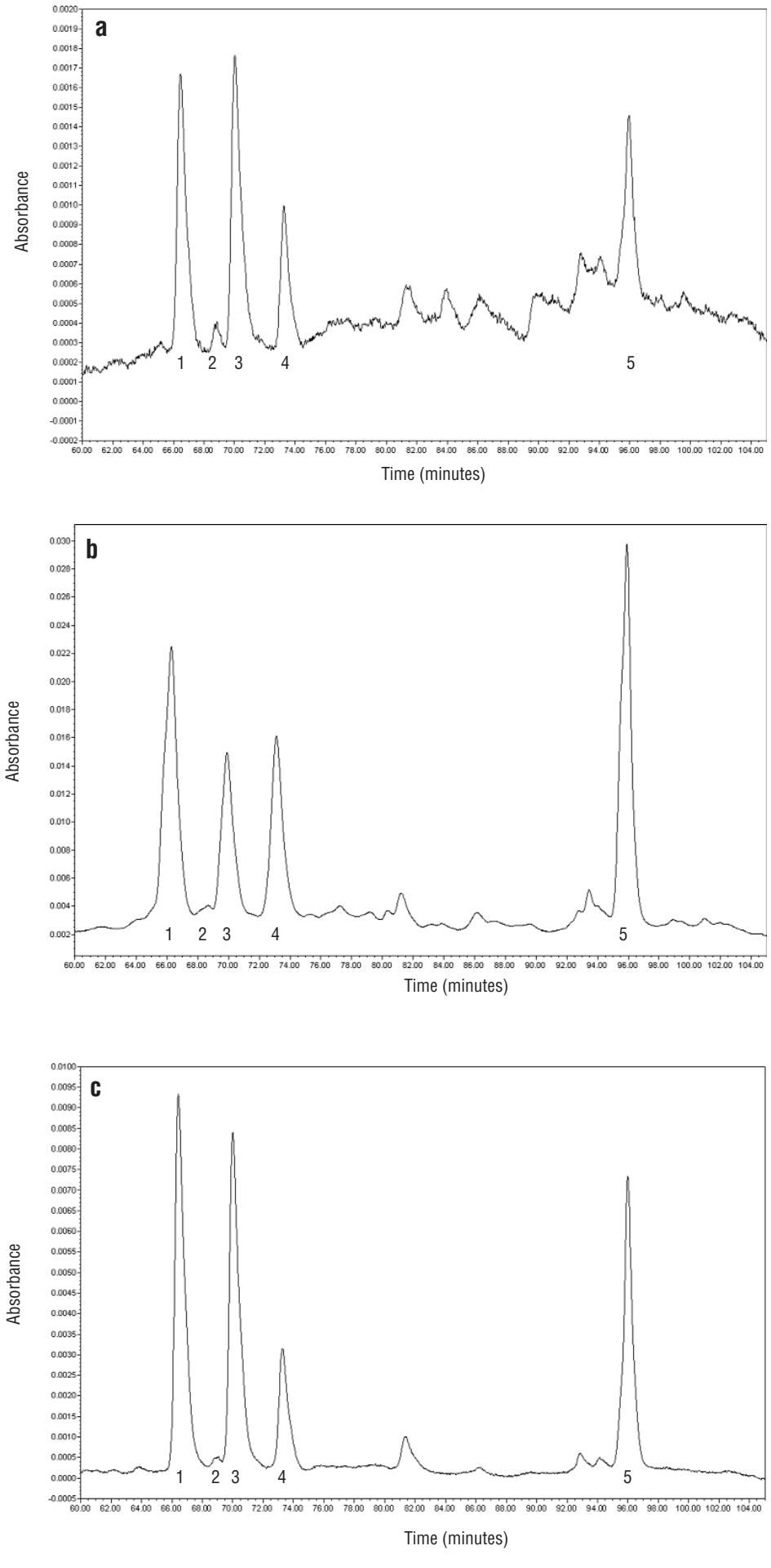


Figure 9. Comparison of the 440 nm chromatograms obtained from the analysis of various (+)-catechin solutions. The solutions are the model white wine system (150 mg/L (+)-catechin) with 0.6 mg/L copper(II) after 12 days at 45°C (**a**), the model wine system (1.16 g/L (+)-catechin) of Osmianski et al. (1996) with 20 mg/L iron(II) after 12 days at 39°C (**b**), and the model white wine system (150 mg/L (+)-catechin) with 0.25 mM glyoxylic acid after several months at 10°C (**c**).

shows that peaks 1–4 are present at identical retention times in all the samples. The LC/MS analysis of all the samples confirmed the presence of a 617 m/z ion for each of the four peaks 1 to 4 (Figure 9a–c). The different conditions used for the copper(II), iron(II) and glyoxylic acid induced reactions most likely contribute to the different peak intensity ratios observed in the chromatograms (Figure 9a–c). Irrespectively, these results confirm that the main coloured pigments generated in the model white wine system both in the absence and presence of copper(II) are xanthylium cations.

Peak 5 in the 440 nm chromatogram of the model white wine system (Figure 6) was observed in all the analysed samples in Figure 9 and its corresponding mass spectrum in the positive ion mode gave a significant ion at 645 m/z. Once again no significant ions were observed in the negative ion mode. The UV/VIS spectrum corresponding to this peak and the ion detected at 645 m/z were both in agreement with the published characteristics of the ethyl ester of the xanthylium cation (Es-Safi et al. 1999a). Similar to the original xanthylium cation, the ethyl ester would also be expected to have six isomeric forms despite the presence of only one peak in the HPLC/DAD.

Role of Cu(II) in xanthylium cation formation

The mechanism for the formation of the xanthylium cations has been described by Es-Safi et al. (1999a, b) as a four-step sequence, involving the oxidative coupling of (+)-catechin via a bridging group. The production of glyoxylic acid, presumably through the oxidative cleavage of tartaric acid (Fulcrand et al. 1997), initiates a reaction between glyoxylic acid and (+)-catechin generating a carboxy-methine linked dimer. After a dehydration step, a xanthene is produced followed by its oxidation to give the xanthylium cation. As all intermediates involved in production of the xanthylium cation are colourless, it is likely that the lag period (Figure 5) involves the initiation steps for the xanthylium cation formation.

Xanthylium cation formation is enhanced by the presence of copper(II) at elevated concentrations in the model white wine system, but copper(II) is clearly not a component of the final product. Rather, of the two 'reactive' rings in (+)-catechin, all chemical modification has occurred at the phloroglucinol-type ring (A ring, Figure 1). This is somewhat surprising as there is considerable published work suggesting the catechol moiety, or B ring, should be capable of complexing with copper(II) ions. The *ortho*-arrangement of the two hydroxy groups in the B ring presents an ideal metal chelation site and catechol itself is an analytical reagent for the determination of copper(II) (van den Berg 1984). Of course, the pH used in previous studies (pH 7–8) was always more basic than that of wine and deprotonation of the hydroxy groups of the B ring would be significant, leading in turn to a greater metal ion chelating propensity.

Irrespective of the absence of copper(II) in the final product, it is apparent (Figure 5) that at concentrations of 0.3 mg/L and higher, copper(II) ions are capable of influencing favourably at least one of four steps in the mechanism for xanthylium cation formation. It has not yet been possible to identify which step or steps the copper(II) may be accelerating. Intriguingly, at a copper(II) concentration of 0.1 mg/L, no enhancement of the browning process was observed (Figure 5). It is not yet certain whether this is due to the low level of copper(II) becoming unreactive, possibly through complex formation, or alternatively, if copper(II) is able to inhibit a step in the browning process and this inhibition is more dominant at lower copper(II) concentrations. The influence of copper(II) on the distribution of reaction products is evident when comparing the 278 nm chromatograms of samples with and without copper(II) (Figure 6). In the 278 nm chromatogram of the copper(II)-free sample (Figure 6a) there are four main peaks with retention times between 35 to 45 minutes that are diminished in the copper(II) containing samples. This suggests that copper(II) is either inhibiting the formation of these particular compounds or is accelerating their degradation. A more detailed study of the role of copper(II) in the mechanism of xanthylium cation formation is presently under way. Importantly our results show that copper(II) is not required for the formation of xanthylium cations from (+)-catechin in a system modelling white wine. Rather, at concentrations resembling those found in white wine, copper(II) accelerates xanthylium cation formation.

These results have illustrated for the first time that xanthylium cations are able to be produced in a system modelling white wine. The critical parameters in this study are the use of lower concentrations of both (+)-catechin and metal ion, the use of copper(II) as the metal ion and a more acidic pH compared with previously reported studies (Oszmianski et al. 1996). Furthermore, there was no evidence for the existence of any coloured compounds that may have been formed from a (+)-catechin derived *ortho*-quinone compound, as occurs in enzymic oxidation. Rather, in chemical, or non-enzymic oxidation, as described here, it is the A-ring that undergoes chemical change. These observations are important in understanding the different types of oxidative browning processes that have been described for white wine. Enzymic oxidation leads to *ortho*-quinones that subsequently react with other wine components to give brown polymers (Singleton 1987). Both non-flavanoids and flavanoids can be involved. However, non-flavanoids do not contain the phloroglucinol-type moiety, as do flavanoids (represented in this work by (+)-catechin) and it is this phloroglucinol unit that is essential for xanthylium cation formation. The work described here, therefore, provides some insight for the observations of Simpson (1982) on the relationship between white wine browning propensity and the concentration of (+)-catechin type compounds.

Effect of oxygen ingress on xanthylium cation formation

Preliminary experiments were conducted on the effect of oxygen ingress on xanthylium cation formation. Two identical model white wine samples were prepared, both with 0.6 mg/L copper(II); one sample was aerated twice

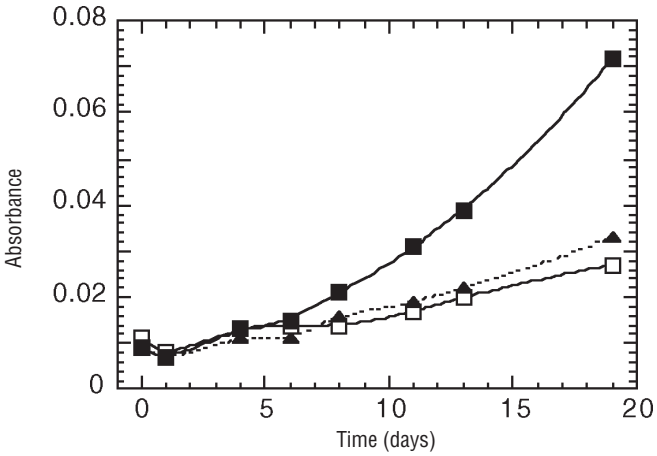


Figure 10. Change in absorbance at 440 nm with time for three different styles of the model white wine (all without copper(II)) during the induced browning process. Model white wine with 12% (v/v) (2.03 M) ethanol (□), 0% (v/v) ethanol (■) and 0% (v/v) ethanol with 2g/L (0.01 M) mannitol (▲).

daily for 24 days while the second sample was only aerated a total of four times in the 24-day period (viz. days 1, 6, 14 and 24). Comparison of the effect of aeration frequency on the absorbance at 440 nm showed that extensive aeration (twice daily) did not markedly influence the absorbance compared with infrequent aeration. The difference in absorbance intensity at 440 nm between the two samples at day 24 was less than 2%. The effect of headspace to sample volume ratio on the rate of browning was examined by comparing absorbance/time plots for two model white wine samples, one with a 200 mL sample volume and 50 mL headspace and the other with a 150 mL sample volume and 100 mL headspace. Both samples were only aerated on absorbance measurement days (i.e. days 1, 6, 13 and 20). As the absorbance difference between the samples was not more than 3% at any stage of the browning process, the results suggest that the variation of the headspace volume did not impact on the 440 nm absorbance reading. In all these experiments, a relatively large headspace to solution volume was used and it is possible that the system was always saturated with respect to its requirements for molecular oxygen. In work with ascorbic acid induced browning of (+)-catechin, Bradshaw et al. (2001) observed that a small headspace to solution volume affected the lag phase only. Further, exclusion of air after the end of the lag phase did not affect the final absorbance reading (Bradshaw et al. 2001), suggesting that oxygen ingress is required only to initiate the browning process. This issue is being refined further in the case of copper(II)-mediated browning.

Influence of ethanol and mannitol on xanthylium cation formation

An investigation into the presence of ethanol on the rate of browning was expected to provide some insight into the possible mechanism of the browning process. To assess the effect of ethanol on the rate of browning in the model white wine, samples without addition of Cu(II) were prepared with and without ethanol (Figure 10). The overall rate of browning increased dramatically in the absence of ethanol. The 440 nm chromatogram and LC/MS data of the ethanol-free model white wine confirmed the production of xanthylium cations in greater amounts than found with a sample containing 12% (v/v) ethanol (Figure 10), implying ethanol is able to inhibit the production of xanthylium cations.

Ethanol is known to react with the hydroxyl radical with a rate constant of 1.9 to 2.2×10^9 (depending on the reaction conditions) (Buxton et al. 1988, Motohashi and Saito 1993). If the inhibitory role of ethanol was via the scavenging of hydroxyl radicals then the addition of mannitol, a known scavenger of this radical (Buxton et al. 1988, Motohashi and Saito 1993), in the absence of ethanol, was expected to also decrease the rate of browning. As shown in Figure 10, the presence of mannitol did decrease the rate of browning. Given the ability of both mannitol and ethanol to suppress the browning process, it is reasonable to assume that the suppression is due to hydroxyl radical scavenging. Ethanol would appear to be a less efficient hydroxyl radical scavenger than mannitol as the extent of suppression is approximately the same (Figure 10), even though ethanol was present in much higher concentration (2.03 M ethanol compared with 0.01 M mannitol). Metal ions, including copper(II), can induce hydroxyl radicals through Fenton type chemistry (Wardman and Candeias 1996) and such a process has been invoked to explain oxidative processes occurring in beer (Chapon and Chapon 1979).

As ethanol is capable of scavenging the hydroxyl radical, a highly complex reaction system must be involved, possibly involving a radical cascade system. It must be pointed out that the xanthylium cation formation is not a chaotic process, as the degree of reproducibility in the absorbance values for equivalent experimental systems is quite high (RSD 1.5%, n = 3). We are presently examining the possible free radical mechanisms that could be involved in this copper(II)-mediated browning reaction.

Conclusions

The main products identified during the browning of the model white wine were xanthylium cations. The existence of xanthylium cations in the model white wine were observed in the absence of copper(II), however the presence of copper(II) above a threshold level led to an increase in concentration of the xanthylium cations. Based on the identification of these products it was concluded that the generation of the xanthylium cations occurred through the reaction of glyoxylic acid with (+)-catechin as proposed by the work of Cheynier's group (Es-Safi et al. 1999a, Oszmianski et al. 1996) on the iron(III)-mediated browning of a model red wine system. There was no clear evidence for the interaction of copper(II) with the catechol moiety of (+)-catechin in the oxidative browning associated with the generation of xanthylium cations.

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The Role of Copper(II) in the Bridging Reactions of (+)-Catechin by Glyoxylic Acid in a Model White Wine

ANDREW C. CLARK,*,† PAUL D. PRENZLER,†,‡ AND GEOFFREY R. SCOLLARY†

National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga, New South Wales 2678, Australia, and School of Science and Technology, Charles Sturt University, Locked Bag 588, Wagga Wagga, New South Wales 2678, Australia

The influence of copper(II) on the bridging reactions between (+)-catechin and glyoxylic acid was studied in a white winelike medium. When the reaction was performed in darkness at 45 °C, copper(II) increased the maximum levels of carboxymethine-linked (+)-catechin dimer and xanthylium cation pigment as monitored by high-performance liquid chromatography/photodiode array detection (HPLC/DAD) and liquid chromatography/mass spectrometry (LC/MS). At 10 °C, similar results were observed except that the xanthene intermediate was monitored and found to also increase in concentration at higher copper(II) concentrations. The kinetics for the formation of these species suggested that copper(II) accelerated the bridging of two (+)-catechin units by glyoxylic acid. The acid group of glyoxylic acid allowed copper(II) to influence this reaction, as no copper(II) enhancement was observed when acetaldehyde was used in place of glyoxylic acid.

KEYWORDS: Copper(II); white wine; (+)-catechin; xanthylium cation; model wine; browning; glyoxylic acid; acetaldehyde; oxidation

INTRODUCTION

The quality of a white wine is in part based on its color; therefore, excessive coloration can often be an indicator of spoilage. Such color changes may often occur well after bottling of the wine; consequently, the implications are not only costly product losses but also compromised consumer confidence.

Metal ions have been observed to accelerate changes in the coloration of model wines. The increased coloration of (+)-catechin-containing model wines, buffered at pH 3.20−3.70 with tartaric acid, in the presence of either added iron(II) or copper(II), has been attributed to the production of xanthylium cation pigments, of which **5** (**Table 1**) is an example (*1*, *2*). The role of the metal ions in the production of xanthylium cations is not entirely understood, although it has been suggested that iron(III), formed from oxidation of iron(II), and copper(II) are involved in the oxidation of tartaric acid to produce glyoxylic acid that can bridge two (+)-catechin units and result in xanthylium cation formation (*3*, *4*).

In the reaction between (+)-catechin **1** and glyoxylic acid **2** (**Scheme 1**), a (+)-catechin/glyoxylic acid adduct initially formed reacts with a further (+)-catechin unit to produce a carboxymethine-linked (+)-catechin dimer (*4*). The carboxymethine bridge may form at positions 8-8 (as shown by **3**), 6-6, or 8-6 between the (+)-catechin units (*5*). Therefore, as the latter isomer has two diastereoisomers, the total number of carboxymethine-linked (+)-catechin dimer isomers formed is four. Each of these isomers may undergo dehydration to form xanthenes that subsequently oxidize to form xanthylium cations. In the case of **3**, the associated xanthene cation and xanthylium cation are **4** and **5**, respectively (*6*). From the four carboxymethine-linked (+)-catechin dimers, a total of six xanthene and xanthylium cation isomers may be formed (*7*).

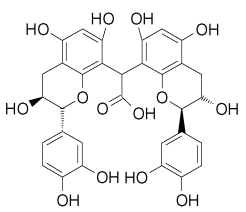
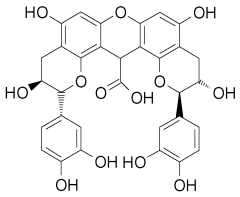
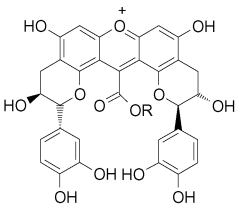
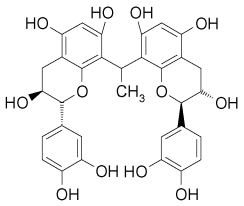
Glyoxal can also react with (+)-catechin to produce identical xanthylium cations to those formed from (+)-catechin and glyoxylic acid (*3*). However, only the reaction between (+)-catechin and glyoxylic acid produced the carboxymethine-linked (+)-catechin dimer, the major intermediate observed in the copper(II) and iron(III)-mediated oxidation of model wines (*3*, *4*).

In model wines containing (+)-catechin, tartaric acid, and copper(II), the production of xanthylium cations was shown to depend on the concentration of copper(II) present (*2*, *3*). However, this influence of copper(II) was only suggested to be a consequence of copper(II) promoting the production of glyoxylic acid from tartaric acid (*3*). Yet, as **Scheme 1** shows, the reaction between (+)-catechin and glyoxylic acid to form the xanthylium cation is a multistep reaction, and copper(II) could influence any one or all of these steps, not just the oxidation of tartaric acid to glyoxylic acid. Preliminary studies have shown that glyoxylic acid can be detected in tartaric acid solutions that are exposed to sunlight (*8*) and that with subsequent addition of (+)-catechin and varying levels of copper(II) to these solutions the formation of xanthylium cations increases significantly with copper(II) concentration (*8*). Al-

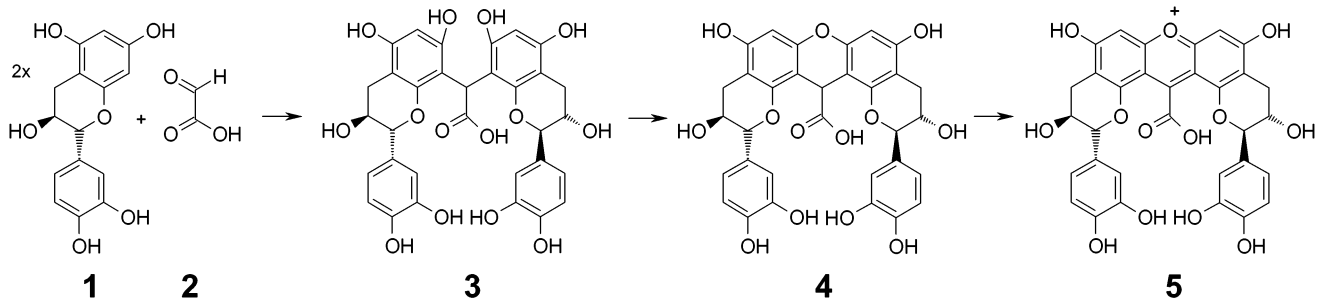
* To whom correspondence should be addressed. Tel: +61 2-69334181. Fax: +61 2-69334068. E-mail: aclark@csu.edu.au.
† National Wine and Grape Industry Centre, Charles Sturt University.
‡ School of Science and Technology, Charles Sturt University.

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Table 1

Compound (one structural isomer shown)	 **3** Carboxymethine-linked (+)-catechin dimer	 **4** Xanthene	 **5** Xanthylium cation	 **7** Methylmethine-linked (+)-catechin dimer
Uv-visible spectra characteristics (maxima and *shoulder* nm)	280	279	R = H: 277, 438-444 and *310* R = CH_2CH_3: 272, 463 and *310*	276-281
LC/MS signal (negative, positive m/z)	635, 637	617, 619	615, 617	605, 607
Peak assignments	**a**, **b**, **c** and **d**	**j**	R = H: **e**, **f**, **g** and **h** R = CH_2CH_3: **i**	**k** and **l**

Scheme 1. Reaction between (+)-Catechin **1** and Glyoxylic Acid **2** To Generate the Carboxymethine-Linked (+)-Catechin Dimer **3** and Xanthene **4** Intermediates as Precursors to the Formation of the Xanthylium Cation **5**



though the initial concentration of glyoxylic acid in these samples was not quantified, this result provides tentative evidence that copper(II) may influence at least one step in **Scheme 1**. Other work in red wine (*9*) has suggested that copper does not catalyze reactions between acetaldehyde and phenolic compounds, and these reactions are similar to the first step in **Scheme 1**.

Therefore, this study was undertaken to more clearly define the role of copper(II) in the reactions between glyoxylic acid and (+)-catechin. The concentrations of (+)-catechin, the xanthylium cation, and intermediates (**Scheme 1**) were followed by HPLC/DAD and LC/MS in the reaction between (+)-catechin and glyoxylic acid at variable copper(II) concentrations. To assess the importance of the acid moiety of glyoxylic acid on the copper(II)-mediated bridging of (+)-catechin, the reaction between acetaldehyde and (+)-catechin was also followed at variable copper(II) concentration.

MATERIALS AND METHODS

Reagents and Apparatus. All glassware and plasticware were soaked for at least 16 h in 10% nitric acid (BDH, AnalaR) and then rinsed with copious amounts of Grade 1 water (ISO 3696). Solutions and dilutions were prepared using Grade 1 water. Copper(II) was added as copper(II) sulfate pentahydrate (BDH, AR). Potassium hydrogen tartrate (>99%) and L-(+)-tartaric acid (>99.5%) were obtained from Sigma. (+)-Catechin monohydrate (Sigma, 98%) was used without further purification. The single wavelength (440 nm) absorbance of samples was recorded on a Unicam 8625 UV−visible spectrophotometer.

HPLC/DAD was conducted on a Waters 2690 Separation Module run by Millenium[32] software and connected to a Waters 996 DAD. The column used was a reverse phase Wakosil C18RS column of particle size 5 μm and 250 mm × 2 mm with a guard column of the same type. The HPLC/DAD analyses were carried out as described previously (*2*).

LC/MS experiments were conducted on a SpectraSYSTEM LC run by Xcalibar software with a P4000 sample pump, UV6000LP UV detector, and a Finnigan AQA quadrapole MS with an electrospray source. The same column was used as in HPLC/DAD experiments. The LC/MS experiments were carried out as published previously (*2*).

Reactions. The model white wine was prepared by adding 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid to aqueous ethanol (12% v/v, 2 L) and stirring overnight at room temperature. (+)-Catechin was added to this solution immediately prior to the preparation of an experiment. The pH of the model white wine was 3.2 ± 0.1. The addition of 0.25 mM glyoxylic acid (Aldrich, 98%) or 1.5 mM acetaldehyde (BDH, >99.5%) was made to the model white wine (150 mL) in 200 mL Schott bottles with screw top lids. The samples were then held in darkness at either 45 or 10 °C, and the sample bottles were only opened on measurement days.

RESULTS AND DISCUSSION

Influence of Copper(II) on the Reaction between Glyoxylic Acid and (+)-Catechin at 45 °C. Glyoxylic acid (0.25 mM)

was added to model white wine solutions containing variable copper(II) concentration, and the resulting solutions were maintained in darkness at 45 °C, a temperature similar to that used in many accelerated oxidation studies (*2*, *7*, *10*, *11*). The copper(II) concentrations adopted in this study, 0, 0.1, 0.3, and 0.6 mg/L, encompassed the levels of copper generally found in white wines (*12*, *13*). The concentration of glyoxylic acid, which provided a (+)-catechin to glyoxylic acid ratio of 2:1, was expected to be sufficient to ensure that the amount of added glyoxylic acid was in excess of that spontaneously generated from the oxidation of tartaric acid in the model white wine (*8*). Separate experiments showed that even though formation of glyoxylic acid is critical to **Scheme 1**, it could not be detected by HPLC/DAD in a 12% aqueous ethanol tartaric acid solution after heating at 45 °C for 3 days, regardless of the presence of copper(II). On the other hand, the addition of 0.25 mM glyoxylic acid to the model white wine was easily detected by HPLC/DAD (peak area, 14.8×10^3). Hence, the addition of 0.25 mM glyoxylic acid would enable a reaction between (+)-catechin and glyoxylic acid that was pseudo-zero order with respect to the formation of glyoxylic acid. Moreover, if copper(II) influenced the production of xanthylium cations or their intermediates under these conditions, then this would provide evidence that copper(II) was accelerating at least one step in **Scheme 1**.

The reaction solutions were analyzed by HPLC/DAD and LC/MS over the 10 day reaction period. In the 278 nm chromatograms of all samples, there were four peaks (peaks a−d, **Figure 1A**) that were observed to increase in peak area over the first few days of the reaction period. However, after 10 days, the 278 nm chromatogram of the samples became much more complicated (**Figure 1B**) and the accurate integration of peaks b−d became difficult. At day 10, well-defined peaks were observed in the 440 nm chromatogram (**Figure 1C**) with corresponding peaks in the 278 nm chromatogram (**Figure 1B**).

On LC/MS analysis of the copper-free sample, each of the four peaks a−d (**Figure 1A**) had associated with them significant *m*/*z* signals at 635 and 637 in the negative and positive ion modes, respectively. These *m*/*z* signals, including a sole maximum at 280 nm in the UV−visible spectrum for each of the peaks, are consistent with the assignment of these peaks as carboxymethine-linked (+)-catechin dimers (**Table 1**). Interestingly, despite the different chromatography conditions utilized in this study, the peak elution pattern and relative peak intensities observed for peaks a−d are similar to those observed for the isomers of the carboxymethine-linked (+)-catechin dimer identified in past studies (*3*, *7*). In all of the remaining samples of varying copper concentration, peaks a−d were assigned as carboxymethine-linked (+)-catechin dimers based on the agreement of their respective retention times, UV−visible spectra, and both negative and positive ion mode LC/MS data with the copper-free sample (**Table 1**).

The peaks e−h in the 440 nm chromatograms were consistent with the profile already observed for xanthylium isomers examined with identical chromatographic conditions (*2*) to that used in this study. The assignment of these peaks (**Table 1**) was confirmed with detection (LC/MS) of ions with *m*/*z* values of 615 and 617 in the negative and positive ion modes, respectively, at the retention times of peaks e−h. The UV−visible absorption spectra of these peaks, all with maxima at 277 nm, another between 438 and 444 nm, and a shoulder at 310 nm, were also consistent with that for the xanthylium cation (i.e., maxima at 273, 308, and 444 nm (*6*)). Peak i had a retention time, UV−visible spectra (i.e., maxima at 272 and 463 nm and

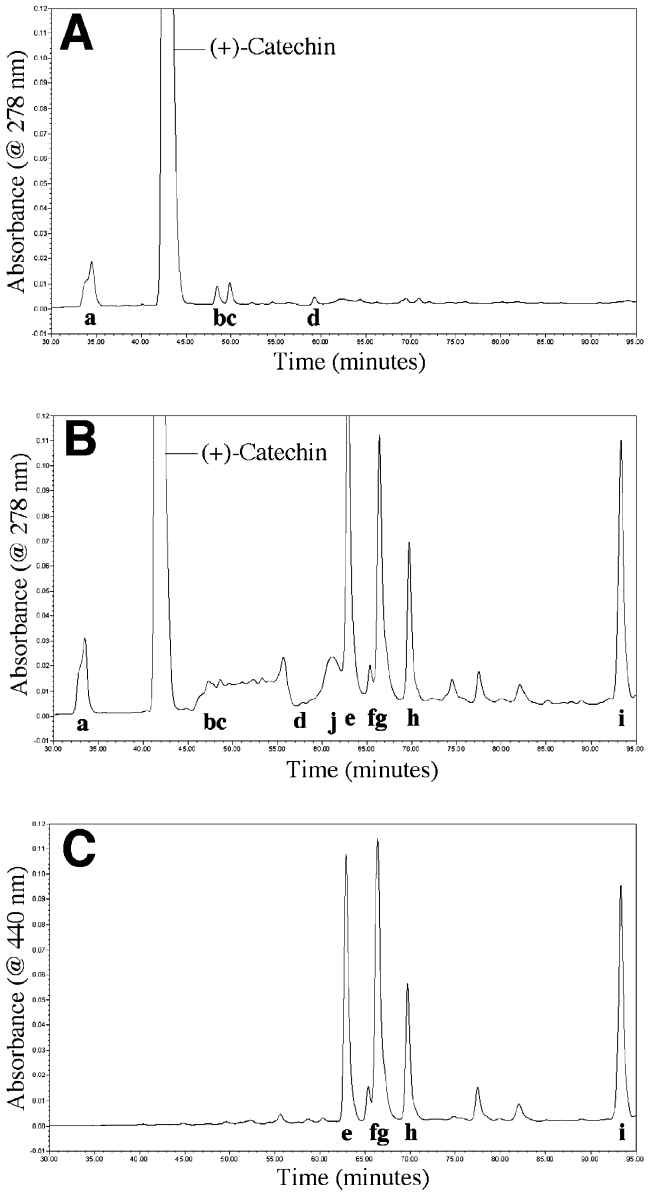


Figure 1. HPLC/DAD chromatograms of the model white wine with 0.25 mM glyoxylic acid and 0.6 mg/L copper(II) at day 1 (**A**; 278 nm) and day 10 (**B**; 278 nm and **C**; 440 nm) of the induced browning process.

shoulder at 310 nm), and LC/MS data (i.e., *m*/*z* signal at 643 and 645 in the negative and positive ion modes, respectively) consistent with an ethyl ester of the xanthylium cation (*2*).

Peak j (**Figure 1B**), with a broad appearance, was observed to have significant ions at 617 and 619 *m*/*z* in the negative and positive ion modes, respectively, and a sole maximum at 279 nm in the UV−visible spectrum. These data suggested that peak j was due to at least one of the six expected xanthene isomers (**Scheme 1**). Unfortunately, peak j was not fully resolved from peak e. The broad appearance of peak j (**Figure 1B**) as well as the peaks in the LC/MS ion chromatogram at 617 and 619 *m*/*z* (negative and positive ion modes, respectively; data not shown) suggested that peak j is most likely due to coelution of more than one xanthene isomer. The LC/MS data also suggested that other xanthene isomers might have been eluting between peaks c and d in an unresolved portion of the 278 nm chromatograms (**Figure 1B**).

The peak area due to (+)-catechin and the peaks a−j in the 278 nm chromatogram (**Figure 1A**) were followed over the 10 day reaction period to define a role of copper(II) in the time−

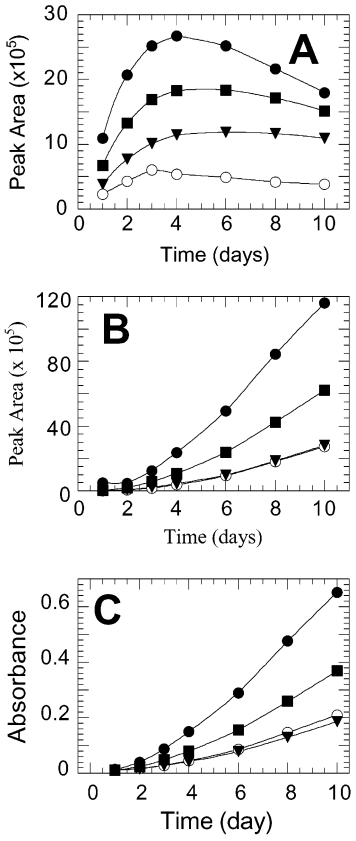


Figure 2. Change in peak areas of peak a (**A**) and peaks e–i (**B**) in the chromatograms of the model white wine with 0.25 mM glyoxylic acid and the change in absorbance at 440 nm (**C**). The copper(II) concentrations were 0 (○), 0.1 (▼), 0.3 (■) and 0.6 (●) mg/L. All samples were held at 45 °C for 10 days.

course dependence of these compounds. The amount of (+)-catechin utilized after the 10 days was 14, 15, 21, and 27% of the original (+)-catechin concentration in the 0, 0.1, 0.3, and 0.6 mg/L copper(II)-containing samples, respectively. This was high as compared to a 1% loss of (+)-catechin observed previously (*2*) in the 0.6 mg/L copper(II)-containing model white wine system without added glyoxylic acid after heating for the same period. The large decrease in (+)-catechin further confirmed that 0.25 mM added glyoxylic acid was in excess of the levels of glyoxylic acid spontaneously produced in the model white wine with 0.6 mg/L copper(II) (*2*). Most importantly, the ability of copper(II) to accelerate the loss of (+)-catechin under these conditions suggested that the consumptive reactions of (+)-catechin were copper(II)-dependent.

The areas of peaks a−d, associated with the carboxymethine-linked (+)-catechin dimer, could be accurately integrated over the first 3 days of the induced browning process, but after this stage, peaks b−d became unresolved from the surrounding peaks. Over the first 3 days of the reaction period (**Figure 2A**), it was evident that peak a increased with the increased concentration of copper(II) in the reaction mixture. Peaks b−d behaved similarly to peak a over this period (data not shown). During days 4−10, it was observed that the area of peak a either leveled out or decreased depending on the concentration of copper(II) in the sample (**Figure 2A**). Considering that the carboxymethine-linked dimer and its subsequent products are the major products formed from (+)-catechin in this work (**Figure 1A**,**B**), the kinetics of (+)-catechin and the carboxymethine-linked (+)-catechin dimer suggest that copper(II) accelerates the reaction between glyoxylic acid and (+)-catechin to form the dimer.

Accurate integration could not be obtained for peak j, which was assigned as a xanthene, throughout the 10 day analysis period, as peak j was not fully resolved from peak e. However, it appeared that peak j was generally larger with higher copper(II) concentrations (data not shown).

The summed intensity of peaks e−h (**Figure 2B**), combined isomers of the xanthylium cation, behaved similarly to the absorbance of the model white wine system at 440 nm (**Figure 2C**). The copper(II) concentrations of 0.3 and 0.6 mg/L copper(II) accelerated both the intensity of absorbance at 440 nm and the summed area of peaks e−h, while at copper(II) concentrations of 0 and 0.1 mg/L, the absorbance values and summed peak areas were similar. This pattern of copper(II) dependency was consistent with that observed for these parameters in the model white wine without added glyoxylic acid (*2*). The area of peak i (ethyl ester of the xanthylium cation) also behaved in a fashion similar to peaks e−h. Therefore, the results show that copper(II) can influence the formation of the xanthylium cation despite this reaction being pseudo-zero order with respect to the formation of glyoxylic acid.

These results demonstrate that copper(II) can influence at least one step of those illustrated in **Scheme 1** for the production of xanthylium cation from (+)-catechin and glyoxylic acid, a role different to the production of glyoxylic acid from tartaric acid (*3*). Although it is clear that copper(II) is promoting the reactions responsible for the production of the carboxymethine-linked (+)-catechin dimer from glyoxylic acid and (+)-catechin, further work is required to assess if copper(II) is influencing the remaining steps of xanthylium cation production (**Scheme 1**).

Influence of Copper(II) on the Bridging of (+)-Catechin by Glyoxylic Acid at 10 °C. The addition of glyoxylic acid to the model white wine at variable copper(II) concentrations was repeated at 10 °C rather than 45 °C. This was performed in an attempt to stabilize the xanthene and allow further insight into the role of copper(II) on the latter stages of **Scheme 1**. Previous work (*3*) had shown decreased reaction rates for **Scheme 1** when solutions of (+)-catechin, tartaric acid, and copper(II) were stored at 20 °C rather than 40 °C.

Throughout a period of 40 days, the model white wine samples were analyzed by HPLC/DAD and LC/MS. After 40 days, the 278 nm chromatograms provided much improved resolution for peaks a−d (**Figure 3A**) as compared to the results obtained using a reaction temperature of 45 °C after 10 days (**Figure 1B**). Once again, peaks a−d provided LC/MS data and UV−visible spectra characteristic of the carboxymethine-linked (+)-catechin dimer (**Table 1**). Peak j also had improved resolution under these conditions as compared to those of **Figure 1B**, mainly due to the smaller intensity of peak e, and the LC/MS data and UV−visible spectra were again consistent with its assignment as a xanthene (**Table 1**). At this lower reaction temperature, the production of the xanthylium cation from the xanthene was slowed considerably with peaks e−h being much less intense than those generated in the experiment at 45 °C. The peaks between c and d in **Figure 1B** were not observed when the reaction was performed at 10 °C (**Figure 3A**).

At 10 °C, copper(II) increased the concentrations of both the carboxymethine-linked (+)-catechin dimer (**Figure 3B**) and xanthene (**Figure 3C**) and also increased the consumption of (+)-catechin from 12 to 19% in the 0−0.6 mg/L copper(II)-containing samples, respectively. The small levels of xanthylium cations also showed copper(II) dependency with the summed areas of peaks e−h having 0.14, 0.21, 0.62, and 1.12×10^5 for

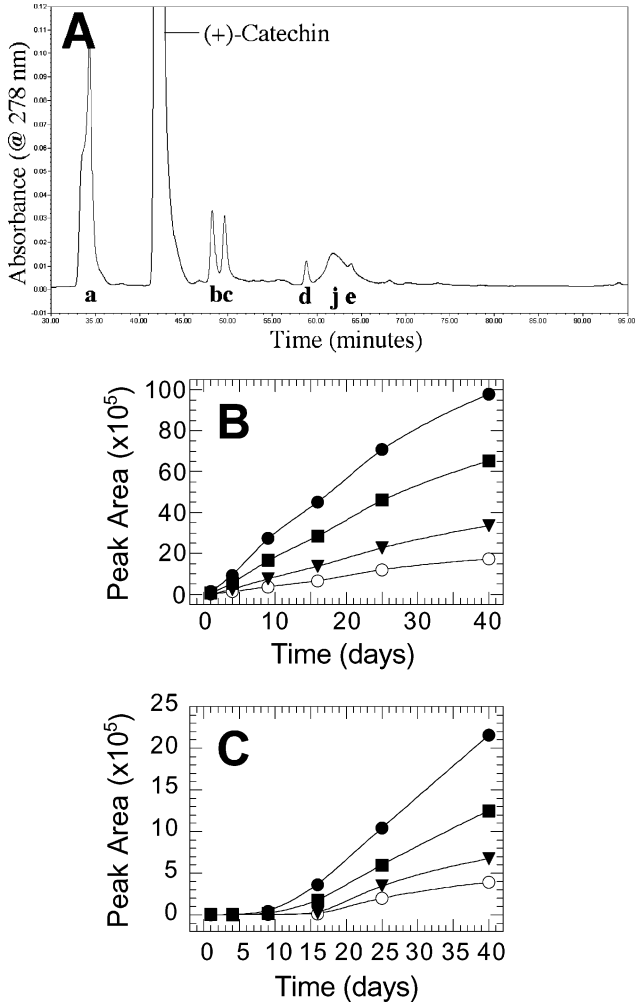
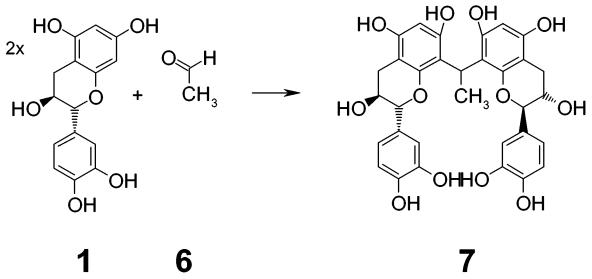


Figure 3. The HPLC/DAD chromatogram (278 nm) of the 0.6 mg/L copper(II)-containing model white wine with 0.25 mM glyoxylic acid (**A**) and the change in peak areas of peaks a–d (**B**) and peak j (**C**). The copper(II) concentrations were 0 (○), 0.1 (▼), 0.3 (■) and 0.6 (●) mg/L. All samples held at 10 °C for 40 days.

Scheme 2. Reaction between (+)-Catechin **1** and Acetaldehyde **6** Resulting in the Formation of a Methylmethine-Linked (+)-Catechin Dimer **7**



the 0, 0.1, 0.3, and 0.6 mg/L copper(II)-containing samples, respectively. Therefore, these results show that copper(II) can influence at least one step in **Scheme 1** at 10 °C as well as at 45 °C. Furthermore, it is evident from the copper(II)-dependent levels of (+)-catechin and the carboxymethine-linked (+)-catechin dimer that the first step in **Scheme 1** is also influenced by copper(II) at 10 °C. As the copper(II) dependency for the concentrations of the carboxymethine-linked (+)-catechin dimer and xanthene were similar (**Figure 3B**,**C**), it is likely that copper(II) accelerated the production of the former, and with

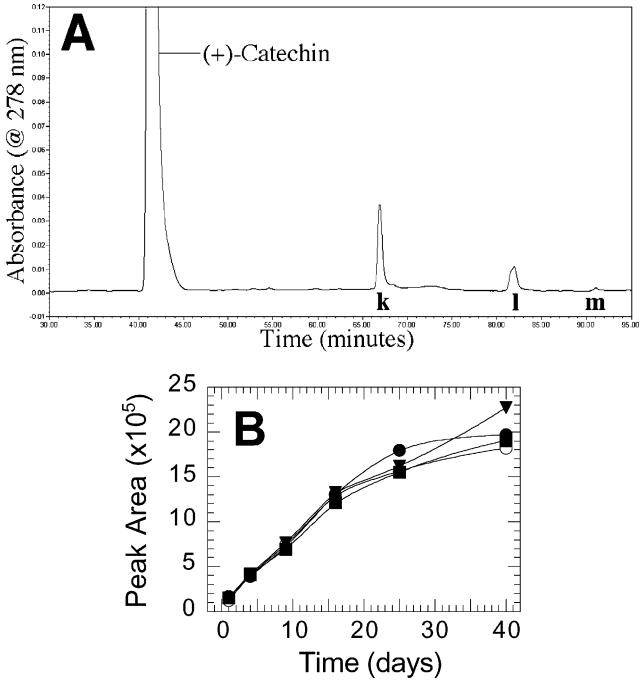


Figure 4. The 278 nm chromatogram of the 0.6 mg/L copper(II)-containing model white wine with 1.5 mM acetaldehyde (**A**) and the change in summed area of peaks k and l (**B**). The copper(II) concentrations were 0 (○), 0.1 (▼), 0.3 (■) and 0.6 (●) mg/L and the samples were held at 10 °C.

subsequent formation of the latter, the copper(II) dependency became common for both (**Scheme 1**). Consequently, there does not appear to be any gross effect of copper(II) on the second step of **Scheme 1**. Given the slow reaction rates and small concentrations of xanthylium cations generated, the influence of copper(II) in the last step of the **Scheme 1** cannot be established with any degree of certainty.

Influence of Copper(II) on the Bridging of (+)-Catechin by Acetaldehyde at 10 °C. The reaction between acetaldehyde and (+)-catechin was monitored at variable copper(II) concentration to allow comparison with the results obtained for the (+)-catechin and glyoxylic acid reaction. In the reaction of acetaldehyde with (+)-catechin, various isomers of a methylmethine-linked (+)-catechin dimer are obtained and the formation of one isomer is shown in **Scheme 2**. However, unlike the carboxymethine-linked (+)-catechin dimer, no evidence has been forthcoming to suggest that the methylmethine-linked (+)-catechin dimer can readily undergo dehydration and oxidation to form equivalent xanthylium cations. The four different isomers of the methylmethine-linked (+)-catechin dimer, including **7**, have been previously studied (*14*).

The reaction between acetaldehyde and (+)-catechin was conducted at 10 °C rather than at 45 °C to minimize loss of the volatile acetaldehyde. As some loss of acetaldehyde was envisaged during sampling, the concentration of acetaldehyde used was considerably higher than that described above for glyoxylic acid (1.5 and 0.25 mM, respectively). In previous studies, the production of methylmethine-linked (+)-catechin dimers has also involved an excess of acetaldehyde (*14*, *15*).

Figure 4A shows the 278 nm chromatogram generated after the model white wine, with 1.5 mM acetaldehyde and 0.6 mg/L copper(II), held at 10 °C for 40 days. Peaks k−m were all observed to increase during the 40 day reaction period in the absence and presence of copper(II) (0.1, 0.3, and 0.6 mg/L).

From the work of Saucier et al. (*14*), whose identical chromatographic conditions were used in this study, the methylmethine-linked (+)-catechin dimers were observed to elute after (+)-catechin with similar intensities as peaks k−m (**Figure 4A**). From the work of Saucier et al. (*14*), it was evident that peaks k and m were due to single isomers, respectively, while peak l was due to two isomers, explaining the broadened profile of this peak. From LC/MS data, peaks k and l were observed to have significant ions at 605 and 607 *m*/*z* (negative and positive ion modes, respectively) and these are characteristic of the methylmethine-linked (+)-catechin dimers (**Table 1**). The ion signal associated with peak m (**Figure 4A**) was not detected by LC/MS. Because of both the inability to confirm the identity of peak m and its comparatively low intensity, only the peak areas of peaks k and l were used to assess the effect of copper(II) on the production of the methylmethine-linked (+)-catechin dimers.

From the results in **Figure 4B**, it is clear that copper(II) does not have any effect on the production of the methylmethine-linked (+)-catechin dimers. Although there is some small difference in the levels of the methylmethine-linked (+)-catechin dimers between the different samples after days 25 and 40, this difference is not related to the concentration of copper(II) in the reaction system. Therefore, this result is consistent with a study performed in red wine (*9*), where the presence of copper(II) in the red wine was suggested to be unable to accelerate the combination of acetaldehyde with phenolic compounds. However, in this red wine study (*9*), the measurements of acetaldehyde, total phenolic compounds, total tannins, and degree of tannin condensation were conducted rather than the identification of specific products, as described here.

A comparison of the data in **Figure 4B** with that in **Figure 3B** suggests that the acid functionality of glyoxylic acid is important in enabling copper(II) to promote the production of the bridged (+)-catechin/aldehyde species. This in turn implies that a copper(II)−glyoxylate complex is probably involved in the production of the carboxymethine-linked (+)-catechin dimers.

CONCLUSION

The role of copper(II) in the production of xanthylium cations from (+)-catechin in winelike conditions has been more clearly defined. Copper(II) is not just a possible oxidation catalyst in the conversion of tartaric acid to glyoxylic acid (*3*), but it is actively involved in the condensation reactions of glyoxylic acid and (+)-catechin. More specifically, it has been demonstrated that the role of copper(II) is important in the formation of carboxymethine-linked (+)-catechin dimer from glyoxylic acid and (+)-catechin; therefore, this implies that copper(II) accelerates the reaction of (+)-catechin with either glyoxylic acid and/or the (+)-catechin/glyoxylic acid adduct. Copper(II) is less important in the conversion of the carboxymethine-linked (+)-catechin dimer to the xanthene, while further work is required to establish the effect of copper(II) on the oxidation of the xanthene to the xanthylium cation.

The acid moiety of glyoxylic acid is crucial for copper(II) to exert an influence on the formation of the carboxymethine-linked (+)-catechin dimer, and consequently, a mechanism involving the complex formation between copper(II) and glyoxylic acid is likely. The results confirm for the first time the ability of a metal ion to accelerate the bridging reaction between (+)-catechin and glyoxylic acid in a winelike medium.

The results of this study allow further insight into reactions that may allow copper(II) to contribute to wine coloration. Tartaric acid additions are frequently made during the production of white wines, and it is not inconceivable that such tartaric acid may have trace amounts of glyoxylic acid impurities, generated either through the storage conditions of tartaric acid (*8*) or during the production of tartaric acid itself. If trace levels of glyoxylic acid are added to wine, then the level and speciation of copper(II) in the wine may be critical with regard to coloration of the wine when compared to wines that have negligible levels of copper(II). The ability of iron(III) to facilitate the bridging reactions of glyoxylic acid with (+)-catechin is not known, but it is expected that iron(III) would behave similarly to copper(II).

ABBREVIATIONS USED

HPLC/DAD, high-performance liquid chromatography/photodiode array detector; LC/MS, liquid chromatography/mass spectrometry; UV, ultraviolet.

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Influence of light exposure, ethanol and copper(II) on the formation of a precursor for xanthylium cations from tartaric acid

ANDREW C. CLARK[1,2] and GEOFFREY R. SCOLLARY[1]

[1] National Wine and Grape Industry Centre, Charles Sturt University, Locked Bag 588, Wagga Wagga NSW 2678, Australia

[2] Corresponding author: Dr Andrew C. Clark, facsimile +61 2 6933 2107, email aclark@csu.edu.au

Abstract

The production of xanthylium cation pigments was greatly increased when an aged, tartaric acid buffered, 12% (v/v) aqueous ethanol solution was used in a model white wine system. This suggested the formation of a precursor to the pigments during the ageing of the tartaric acid solution. On examining factors responsible for the generation of tartaric acid oxidation products in wine-like solutions it was observed that on exposure of samples to sunlight, glyoxylic acid, a known precursor to xanthylium cations, was produced. The production of glyoxylic acid was achieved in both the absence and presence of ethanol and copper(II). Hydrogen peroxide was also detected in these solutions. The results were consistent with the presence of glyoxylic acid in the aged tartaric acid buffered, 12% (v/v) aqueous ethanol solution that had frequent aeration and periodic exposure to sunlight throughout its storage. Studies on the role of hydrogen peroxide in the production of glyoxylic acid were also investigated. On the addition of hydrogen peroxide to tartaric acid solutions, with heating at 45°C in darkness, glyoxylic acid was only determined in solutions without ethanol.

Abbreviations

HPLC/DAD high performance liquid chromatography/photodiode array detector; **LC/MS** liquid chromatography/mass spectrometry; **MS** mass spectrometry; **UV** ultra violet; **VIS** visible

Keywords: *browning, (+)-catechin, glyoxylic acid, hydrogen peroxide, model wine, oxidation, sunlight, tartaric acid, white wine, xanthylium cation*

Introduction

The oxidative spoilage of white wine is usually concomitant with an increased intensity of colour of the wine as monitored by its absorbance at 420 nm (Simpson 1982). This oxidative process can occur after bottling and often a random selection of wine bottles is affected. The random nature of oxidative spoilage is not understood, but various factors such as the exposure of certain bottles to excessive temperature, sunlight or molecular oxygen compared to other bottles have occasionally been suggested by winemakers.

For white wines that have undergone an induced oxidation process, the presence of flavanols, such as (+)-catechin, has been correlated with an increased level of browning (Simpson 1982). This non-enzymic mechanism of browning has been the focus of much current research (Singleton 1987, Oszmianski et al. 1996, Es-Safi et al. 2000, Clark and Scollary 2002).

In a model white wine system consisting of a 12% (v/v) aqueous ethanol tartaric acid buffered medium containing (+)-catechin, the pigments generated after an induced oxidative process were identified as xanthylium cations (Clark and Scollary 2002). These compounds absorb in the visible region with absorbance maxima in the range of 440–450 nm. The xanthylium cations were produced through the reaction between glyoxylic acid and (+)-catechin. The presence of tartaric acid was crucial for the formation of xanthylium cations in a (+)-catechin-containing model wine media, implying the formation of glyoxylic acid from tartaric acid (Fulcrand et al. 1997). The production of xanthylium cations was accelerated in the presence of iron(III) (Oszmianski et al. 1996, Es-Safi et al. 1999a) and copper(II) (Clark and Scollary 2002), provided a sufficient concentration of the latter heavy metal ion was present.

The chemistry of xanthylium cations has been extensively studied (Es-Safi et al. 1999a, 2000). Six different structural isomers have been characterised and the corre-

sponding ethyl esters identified. The mechanism for the production of these isomeric pigments from glyoxylic acid and (+)-catechin is generally understood but the production of glyoxylic acid from tartaric acid is less well known.

Fenton (1894) in a classic study described the oxidation of tartaric acid in the presence of iron(II) and hydrogen peroxide to give a product that was subsequently suggested to be dihydroxymaleic acid (Fenton 1905). Later, it was shown that the crystalline hydrate of 'dihydroxymaleic acid' was actually dihydroxyfumaric acid (Gupta 1953), that is, the trans isomer of dihydroxymaleic acid. Fenton (1905) also mentioned the ability of dihydroxymaleic acid to exist in tautomeric equilibrium with hydroxyoxaloacetic acid in solution. Studies of dihydroxymaleic acid, and its isomers, showed these compounds to give a variety of degradation products involving both decarboxylative and oxidative mechanisms (Wieland and Franke 1928, Baraud 1954).

Wieland and Franke (1928) examined the oxidation of tartaric acid in a study investigating the activation of molecular oxygen by the iron(II)/iron(III) redox couple. In the presence of iron(II) and molecular oxygen, tartaric acid was oxidised to dihydroxymaleic acid that then underwent further oxidation by iron(III) to form diketosuccinic acid. This product readily degraded to oxalic acid either directly or via a mesoxalic acid intermediate. The spontaneous autoxidation of dihydroxymaleic acid to produce diketosuccinic acid was also observed. Oxidation of tartaric acid occurred in the presence of copper(II) sulfate, but to a lesser extent than that induced by the iron(II)/iron(III) couple. Baraud (1954) followed the compounds formed in an aqueous solution of tartaric acid in the presence of iron(II). After thirteen months, the solution contained (in order of concentration) oxalic acid, glyoxylic acid, glycol aldehyde, glyoxal, mesoxalic acid and diketosuccinic acid. All these products were explained by oxidation and decarboxylation processes. A suggested mechanism for the formation of glyoxylic acid in the presence of the iron(II)/iron(III) couple involved the oxidation of tartaric acid to dihydroxymaleic acid, the oxidation of the latter to diketosuccinic acid followed by decarboxylation to give mesoxalic acid semialdehyde. The mesoxalic acid semialdehyde then underwent a proposed oxidation to generate mesoxalic acid, the latter decarboxylating to form glyoxylic acid. The oxidation of glycol aldehyde to glyoxylic acid was also suggested.

This present study was performed in order to investigate the generation of glyoxylic acid from tartaric acid under wine-like conditions, as glyoxylic acid production is relevant to the generation of pigments that absorb at 440 nm in model wine systems. The conditions conducive to glyoxylic acid formation would provide insight into factors that may contribute to the spoilage of white wine. Previously it was observed that xanthylium cations can be produced in the absence of copper(II) (Clark and Scollary 2002) and thus particular attention was paid to the generation of glyoxylic acid in 12% aqueous ethanol tartaric acid media without the presence of heavy metal ions. Further, the ability of low concentrations of copper(II) to promote the formation of glyoxylic acid was investigated in order to assess whether it is in this manner that copper(II) is able to enhance xanthylium cation production (Clark and Scollary 2002).

Materials and methods

Instrumentation and software

Absorbance and pH measurements were conducted as described by Clark and Scollary (2002).

HPLC/DAD was conducted on a Waters 2690 Separation Module run by Millenium^{32} software and connected to a Waters 996 photodiode array detector based on the method by Frayne (1986). Samples were analysed using a micro guard cation H^+ cartridge guard column (Bio-Rad Laboratories) and a 300 mm × 7.8 mm Aminex HPX-87H organic acid analysis cation exchange column (Bio-Rad Laboratories).

LC/MS work was conducted on a SpectraSYSTEM LC run by Xcalibar software with a P4000 sample pump, UV6000LP UV detector and a Finnigan AQA quadrapole MS with an electrospray source. The same column was used as in HPLC/DAD experiments.

The HPLC/DAD and LC/MS determination of the xanthylium cation pigments was conducted in the manner described by Clark and Scollary (2002).

Square wave cathodic voltammetry was performed on a Radiometer (Lyon, France) TraceLab50 voltammetric system that included a Radiometer MDE150 polarographic stand and a Radiometer POL150 polarographic analyser. The experiments were managed with the Radiometer Tracemaster5 (version 2.03) software that was installed on an IBM compatible computer. A three electrode system was used that consisted of a hanging drop electrode as the working electrode, a silver/silver chloride reference electrode with a filling solution of 3 M potassium chloride, and a platinum metal counter electrode. All potentials are quoted with respect to this reference electrode. The measurement of hydrogen peroxide was conducted as described by Bradshaw et al. (2002).

Tartaric acid solutions

1. Aqueous ethanol tartaric acid solution

The aqueous ethanol tartaric acid solution was prepared by adding approximately 10 g of potassium hydrogen tartrate (Sigma, > 99%) to aqueous ethanol (12% v/v, 2L) and stirring overnight at room temperature. Excess potassium hydrogen tartrate was removed from the saturated solution, by filtration through a 0.45 µm Sartorius cellulose acetate filter fitted to an all glass filter unit. The pH of the solution was then adjusted to pH 3.20 with aqueous tartaric acid (300 g / L, Sigma, > 99.5%).

After the filtration of the saturated potassium hydrogen tartrate solution and prior to the addition of tartaric acid, the level of tartaric acid in solution was quantified by HPLC/DAD and found to be 0.017 ± 0.001 M.

2. Ethanol-free tartaric acid solution

Ethanol-free tartaric acid solution was prepared by dissolving 0.017 M potassium hydrogen tartrate in water.

The solution was filtered and tartaric acid added in an amount equal to that added to the aqueous ethanol tartaric acid solution.

3. Model white wine and the induced browning process
The model white wine was prepared immediately before the commencement of the induced browning process by the addition of 150 mg/L (+)-catechin, as (+)-catechin monohydrate (Sigma, 98%), to the aqueous ethanol tartaric acid solution. In experiments requiring copper(II), the metal ion was added as copper(II) sulfate pentahydrate (BDH, AR). The induced browning process involved heating at 45°C in darkness as outlined in Clark and Scollary (2002).

Analytical HPLC/DAD and LC/MS analyses
The HPLC/DAD flow rate was 0.70 mL/min with an isocratic elution of 0.085% phosphoric acid in water. The sample volume injected was 10 µL and the detection was performed at 210 nm. Glyoxylic acid monohydrate (Aldrich, 98%) was used as a standard.

LC/MS work was conducted both in the positive ion mode, with an ion spray voltage of +4 kV and orifice voltage of +30 V, and in the negative ion mode, with an ion spray voltage of –4 kV and orifice voltage of –30 V. UV detection at 210 nm was performed with an isocratic elution of 0.05% acetic acid in water. The same column and flow rate were used as for HPLC/DAD experiments. The sample injection was 20 µL.

Results and discussion

Browning of model white wine systems with 0 mg/L copper(II)
An aqueous ethanol tartaric acid solution was prepared and stored for a period of six months mostly in the dark but with periodic exposure to sunlight. This aged solution had a continued supply of molecular oxygen through intermittent exposure to the atmosphere. After the addition of (+)-catechin (150 mg/L) to this aged solution, the resulting sample, referred to as a model white wine, underwent the induced browning process, that is, heating at 45°C in darkness for 20 days. After this process (Experiment 1, Table 1) it was found that the 440 nm absorbance was much higher in this model white wine compared to another model white wine prepared with an entirely fresh aqueous ethanol tartaric acid solution (Experiment 2, Table 1). This result showed that the age of the aqueous ethanol tartaric acid solution had a large impact on the extent of browning of the resulting model white wine system.

The pigments generated in the model white wine system prepared entirely fresh have been shown to be xanthylium cations (Clark and Scollary 2002). When the model white wines resulting from Experiments 1 and 2 (Table 1) were analysed by HPLC/DAD, all significant peaks in the respective 440 nm chromatograms were at identical retention times. The main difference was the increased intensity of the peaks in the chromatogram of the model white wine from Experiment 1. From the LC/MS analysis, the most intense peaks in the 440 nm chromatogram were associated with ions at 617 m/z in the positive ion mode, characteristic of the xanthylium cation (Clark and Scollary 2002). The only other intense peak was associated with an ion at 645 m/z in the positive ion mode, characteristic of the ethyl ester of the xanthylium cations. This confirmed that the pigments generated as a consequence of Experiment 1 (Table 1) were xanthylium cations.

Table 1. Summary of conditions applied in, and results from the induced browning experiments with model white wines.

Exp. no.	Conditions applied in the preparation of the12% aqueous ethanol tartaric acid solution	(+)-Catechin conc.[1] (mg/L)	Copper(II) conc.[1] (mg/L)	Absorbance after induced browning[2]
1	6 month, periodic sunlight	150	0	0.131
2	Fresh	150	0	0.042
3	6 month, periodic sunlight	150	0.6	0.242
4	Fresh	150	0.6	0.052
5	7 days at 45°C, darkness	150	0	0.043
6	7 days at 45°C, darkness	150	0.6	0.053

1. The addition of (+)-catechin and copper(II) was made after the preparation of the 12% aqueous ethanol tartaric acid solution and prior to the induced browning process.
2. The induced browning process consisted of heating at 45°C in darkness for 20 days.

The enhanced concentrations of the xanthylium cations in the model white wine system of Experiment 1 compared to Experiment 2 (Table 1) suggest that a precursor to the final xanthylium cation pigment is formed in the aged aqueous ethanol tartaric acid solution.

Browning of model white wine systems with 0.6 mg/L copper(II)
Experiments 1 and 2 were repeated with the inclusion of 0.6 mg/L copper(II), which was added after (+)-catechin but immediately prior to the induced browning process. In the presence of copper(II), the model white wine with the aged aqueous ethanol tartaric acid solution (Experiment 3, Table 1) had a more intense 440 nm absorbance than the freshly prepared sample (Experiment 4, Table 1). Furthermore, the absorbance at 440 nm was higher in the sample prepared with copper(II) than in that without copper(II) (Experiments 1 and 3, Table 1). Although this influence of copper(II) was expected for Experiments 2 and 4 (Table 1) from previous work (Clark and Scollary 2002), it had not been observed for aged aqueous ethanol tartaric acid solutions (Experiments 1 and 3, Table 1). The HPLC/DAD and LC/MS analysis of the copper(II)-containing model white wine systems confirmed that the pigments generated were again xanthylium cations. Therefore, it appears that despite the presence of a precursor to xanthylium cations in the aqueous ethanol tartaric acid solution, copper(II) is still able to accelerate the formation of xanthylium cations during the induced browning of the model white wine system.

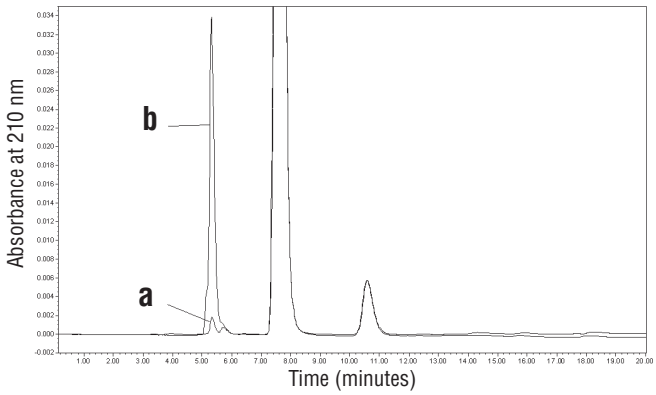


Figure 1. HPLC chromatograms of the aqueous ethanol tartaric acid solution used in Experiments 1 and 3 in Table 1 (**a**), and Experiment 7 in Table 2 (**b**). See text for the identification of peaks. Both chromatograms were obtained by HPLC/DAD with detection at 210 nm.

Influence of ethyl tartrate on the browning of the model white wine

The chromatogram of the aged aqueous ethanol tartaric acid solution utilised in Experiments 1 and 3 (Table 1) is shown in Figure 1, chromatogram a. The peak eluting at 7.6 minutes is due to tartaric acid. The peak at 10.6 minutes had a peak area of 147×10^3 (arbitrary units), much greater than that of the comparable peak in the chromatogram of a freshly prepared aqueous ethanol tartaric acid solution (4×10^3 arbitrary units). An LC/MS chromatogram indicated that this peak had m/z values of 179 and 177 in the positive and negative ion mode respectively, corresponding to the mono-ethyl ester of tartaric acid.

The esterified (ethyl ester) form of the xanthylium cations produced during the browning of (+)-catechin has been identified in both the iron(III)-mediated (Es-Safi et al. 1999a) and copper(II)-mediated (Clark and Scollary 2002) systems. To investigate whether the presence of ethyl tartrate in the aqueous ethanol tartaric acid solution was the cause of the more intense browning in Table 1, Experiments 5 and 6 (Table 1) were performed. This involved heating an aqueous ethanol tartaric acid solution for 7 days to increase the levels of ethyl tartrate to over 15 times that found in the freshly prepared solution. After the addition of (+)-catechin, and copper(II) in the case of Experiment 6 (Table 1), the induced browning process was conducted on these samples. However, in comparing the 440 absorbances obtained from Experiments 2 and 5 (Table 1), it is clear that the presence of ethyl tartrate in the starting system is not the cause of enhanced browning observed in Experiment 1 (Table 1). This was also the case in the presence of copper(II) (Experiments 4 and 6, Table 1).

The ethyl ester of the xanthylium cation has been identified as a pigment produced in the browning of the model white wine system (Clark and Scollary 2002). During investigations into the formation of the ethyl ester of the xanthylium cation, it was shown by Es-Safi et al. (1999a) that esterification was achieved before cyclisation of the carboxymethine linked (+)-catechin dimer.

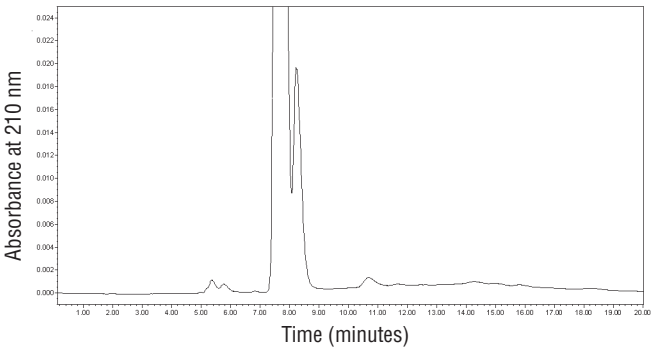


Figure 2. HPLC chromatogram from the HPLC/DAD with UV detection at 210 nm of 0.006 M glyoxylic acid in a freshly prepared aqueous ethanol tartaric acid solution.

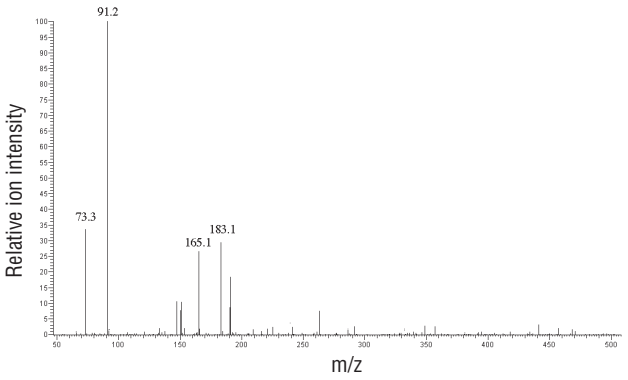


Figure 3. The mass spectrum of glyoxylic recorded in the 12% aqueous ethanol tartaric acid solution.

Therefore, the esterification of tartaric acid, as observed in this study, provides a potential stage at which the acid moiety of the xanthylium cation may have been originally esterified. Nevertheless, it appears that although heating was sufficient to increase the concentration of ethyl tartrate, it was not sufficient to cause the production of the precursors to xanthylium cations.

Glyoxylic acid detection in aqueous ethanol tartaric acid solutions

Prior to examining the generation of glyoxylic acid (Figure 4a), a known precursor of the xanthylium cation pigments, the measurement of glyoxylic acid by HPLC/DAD and LC/MS was investigated. The addition of glyoxylic acid to a freshly prepared aqueous ethanol tartaric acid solution yielded the chromatogram in Figure 2. Glyoxylic acid eluted at 8.3 minutes but was not well resolved from tartaric acid. In some experiments involving the production of glyoxylic acid, it was necessary to use two columns to improve peak resolution and thereby allow low concentrations of glyoxylic acid to be clearly identified in the presence of a high concentration of tartaric acid.

Intriguingly, the mass spectrum for the peak eluting at 8.3 minutes in the 210 nm chromatogram (Figure 2) has its most signification ion at an m/z value of 91 (negative ion mode) (Figure 3). This indicates an association between the glyoxylic acid anion and water (73 + 18 m/z). Although this ion could potentially be due to a

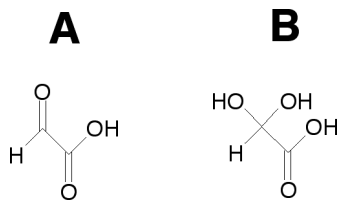


Figure 4. The structure of glyoxylic acid (**A**; 74 Da) and its hydrated form (**B**; 92 Da).

non-covalent adduct there is some evidence to suggest this is not the case. The species responsible for the ion at a value of 91 m/z appears able to form clusters with glyoxylic acid (74 Da) and also with a species of molecular mass 92 Da to generate the signals at m/z values of 165 and 183 respectively (Figure 3). As appreciable ion signals are not usually generated for tri- or tetra-molecular clusters, the ion responsible for the signal at the m/z value of 91 is therefore not likely to be a non-covalent adduct. Alternatively, as glyoxylic acid can readily undergo hydration in aqueous solution (Leitzke et al. 2001) to generate a di-alcohol of molecular mass 92 Da (Figure 4 B), such hydration would also be expected in the 12% aqueous ethanol medium. The ion at m/z 183 (Figure 3) would then be explained by the non-covalent adduct formed between two of these hydrated glyoxylic acid species.

Tartaric acid solutions in sunlight: production of glyoxylic acid and hydrogen peroxide

In the chromatogram of the aged aqueous ethanol tartaric acid solution (Figure 1a, Experiments 1 and 3, Table 2), there was a pair of peaks eluting around 5.3 and 5.7 minutes in addition to the tartaric acid and ethyl tartrate peaks. When this same aged aqueous ethanol tartaric acid solution was re-analysed by HPLC/DAD after a further two weeks of exposure to air and intermittent sunlight (Experiment 7, Table 2), the chromatogram (Figure 1b) showed a marked increase in the peak at 5.3 minutes. From the studies made to detect glyoxylic acid discussed above, it was clear that this peak was not due to glyoxylic acid.

Experiments were therefore conducted to both identify the species responsible for the peak at 5.3 minutes and determine any influence this species had on the production of glyoxylic acid in tartaric acid media. Furthermore, the effect of copper(II) and ethanol on the production of this species, and of glyoxylic acid, were also determined.

A freshly prepared aqueous ethanol tartaric acid solution was exposed to sunlight for three days (Experiment 8, Table 2). HPLC analysis (with detection at 210nm) of this experiment solution showed the peak with the retention time of 5.3 minutes together with a peak at the retention time identical to that of glyoxylic acid. The LC/MS data were consistent with the identity of this latter peak being due to the presence of glyoxylic acid.

When the experiment was repeated in the presence of copper(II) (Experiment 9, Table 2), the peak at the retention time of 5.3 minutes was observed but at a lower intensity than in Experiment 8. Furthermore, no peak was observed for glyoxylic acid in the 210 nm chro-

Table 2. Summary of conditions applied in, and results from the experiments on tartaric acid solutions[1]

Exp. no.	Age of tartaric acid solution[3]		Additives during ageing of solutions	Conditions during ageing of solutions	H_2O_2 detected after ageing (Peak area × 10^3)[4]	Glyoxylic acid detected after ageing[5]
	Prepared with 12% ethanol	Prepared ethanol-free				
1 and 3[2]	6 months old	NA	none	Periodic sunlight	21	No
2 and 4[2]	Fresh	NA	none	None	low	No
5 and 6[2]	7 days old	NA	none	45°C, darkness	low	No
7	6.5 months old	NA	none	Periodic sunlight	4798	No
8	3 days old	NA	none	Sunlight	19576	Yes (UV, MS)
9	3 days old	NA	Cu(II)	Sunlight	low	Yes (MS)
10	NA	3 days old	none	Sunlight	6924	Yes (UV, MS)
11	NA	3 days old	Cu(II)	Sunlight	485	Yes (MS)
12	3 days old	NA	none	45°C, darkness	low	No
13	3 days old	NA	Cu(II)	45°C, darkness	low	No
14	3 days old	NA	H_2O_2	45°C, darkness	48379	No
15	3 days old	NA	H_2O_2 and Cu(II)	45°C, darkness	8846	No
16	NA	3 days old	none	45°C, darkness	low	No
17	NA	3 days old	Cu(II)	45°C, darkness	low	No
18	NA	3 days old	H_2O_2	45°C, darkness	39018	Yes (MS)
19	NA	3 days old	H_2O_2 and Cu(II)	45°C, darkness	1111	Yes (MS)

1. Experiments 1–6 are the same as those in Table 1 but analyses reported here were made prior to the addition of (+)-catechin.
2. The peak areas (× 10^3) for ethyl tartrate in Experiments 1 and 3 were 147, in Experiments 2 and 4 were 4, and in Experiments 5 and 6 were 62.
3. All these solutions had frequent exposure to air. NA: not applicable.
4. Peak areas for hydrogen peroxide less than 20 × 10^3 (arbitrary units) are termed 'low'.
5. UV (210 nm) was used for the HPLC detection of glyoxylic acid in Experiments 1–6 and both UV (210 nm) and MS (91 m/z) for the remaining experiments. The detector responsible for the positive detection of glyoxylic acid is indicated in parentheses.

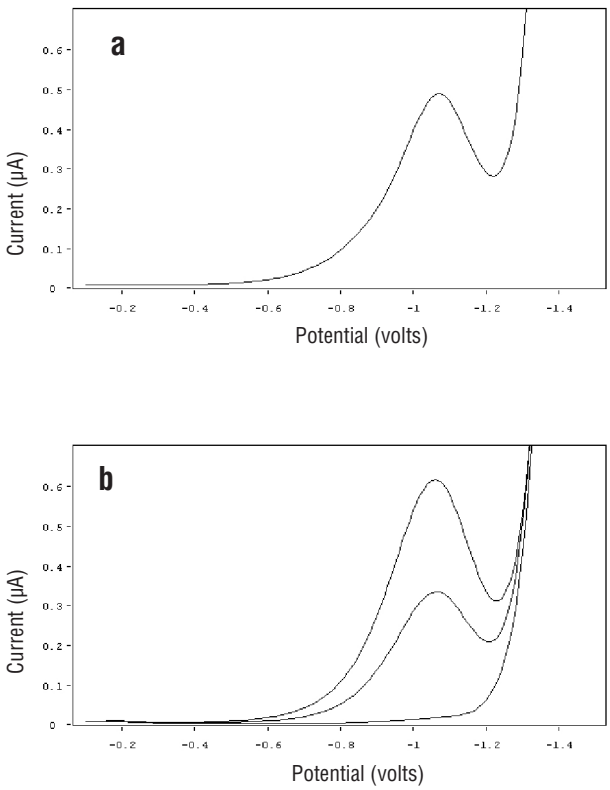


Figure 5. The square wave voltammetric analysis of an aqueous ethanol tartaric acid solution after exposure to sunlight (Experiment 8, Table 2) (**a**) and hydrogen peroxide standards of 0, 100 and 200 μM (**b**) prepared in the aqueous ethanol tartaric acid medium.

matogram although it was detected by LC/MS, indicating that its concentration was low.

The ability of copper(II) to inhibit the accumulation of the species responsible for the peak at 5.3 minutes was consistent with the action of copper(II) on hydrogen peroxide. Copper(II) is able to remove hydrogen peroxide by a Haber-Willslätter mechanism that proposes the production of reactive hydroxyl radicals (Haber and Willslätter 1931, Wardman and Candeias 1996). As hydrogen peroxide is often implicated in oxidation mechanisms, the ability of hydrogen peroxide to be detected in the 210 nm chromatograms of experimental samples was investigated. When hydrogen peroxide was added to freshly prepared aqueous ethanol tartaric acid and the solution analysed, the chromatogram showed a peak eluting prior to tartaric acid at a retention time consistent with the peak at 5.3 minutes. A peak with the same retention time could be generated for hydrogen peroxide in water. In both water and the aqueous ethanol tartaric acid solutions the peak at 5.3 minutes increased in proportion to the concentration of added hydrogen peroxide. This suggested that the unknown peak generated in the aqueous ethanol tartaric acid solution may have been due to hydrogen peroxide.

A square wave voltammetric analysis of the sample from Experiment 8 (Table 2) was conducted in order to confirm that hydrogen peroxide was indeed present. The resulting square wave voltammogram (Figure 5a) contained a peak at –1.08 V, corresponding to that for standards prepared by adding hydrogen peroxide to the aqueous ethanol tartaric acid solution (Figure 5b). Using a calibration curve constructed by incremental additions (100 μM) of hydrogen peroxide to freshly prepared aqueous ethanol tartaric acid solution, the concentration of hydrogen peroxide in the sample from Experiment 8 (Table 2) was calculated to be 140 ± 40 μM (n = 2).

These data confirm that the chromatography peak eluting at 5.3 min is attributable to hydrogen peroxide, the spontaneous production of which is assumed to result from the exposure of an aqueous ethanol tartaric acid solution to sunlight. The accurate quantification of hydrogen peroxide by analysis of the peak eluting at 5.3 min is the subject of further study, as it is somewhat surprising that hydrogen peroxide would chromatograph under the conditions employed here. For the purpose of the present work, the peak at 5.3 minutes was used only in a semi-quantitative fashion to indicate gross differences between the hydrogen peroxide concentrations in various reaction systems. The area of the peak at 5.3 minutes was termed low (Table 2) when the magnitude of the peak was below 20×10^3, as at these lower magnitudes the background signal interfered with accurate quantification of the peak.

Experiments 10 and 11 (Table 2) were conducted to investigate the effect of an absence of ethanol on the production of glyoxylic acid and hydrogen peroxide in tartaric acid solutions that were exposed to sunlight. Once again, glyoxylic acid was detected, regardless of the presence or absence of copper(II), however the peak area for hydrogen peroxide was much larger when copper(II) was not present.

The mechanism responsible for the production of hydrogen peroxide in the tartaric acid buffered solutions is not certain. Past work (Holroyd and Bielski 1978, Bielski and Gebicki 1982) has demonstrated the production of superoxide radicals, which disproportionate to form hydrogen peroxide, in aqueous and aqueous ethanol solutions that were subjected to vacuum-UV photolysis. However, the 160–180 nm wavelengths used in these published studies are much shorter than the wavelengths expected to impinge upon the samples exposed to sunlight. Irrespective of the mechanism responsible for the production of hydrogen peroxide, exposure to UV light is known to degrade hydrogen peroxide into hydroxyl radicals (Legrini et al. 1993) that could consequently oxidise tartaric acid.

The results of this work suggest that the storage conditions of aqueous ethanol tartaric acid solutions, including both sunlight exposure and frequent aeration, are conducive to the production of glyoxylic acid, and this would account for the enhanced level of absorbance at 440 nm for Experiments 1 and 3 (Table 1). Irrespectively, the inability to detect glyoxylic acid in some aqueous ethanol tartaric acid solution may be due either to the low levels of glyoxylic acid produced or as a consequence of its instability in this medium. For instance, Baraud (1954) suggested the oxidation of glyoxylic acid in the presence of molecular oxygen and iron(III). Furthermore, it is also known that hydrogen peroxide

readily oxidises glyoxylic acid to formic acid (Leitzke et al. 2001). To detect glyoxylic acid formed in the presence of hydrogen peroxide, the rate of glyoxylic acid production is required to be in excess of the rate of oxidative removal of glyoxylic acid. The glyoxylic acid concentration would be expected to fluctuate in the aqueous ethanol tartaric acid solution depending on the intensity and period of sunlight exposure, as this is the mode by which hydrogen peroxide is produced. Currently, conditions conducive to the formation and stability of glyoxylic acid in model white wine solutions are being investigated. Although no formic acid was observed in the LC/MS analyses of any of the solutions studied here, the chromatographic conditions were not optimised for this analyte and therefore any formic acid present may have escaped detection.

Tartaric acid solutions with added hydrogen peroxide

As a link seemed to exist between the presence of hydrogen peroxide and glyoxylic acid in tartaric acid solutions exposed to sunlight, the ability of hydrogen peroxide to promote glyoxylic acid formation was investigated. The influence of both ethanol and copper(II) on the production of glyoxylic acid from various tartaric acid solutions was also investigated. Experiments 12–19 (Table 2) involved the heating of tartaric acid solutions in darkness at 45°C for 3 days in the presence and absence of copper(II), ethanol and hydrogen peroxide.

From Experiments 12–15 (Table 2) it is clear that glyoxylic acid could not be detected in the presence of ethanol despite the addition of 0.6 mg/L copper(II) and/or 0.001 M hydrogen peroxide. Furthermore, apart from the expected peaks due to tartaric acid, ethyl tartrate and hydrogen peroxide, no other peak of sufficient peak area to allow confidence in assignment was observed in the chromatograms made with detection at 210 nm for any of these samples (Experiments 12–15, Table 2). In the chromatograms generated from Experiments 14 and 15 (Table 2), the peak attributed to hydrogen peroxide was considerably smaller in the presence of copper(II), confirming earlier observations.

In contrast, in the experiments conducted in the absence of ethanol (Experiments 16–19, Table 2) the presence of glyoxylic acid was detected by MS in the samples containing added hydrogen peroxide. Also, in the chromatograms made with detection at 210 nm on the solutions from Experiments 18 and 19 (Table 2), the peak attributed to hydrogen peroxide was again considerably smaller in the presence of copper(II) (Table 2).

A comparison of the results from Experiments 14 and 18 (Table 2) suggests that ethanol is capable of inhibiting the accumulation of glyoxylic acid. From previous work (Clark and Scollary 2002), a link between the production of glyoxylic acid and the presence of hydroxyl radicals has been suggested. During the heating of hydrogen peroxide, the thermolysis of hydrogen peroxide may allow the production of hydroxyl radicals. A possible mechanism for the inhibition of glyoxylic acid production by ethanol may involve oxidation of the latter by the hydroxyl radical in preference to tartaric acid due to the far greater concentration of ethanol in the aqueous ethanol tartaric acid medium. The net product in the reaction between ethanol and hydroxyl radicals is acetaldehyde (Kaneda et al. 1988). Such an outcome – that is, the consumption of a radical and its replacement by a comparatively stable compound – may explain the ability of ethanol to act as a radical quencher.

As hydrogen peroxide is a relatively stable peroxide, especially compared to alkoxy peroxides, the rate of thermolysis at 45°C would be expected to be low. This is consistent with the small amounts of glyoxylic acid produced in the ethanol-free medium to which hydrogen peroxide had been added. The increased levels of glyoxylic acid produced in the sunlight exposed tartaric acid samples (Experiments 8 and 10, Table 2) may have been as a consequence of the greater production of radicals in the sunlight-induced rather than thermolysis-induced degradation of hydrogen peroxide.

Although the production of glyoxylic acid was observed in several samples to which hydrogen peroxide had been added, it only occurred in those with properties that are not generally applicable to wine; that is, in ethanol-free samples at elevated hydrogen peroxide concentrations. With regard to hydrogen peroxide, a suggested source in wine and juice is its production as a by-product of catecholate autoxidation (Singleton 1987). However, in aqueous ethanol tartaric acid solutions such as those in Table 2, which do not contain catecholate-containing compounds, there is no obvious source of hydrogen peroxide. This is consistent with the inability to detect appreciable amounts hydrogen peroxide formed in the aqueous ethanol tartaric acid solution, or its ethanol-free variant, when heated in darkness at 45°C (Experiments 12 and 16, Table 2).

No direct link was observed between the presence of copper(II) and glyoxylic acid production and accumulation in the aqueous ethanol tartaric acid medium. The main role of copper(II) appeared to be both accelerating the removal of added hydrogen peroxide and decreasing the amount of hydrogen peroxide accumulated in the presence of sunlight. However, if hydroxyl radicals were the sole factor responsible for glyoxylic acid production from the oxidation of tartaric acid, then more glyoxylic acid would have been formed in the presence of copper(II) and this was not the case. It appears therefore that the production of glyoxylic acid involves a multi-step mechanism involving more than just the participation of radical species.

In regard to the production of pigments in the model white wine, it appears that copper(II) may influence another stage in the mechanism for the production of xanthylium cations rather than only the production of glyoxylic acid from tartaric acid. This is supported by the results of Experiments 1 and 3 (Table 1), wherein increased amounts of xanthylium cations were produced in the presence of copper(II) despite the likelihood that the initial concentration of glyoxylic acid in these samples was identical. Further studies on the effect of copper(II) on the formation of the xanthylium cations are currently in progress.

Conclusion

The results show the need for careful and reproducible conditions when attempting to model the oxidation of white wine. As observed in this study, what appears to be a simple parameter, the age of the aqueous ethanol tartaric acid solution, has a significant impact on the extent of oxidative browning. This oxidative browning is likely to be a consequence of the sunlight-induced oxidation of an aerated tartaric acid solution that led to the formation of glyoxylic acid, a known precursor of the xanthylium cation pigments. There seems to be a link between glyoxylic acid and hydrogen peroxide that is more apparent in the presence of sunlight, but the exact nature of this relationship is uncertain at this stage. These results have implications for the potential link between random oxidation and browning of white wine, as oxygen ingress and light exposure are conditions conducive to glyoxylic acid formation.

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