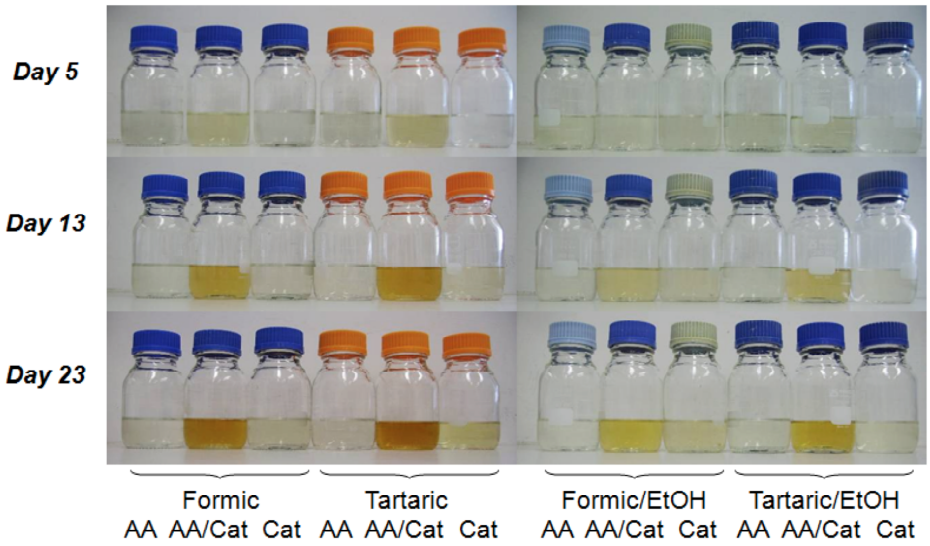
NATIONAL Wine & Grape INDUSTRY CENTRE

Australian Government
Grape and Wine Research and Development Corporation

Chemistry of sulfur dioxide and ascorbic acid as an anti-oxidant system in white wine



FINAL REPORT to
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Chemistry of sulfur dioxide and ascorbic acid as an anti-oxidant system in white wine

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ABSTRACT

This project has examined in detail the chemistry of ascorbic acid and sulfur dioxide as an antioxidant system in conditions relevant to white wine. Model wine systems were utilised in order to gauge the impact of a variety of wine components on the chemistry of ascorbic acid and its degradation products. A variety of analytical and organic chemistry techniques were employed to monitor, isolate and identify products arising from ascorbic acid itself and in combination with wine components. The main degradation products of ascorbic acid were identified and the mechanisms by which ascorbic acid may induce or inhibit oxidative colouration were explained. Importantly a precursor to pigment production, formed from a degradation product of ascorbic acid and a catechin-type phenolic compound, has been identified. The impact of a variety of wine components, including sulfur dioxide, on the reaction products of ascorbic acid was established. In the presence of metal ions, the consumption ratio of ascorbic acid to either free or total sulfur dioxide was 1:1 indicating that, other than hydrogen peroxide, the degradation products of ascorbic acid were not binding significantly to sulfur dioxide. The winemaking implications of these results are discussed and recommendations for future work outlined.

EXECUTIVE SUMMARY

Ascorbic acid is well known for its use as a complementary antioxidant to sulfur dioxide in white wine. However, the fate of ascorbic acid and its degradation products that are formed under wine conditions are not well understood. Furthermore, there appears to be some ambiguities in the role of ascorbic acid as an antioxidant, particularly in its role in preventing the oxidative colouration of white wine. Such oxidative colouration usually results in the conversion of the white wine from its normal pale yellow or straw colour to a darker yellow or even brown colour.

This project was conducted to gain more insight into the chemistry of ascorbic acid and its degradation products under conditions relevant to white wine. The extensive chemical literature on ascorbic acid was surveyed and a comprehensive review written on ascorbic acid and its chemistry in a wine-like environment. This review is the first cohesive overview of published work on ascorbic acid with the specific focus of its chemistry in wine.

Experiments were conducted on model wine systems utilising a range of predominantly analytical and organic chemistry techniques. Several of the main terminal degradation products of ascorbic acid, in the model wine system adopted, were identified as 3-hydroxy-2-pyrone, 2-furoic acid and furfural. The mechanism for the production of these compounds was identified and involved an oxidative pathway for the first two compounds and an oxygen-independent pathway for the latter.

The manner by which ascorbic acid contributes to colour development in the absence of sulfur dioxide was also explained in the model wine system containing catechin-type phenolic compounds. Whilst present in solution, ascorbic acid induced a faint green/yellow colouration that was due to its own degradation products. In this period, ascorbic acid did indeed prevent the production of any (+)-catechin-derived coloured compounds that would have otherwise formed. However, once the ascorbic acid was fully depleted, pigmented phenolic compounds (i.e. (+)-catechin-derived) were generated at an accelerated rate via two mechanisms. One stemmed from the hydrogen peroxide generated from ascorbic acid, whilst the other depended on an aldehyde degradation product of ascorbic acid (i.e. xylosone). Both of these mechanisms could generate the same yellow xanthylium cation pigments. In the case of the mechanism involving xylosone, an intermediate compound was isolated, characterised and reported for the first time.

The complexity of the model wine system was increased to observe the impact of a range of components on the oxidative pigment production. Importantly, the presence of a non-flavonoid phenolic compound (i.e. caffeic acid), normally extracted into wine from the pulp of the grape (albeit in an esterified form with tartaric acid), had an important impact on the colour of the model wine system. Although it only had a small impact on colour formation when it was the sole phenolic compound in the model wine system, it induced red colouration when in the presence of (+)-catechin. This red colouration in combination with the yellow xanthylium cation pigments meant that the presence of caffeic acid led to the formation of yellow/red or orange colour in the model wine system as it became oxidised. Once again, ascorbic acid was efficient at preventing the production of phenolic pigments while it remained in solution, but then led to accelerated production of the yellow/red pigments once it was depleted. This latter effect was attributed in part to the ability of ascorbic acid to induce the oxidative cleavage of caffeic acid, and the subsequent aldehydic product reacting with (+)-catechin to provide a red coloured compound.

The efficiency of ascorbic acid to inhibit pigment formation was compared to erythorbic acid,

the latter being an isomer (i.e. diastereoisomer) of ascorbic acid. Certain advantages were observed for erythorbic acid compared to ascorbic acid, including the accelerated oxidative decay of erythorbic acid, implying that it may be reacting more efficiently with either oxygen and/or hydrogen peroxide. Erythorbic acid also induced the formation of less phenolic pigments once it was fully degraded in the model wine system. The complexity of the oxidation chemistry was apparent from the significant impact of the specific flavanol/antioxidant combinations on the rate of oxidative colouration intensity in the model wine system.

Ascorbic acid was shown to be significantly less efficient at scavenging oxygen at low metal concentrations. This result places the metal ion content in wine in a new light and may mean that speciation of metal ions in wine may provide insight into the efficiency of ascorbic acid as an oxygen scavenger in a particular wine. Furthermore, based on the consumption ratios of sulfur dioxide to ascorbic acid, it was found that dehydroascorbic acid, and its subsequent degradation products, are not significant binders of 'free' sulfur dioxide. This in turn meant that the amount of 'free' sulfur dioxide consumed per oxygen consumed is lower in the presence of ascorbic acid. Also, provided that the sulfur dioxide to ascorbic acid ratio was high (i.e. 2:1), sulfur dioxide significantly inhibited the production of pigmented phenolic compounds.

The ability of glutathione to aid sulfur dioxide in scavenging the degradation products of ascorbic acid was investigated. The results showed that provided the glutathione concentration is sufficiently high, it can indeed bind the aldehydic degradation products involved in pigment production. Furthermore, glutathione inhibited the formation of these pigments when incubated in pigment precursor solutions. During storage of the model white wine in oxidising conditions, the presence of glutathione at high concentrations led to a protective effect against oxidative colouration followed by a period of accelerated colouration. Further work is required to assess at what glutathione concentrations the inhibition of oxidative colouration is significant.

The majority of these results were obtained by Ms Célia Barril at the National Wine and Grape Industry Centre (NWGIC) as her PhD studies, which formed a cotutelle arrangement between AgroParisTech (Paris) and Charles Sturt University (Wagga Wagga). The studies were predominantly funded by Australia's grapegrowers and winemakers through their investment body the Australian Grape and Wine Research and Development Corporation (GWRDC). Some additional funding for the work was also contributed by the NWGIC/Charles Sturt University. Collaborative work was performed by Assoc. Prof. Peter Karuso from Macquarie University, and Dr Julie Niere and Dr Daniel Dias from the Royal Melbourne Institute of Technology. Visiting research students from The University of Bologna (Italy), Forschungsanstalt Geisenheim (Germany), and The University of Poitiers (France), also participated in the research.

BACKGROUND

The browning of white wines is not a new phenomenon, but remains one of the major problems experienced by the wine industry. The colour alteration resulting from browning is often accompanied by an off-flavour development, as well as a loss of fresh bouquet of the wine. The image of the wine industry depends upon the production of wine free from quality defects and consequently, considerable attention has been paid to understanding the chemical processes that cause white wine browning.

There are two discrete processes that have been examined: enzymatic and non-enzymatic browning. In the enzymatic browning process, polyphenoloxidase enzymes convert *o*-diphenols to *o*-quinones that, in turn, react with a range of wine components to give brown compounds (Singleton 1987). The on-set and extent of enzymatic browning is assessed by an increase in absorbance at 420 nm (Iland et al. 2000). It is often assumed that the non-enzymatic browning process is identical to the enzymatic process, but recent work on modelling the non-enzymatic process has shown that xanthylium cations are formed through an oxidative bridging process involving the phloroglucinol ring and not the catechol ring of (+)-catechin (Es-Safi et al. 1999a, 1999b, 2000, Clark and Scollary 2002). The xanthylium cations absorb in the 420 – 440 nm range, similar to that found in the enzymatic browning process.

Protection of a wine against spoilage is generally achieved through sulfur dioxide's ability to act as an anti-microbial agent, as an antioxidant and as a destabiliser of disulfide bridges in enzymes, the latter inhibiting enzymatic browning processes (Chapon et al. 1982, Sapers 1993). The anti-microbial activity of sulfur dioxide depends on the availability of molecular SO_2, as this is the only form of sulfur dioxide showing any effect on microbial activity (Zoecklein et al. 1995). Sulfur dioxide's antioxidant role mainly relates to its ability to scavenge hydrogen peroxide, with a more minor role in scavenging radicals and molecular oxygen. In addition, sulfur dioxide can bind acetaldehyde, known for its capacity to bridge phenolic compounds, a precursor step in some browning processes (Chapon et al. 1982, Sapers 1993, Zoecklein et al. 1995).

The widespread use of sulfur dioxide has led to some criticism of wine as a life style beverage as sulfur dioxide has been linked to severe allergy-like reactions in some asthmatics (Sapers 1993, Zoecklein et al. 1995, Rose 1993). While no ready replacement for the anti-microbial role of sulfur dioxide was available, it was argued (Sapers 1993, Bauernfeind and Pinkert 1970) that ascorbic acid and its optical isomer, erythorbic acid, had the potential to replace sulfur dioxide as the antioxidant in white wine.

However, complete replacement of sulfur dioxide by ascorbic acid in white wine is not recommended as the aerobic oxidation of ascorbic acid produces hydrogen peroxide, considered to be a major oxidant in wine (Zoecklein et al. 1995, Bradshaw et al. 2002, Rankine 1989, Sansal and Somer 1997, Zambonin and Losito 1997). Rather, ascorbic acid is used as a complement to sulfur dioxide as both molecular sulfur dioxide and hydrogen sulfite, the two active forms of sulfur dioxide at wine pH, are both capable of quenching hydrogen peroxide (Zoecklein et al. 1995, Bauernfeind and Pinkert 1970, Rankine 1989, Ough 1992, Peng et al. 1998).

The combined use of ascorbic acid and sulfur dioxide therefore requires considerable care as oxidation of ascorbic acid leads to a loss of sulfur dioxide that, in turn, leads to a reduction in the main anti-microbial component.

There is, however, consistent questioning regarding the complementary role of ascorbic acid and sulfur dioxide as an anti-oxidant system in white wines. Increasing evidence, both published and anecdotal, indicates that ascorbic acid can act as a pro-oxidant leading to elevated levels of oxidative spoilage, even in the presence of sulfur dioxide. Marks and Morris (1993) reported there was no benefit from including ascorbic acid in wines where sulfur dioxide had already been used. In fact, wine samples subjected to accelerated browning, through increased temperature and high levels of oxygen, demonstrated that treatments with ascorbic acid ultimately had the highest potential for browning.

Bradshaw et al. (2001) have demonstrated that the addition of ascorbic acid to a wine-like system resulted in an increased rate of browning of (+)-catechin, the oxidisable polyphenolic compound used in the model system. The onset of this ascorbic acid-induced browning of (+)-catechin was found to be dependent on the presence of oxygen, although past the 'lag period', the process was independent of oxygen. Further, Bradshaw et al. (2001), have questioned whether hydrogen peroxide is as strong a colourant precursor in wine-based systems as has been previously thought.

Similar observations to Bradshaw et al. (2001) were noted by Peng et al. (1998, 1999) who reported that the addition of ascorbic acid to either model wine systems or white wines resulted in an initial decrease in both the brown colour and the redox potential of the samples. However, after a short period of time, a reversal of the effects was observed, with a reported increase in the browning as well as the redox potential of the samples. Further, the presence of oxygen was found to enhance the browning phenomenon, while the addition of sulfur dioxide alone led to a reduced rate of spoilage of the wine samples. Intriguingly, Peng and co-workers claimed that ascorbic acid promoted the browning of wine regardless of the concentration of sulfur dioxide used.

Buettner and Jurkiewicz (1996) have suggested that ascorbic acid may demonstrate a "crossover" effect depending upon the level of ascorbic acid. At higher concentrations, ascorbic acid acts as an antioxidant, while at lower concentrations, a pro-oxidant influence is exhibited. The crossover effect was proposed to result from the production of radical species from the oxidation of ascorbic acid (Buettner and Jurkiewicz 1996).

In our work (GWRDC/UM96/1), we have demonstrated that the addition of ascorbic acid resulted in an initial inhibition of browning followed by an enhanced rate of browning for a model wine system containing the oxidisable polyphenolic substrate (+)-catechin (Bradshaw et al. 2001) as measured by the absorbance at 440 nm (A_{440}).

From the aerobic degradation of ascorbic acid (Equation 1), it is expected that the ratio of ascorbic acid decay to sulfur dioxide consumption will be around 1:2, assuming that hydrogen peroxide and dehydroascorbic acid (or its degradation products), will each consume one molar equivalent of sulfur dioxide. It has been reported that the actual molar ratio of ascorbic acid to sulfur dioxide depletion in an aerated model wine system at 45 °C, without added metal ions, was 1:1.7 (Bradshaw et al. 2004). The study by Bradshaw et al. (2004) did not provide a measure of bound hydrogen sulfite and therefore it was not possible to gain further insight into the mechanisms by which sulfur dioxide is consumed (i.e. reversible versus irreversible binding).

However, the reported ratio of 1:1.7 does suggest that a similar amount of sulfur dioxide is utilised upon the ingress of a set amount of oxygen into white wine regardless of the presence of ascorbic acid. Such a result is consistent with the sulfur dioxide levels in Riesling and Chardonnay wines, bottled for three-years with and without ascorbic acid that showed slightly higher sulfur dioxide concentrations in the wines to which ascorbic acid was added at

bottling (Skouroumounis et al. 2005). On the other hand, in the presence of ascorbic acid, the rate of sulfur dioxide consumption would be expected to increase for a set amount of oxygen ingress into the wine. This is due to the ability of ascorbic acid to react with oxygen more rapidly than wine phenolic compounds and hence increase the rate of production of hydrogen peroxide, which in turn would be scavenged by sulfur dioxide.

At a sulfur dioxide to ascorbic acid mole ratio of 0.8:1.0, sulfur dioxide extended the delay prior to the onset of (+)-catechin browning as measured by the increase in absorbance at 440 nm from 2 to 4 days. The lengthening of the pre-browning period was correlated with an increase in the time required to oxidise ascorbic acid completely, the sequence of events being loss of sulfur dioxide (Day 3), loss of ascorbic acid (Day 4) and on-set of (+)-catechin browning (Day 4). Increasing the sulfur dioxide to ascorbic acid mole ratio to 3:1 inhibited the onset of (+)-catechin browning over the 14 day reaction period.

Intriguingly, sulfur dioxide alone (that is, in the absence of ascorbic acid) totally inhibited the browning of (+)-catechin. Therefore, there is an apparent contradiction that must be resolved in a winemaking context. That is, sulfur dioxide with limited antioxidant capacity inhibits the browning of (+)-catechin whereas ascorbic acid, at least when fully degraded, enhances the degree of (+)-catechin browning.

There is a clear need to understand the sulfur dioxide / ascorbic acid reaction system to allow guidelines for their proper use in winemaking to be established. It is known that dehydroascorbic acid undergoes further reaction in the model wine base (Bradshaw et al. 2001), but its breakdown products in this matrix have not been identified. There have been several studies on the degradation of ascorbic acid and dehydroascorbic acid in aqueous solutions, some at a pH relevant to wine, but not in the presence of ethanol. Recently Yuan and Chen (1998) reported that at low pH, furfural, 2-furoic acid and 3-hydroxy-2-pyrone are formed from ascorbic acid and previously Niemela (1987) identified threonic, oxalic and threo-2-pentulosonic acids as the major oxidative degradation products. The role of ethanol in the formation of some degradation products of ascorbic acid cannot be ignored, especially as it has been shown to combine with ascorbic acid to form sotolon (König et al.1999).

It is well known in wine chemistry that sulfur dioxide binds to aldehydic functional groups and many of the degradation products of ascorbic acid possess an aldehydic functional group capable of binding sulfur dioxide. It must also be noted that glyoxylic acid, the initiator of xanthylium cation formation (Es-Safi et al. 1999a, 1999b, 2000, Clark and Scollary 2002) contains an aldehydic functional group. Binding of sulfur dioxide to one or more of these aldehydic compounds would contribute to the loss of sulfur dioxide. Alternatively, if a radical species is formed during the oxidation of ascorbic acid, as proposed by Buettner and Jurkiewicz (1996), then a radical cascade reaction could be responsible for the rapid consumption of sulfur dioxide.

PROJECT AIMS AND PERFORMANCE TARGETS

The original objectives and outputs of this project were the following:

OBJECTIVES:

- To examine the chemistry of ascorbic acid and sulfur dioxide as an antioxidant system in a model white wine system.
- To establish a chemical basis for the enhanced loss of sulfur dioxide in the presence of ascorbic acid.
- To apply the results from model studies to the behaviour of ascorbic acid and sulfur dioxide in white wines.
- To propose criteria for the proper use of ascorbic acid and sulfur dioxide in white wine production.

Outputs and Performance Targets April – June 2005

Outputs	Performance Targets
1. PhD candidate	Candidate to commence on April 1, 2005
2. Project design	Project design completed by June, 2005
3. Literature review	Literature review commenced by May 2005
4. Model studies	Chromatographic studies commenced in May 2005

Outputs and Performance Targets July 2005 – June 2006

Outputs	Performance Targets
1. Literature Review	Literature review completed by August 2005
2. White wine trials	First trials in vintage 2006
3. Model studies	Degradation products of ascorbic acid separated and characterised by April 2006
4. Chemistry of SO_2/ascorbic acid	Study of the interaction between ascorbic acid degradation products and sulfur dioxide commenced by May 2006

Outputs and Performance Targets July 2006 – June 2007

Outputs	Performance Targets
1. Wine ageing study:	Comparison of bottles versus barrels completed by June 2007
2. White wine trials	Second trials in vintage 2007
3. Chemistry of SO_2/ascorbic acid	Study of the interaction between ascorbic acid degradation products and sulfur dioxide completed by May 2007
4. Publications	One refereed publication and one technical paper by June 2007

Outputs and Performance Targets July 2007 – March 2008

Outputs	Performance Targets
1. Winemaking guidelines	Guidelines completed and published by March 2008
2. White wine trials	Completion from vintage 07
3. Publications	One technical and one refereed publication by March 2008
4. Conference presentation	AWITC in July, 2007; *In Vino Analytica Scientia*, July 2007
4. Final report	Final report submitted by March 2008
5. PhD thesis	PhD thesis submitted by March 2008

A number of variations were approved by GWRDC during the project lifetime that included:

1. Variation in original project start and termination dates, and variation in output and performance targets (see GWRDC confirmation letter of 11 August 2005)

2. Variation in project end date, 6-month extension to PhD stipend payment, and change of project supervisor (see GWRDC confirmation letter of 6 March 2008)
3. Variation in project end date and mode of enrolment of PhD student (i.e. full-time to part-time). Variation in planned project outputs – Letter from GWRDC 11 September 2008.
4. Variation in project end date (see GWRDC confirmation letter of 11 June 2008).
5. Variation in project end date (see GWRDC confirmation letter of 2 March 2010).

Based on these project variations, the new project end date became 31 March 2010, and approval was granted for revised project outputs. This included additional publications on the chemistry of molecular oxygen, ascorbic acid and sulfur dioxide in model wine systems instead of the white wine trials (i.e. 05 to 08 above). The delay and eventual inability to conduct the white wine trials was highlighted in the progress reports from 05/06 to 08/09.

Based on this, the planned outputs in place of those for July 07 to March 08 (above) became:

OUTPUTS	PERFORMANCE TARGETS
Winemaking guidelines	No longer part of revised outputs
White wine trials	No longer part of revised outputs
Publications	1 technical and 3 referred publications
Conference presentations	AWTIC in July, 2007; In Vino Analytica Scientia, July 2007 and July 2009
Final report	Final report submitted 31 March 2010
PhD thesis	PhD submitted April 2010

The outputs above are discussed throughout the following sections of this report.

METHOD

The oxidation of white wine was predominantly followed by employing model wine systems. The composition of the model wine system was varied to establish the impact of various components on the wine oxidation process, however, the main model wine systems adopted consisted of a 12% aqueous ethanol solution, buffered with tartaric acid and adjusted to pH 3.2. The tartaric acid-buffered solution was prepared by dissolving 2.09 g of potassium bitartrate and 0.990 g of potassium hydroxide in 1 L of 12% (v/v) aqueous ethanol, resulting in a tartaric acid concentration of 0.011 M and a potassium concentration of 0.026 M (1 g L^{-1}). The pH of the buffered solution was adjusted to 3.2 with 10% (v/v) sulfuric acid using a Cyberscan 510 ion pH meter and a EUTECH Instruments pH electrode.

Additional components or variations to the model wine system are discussed throughout the report and include ascorbic acid, sulfur dioxide, the wine phenolic compounds caffeic acid and (+)-catechin, glutathione, the metal ions copper and iron as well as formic acid-buffered model wine systems and the presence or absence of ethanol.

The analytical techniques employed to measure the model wine solution and its components during storage were numerous and diverse. It mainly included spectroscopy (UV/Vis, NMR, colorimetric), and chromatographic (HPLC, LC-MS, LC-H^1-NMR) techniques. The details of the specific analysis methods are included in the attached papers at the end of this report.

RESULTS AND DISCUSSION

The results of this project and their interpretation are presented in this section as independent studies. The interaction between the studies is described in the project outcomes and conclusions.

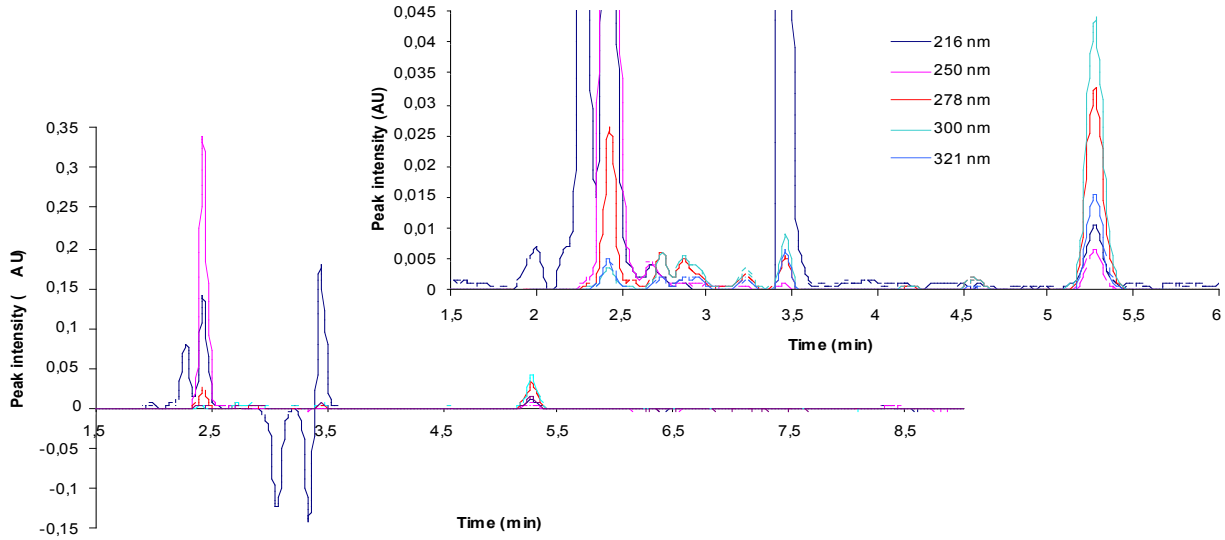
1. The degradation of ascorbic acid in a model wine: identifying the degradation compounds

Bradshaw (GWRDC/UM96/1) described the decay of ascorbic acid, proposing that one or more of the degradation compounds of ascorbic acid may be responsible for the observed enhanced browning of catechin-model wine systems containing ascorbic acid. In this Section, we describe approaches to identifying the degradation products.

The degradation conditions employed in the previous GWRDC report (UM96/1) consisted of 0.5 g/L ascorbic acid dissolved in a model wine system, containing 0.011M tartaric acid and 0.026M potassium in a 12% (v/v) aqueous ethanol solution, buffered to a pH of 3.20. The solution was left to react in darkness at 45 °C, in order to model accelerated ageing conditions, and aerated on a daily basis to replenish the oxygen content and achieve oxygen-saturated atmosphere.

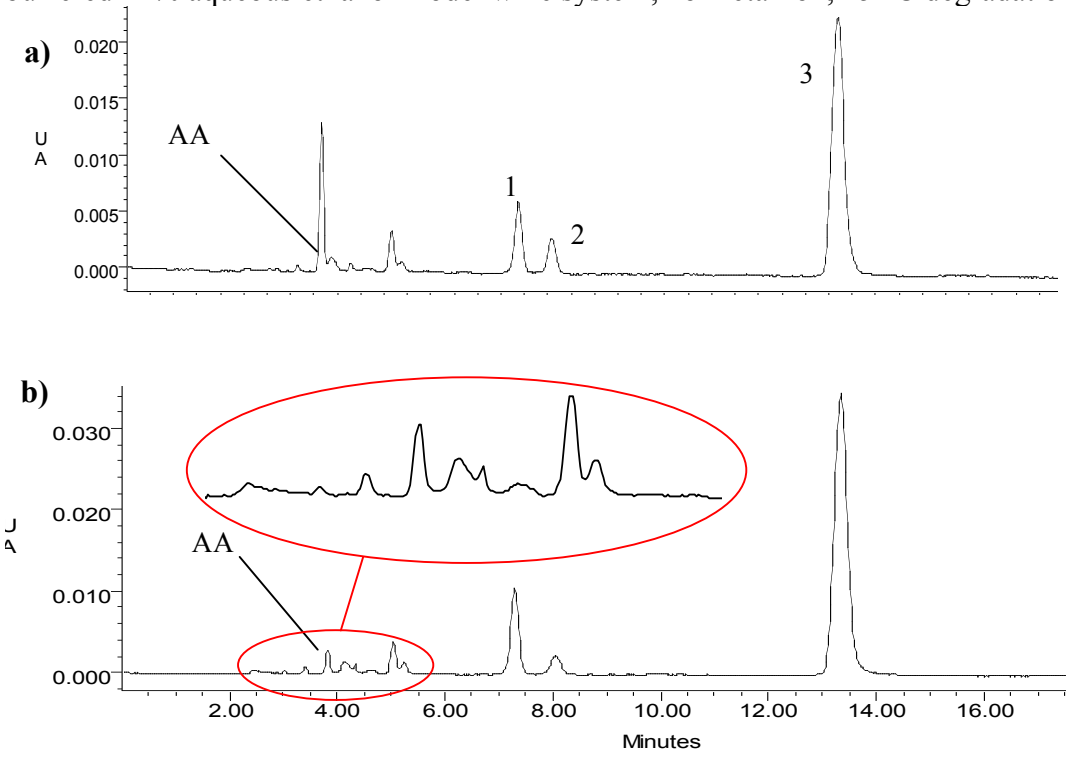
The first step consisted in the optimisation of the chromatographic method so the peak separation was improved and allowed better monitoring of single peaks and possible fraction collection. Figure 1.1 shows the chromatograms that could be obtained with the chromatographic method employed in the GWRDC CSU01/02 report. It is evident from Figure 1.1 that many peaks are not well defined.

Figure 1.1 Chromatogram at various wavelengths of an ascorbic acid solution after 2 days of degradation under accelerated ageing conditions. [AA] = 0.5 g/L, tartaric acid-buffered 12% aqueous ethanol model wine system, no metal ion, 45 °C degradation.



In order to improve the chromatographic detection, factors such as the polarity of the mobile phase, the acid strength and the gradient ramp were investigated by injecting an ascorbic acid solution after 3 or 4 days degradation under accelerated ageing conditions. Figure 1.2 shows chromatograms (C18 reverse phase column, 280 nm detection) with the best separation that could be achieved, although there are still peaks at the beginning of the chromatogram which are not well defined (Figure 1.2b).

Figure 1.2 Chromatogram at 280 nm of an ascorbic acid solution after a) 3 days and b) 4 days of degradation under accelerated ageing conditions. [AA] = 0.5 g/L, tartaric acid-buffered 12% aqueous ethanol model wine system, no metal ion, 45 °C degradation.



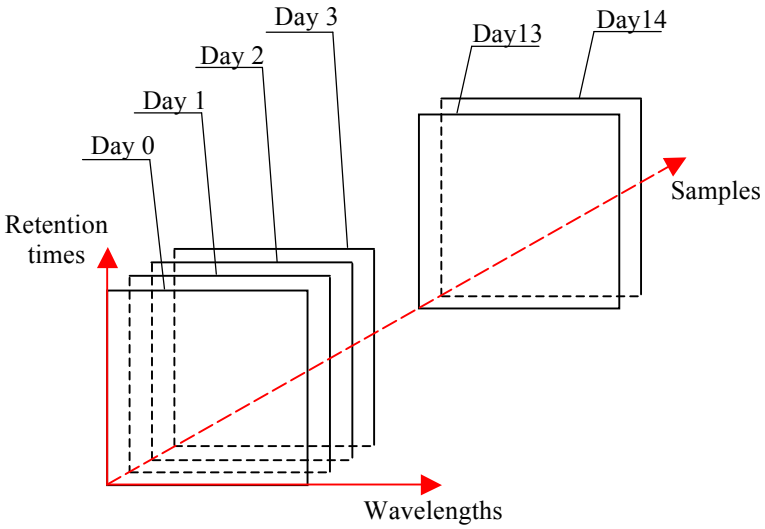
On day 3, ascorbic acid is still present (labelled AA in Figure 1.2a) while several other peaks can also be observed, indicating the extensive degradation of ascorbic acid that has occurred. On day 4 (Figure 1.2b), most of the ascorbic acid has decayed and the other peaks have increased in intensity. The later peaks (1, 2 and 3), the least polar degradation products, were the most stable over time and well separated under the conditions used and shown on the chromatograms. However, the group of peaks eluting early, most polar compounds, (encircled in Figure 1.2b) were not very stable over time and hard to monitor due to their co-elution. As a result, most of the degradation products of ascorbic acid could not be identified using this HPLC-DAD technique.

Before trying several other techniques to identify the ascorbic acid degradation products, the impact of the reaction media and accelerated ageing conditions on the degradation of ascorbic acid were assessed. A wide range of degradation factors were investigated, including:
- the degradation temperature: 20 or 45 °C;
- the initial ascorbic acid concentration: 0.5, 2.0 and 5.0 g/L or 2.8, 11.2 and 28 mM respectively;
- the supporting model wine buffer: tartaric acid or formic acid;
- the presence or absence of ethanol;
- the presence of absence of metal ions: iron(II) and/or copper(II).

In all model wine systems mentioned above, the same general chromatographic profile was observed. Among these conditions, only the supporting buffer showed little impact. The other factors, however, influenced the kinetics of formation and/or decay of the degradation products, but not the actual identity of the degradation products formed.
Due to the complexity of the ascorbic acid degradation, as shown by the chromatographic profiles, especially at the beginning of the run (Figure 1.1 and 1.2), chemometrics were applied to the chromatographic data to try and uncouple the co-eluted peaks and generate kinetic data for the formation and/or decay of the peaks.

Multi-way data are characterised by several sets of variables that are measured in a crossed fashion. Chemical examples could be any kind of spectrum measured chromatographically for several samples, such as UV-visible spectra measured by HPLC during 14 days of the ascorbic acid degradation in our case. Determining such variables will give rise to three-way data, *i.e.* the data can be arranged in a cube instead of a matrix as in standard multivariate data sets. Figure 1.3 represents the "retention-time-versus-wavelength-versus-sample" data, the retention times going up to 15 minutes, the wavelengths going from 200 to 500 nm and the samples being each of the 14 day injections.

Figure 1.3 Representation of the UV-visible data measured by HPLC-DAD for the ascorbic acid degradation products over 14 days of injection.

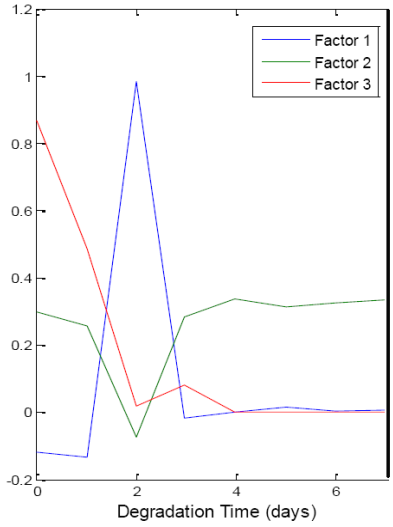


The PARAllel FACtor analysis (PARAFAC) is one of the several decomposition methods for multi-way data and is used, among other things, to decompose spectra into the contributions of individual source spectra, *i.e.* pure spectra can be recovered from multi-way spectral data. This factor analysis method is a generalisation of the Principal Component Analysis (PCA) to higher order arrays. PARAFAC and two-way PCA are respectively multi- and bi-linear decomposition methods, which decompose the array into sets of scores and loadings that hopefully describe the data in a more condensed form than the original data array. However, with three-way data, one cannot as in PCA estimate components successively as this will give a model with poorer fit, than if the simultaneous solution is estimated, giving a rather more robust, adequate and interpretable model. In addition, multi-way methods are less sensitive to noise.

A three-component, or three-factor (new variables) model was chosen to fit the initial chromatographic data (Figure 1.1). Each loading, factor coordinates, *i.e.* importance/weight of each variable in the principal components, were graphically represented for each mode (sample, retention time and wavelength) in order to assess the three factors to their corresponding meaning.

The plot of the sample loadings (Figure 1.4) shows the kinetic evolution of the three factors as the samples correspond to the injections of the ascorbic acid degradation solution day after day. The loadings are shown from day 0 to day 7 and remained consistent until day 14. The different evolutions of the three factors shown in Figure 1.4 allow the possibility for relating the different factors to the various compounds involved in ascorbic acid degradation.

Figure 1.4 Sample mode loading plot of the three factors used in the PARAFAC model.



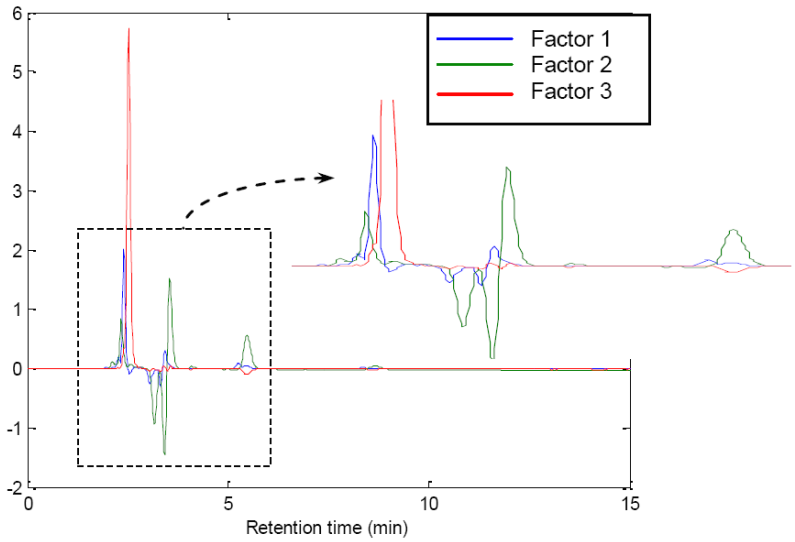
It seems that the factor 3 (in red, Figure 1.4), whose general trend is a total disappearance in four days, relates to the ascorbic acid starting reagent. This supposition is in accordance with previous kinetic observations (Bradshaw et al. 2003) that highlighted that 0.5 g/L ascorbic acid was totally degraded in four days at 45 °C.

Factor 2 (in green, Figure 1.4), which remains stable from the fourth day of degradation, may well correspond to ascorbic acid degradation products. However, these products seem to appear as soon as the degradation solution was prepared (day 0) as the factor is at a level equivalent to the final amount obtained when all the ascorbic acid has reacted (Day 4). The behaviour of the factor response for the day-2-sample does not follow the general trend and is discussed below.

Factor 1 (in blue, Figure 1.4), which shows a rise from day 1 to reach a maximum at day 2 and then a decrease down to day 3, might well reflect an intermediate degradation product of the ascorbic acid. There were however several experimental difficulties with the day-2 sample in this experiment that unfortunately minimises the effectiveness of placing a detailed interpretation of the behaviour of factor 1.

The retention time mode loadings were plotted for the three factors and this generated the chromatographic profiles represented in Figure 1.5. By comparing the retention time of the unique peak given by factor 3, 2.52 min (Table 1.1), it re-enforces that this factor stands for the ascorbic acid as it is very close to the actual ascorbic acid retention time observed on the chromatograms shown in Figure 1.1. This is in accordance with what was found on the sample mode plot (Figure 1.4).

Figure 1.5 Retention time mode loading plot of the three factors used in the PARAFAC model



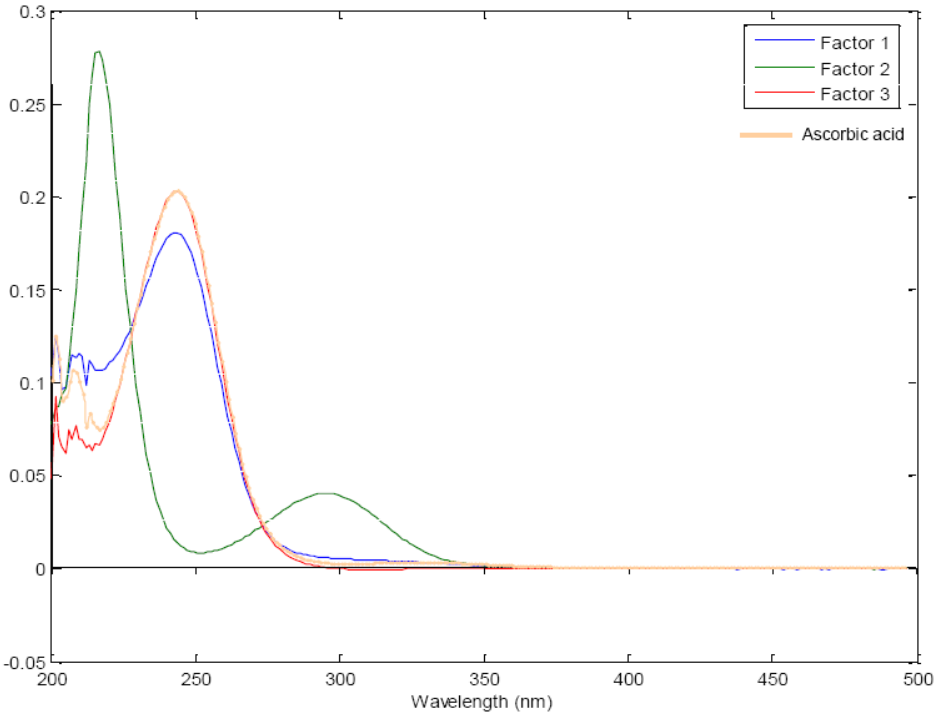
Factor 2 gave several peaks including two negative peaks, similar to those observed in the chromatogram represented in Figure 1.1 as well as some of the major degradation peaks at 45 °C. A comparison of the retention times is detailed in Table 1.1 showing that despite a systematic shift of about 10 seconds (except for peak at 5.49 minutes) the peaks shown by factor 2 rather match the major degradation products.

Table 1.1 Retention time comparison between the PARAFAC model and HPLC-DAD chromatogram in Figure 1.1.

Factor	Peak Rt (min)		Comments
	PARAFAC model	Chromatogram in Figure 1.1	
3	2.52	2.42	Ascorbic acid peak
2	3.15	3.06	Negative peaks
	3.40	3.34	
	2.31	2.27	Degradation peaks
	3.54	3.43	
	5.49	5.27	

The plot of the wavelength loadings (Figure 1.6) shows the UV-visible spectra of the three factors. Providing that each factor stands for one compound, then pure spectra could be extracted from the combined spectra of the degradation mixture.

Figure 1.6 Wavelength mode loading plot of the three factors used in the PARAFAC model



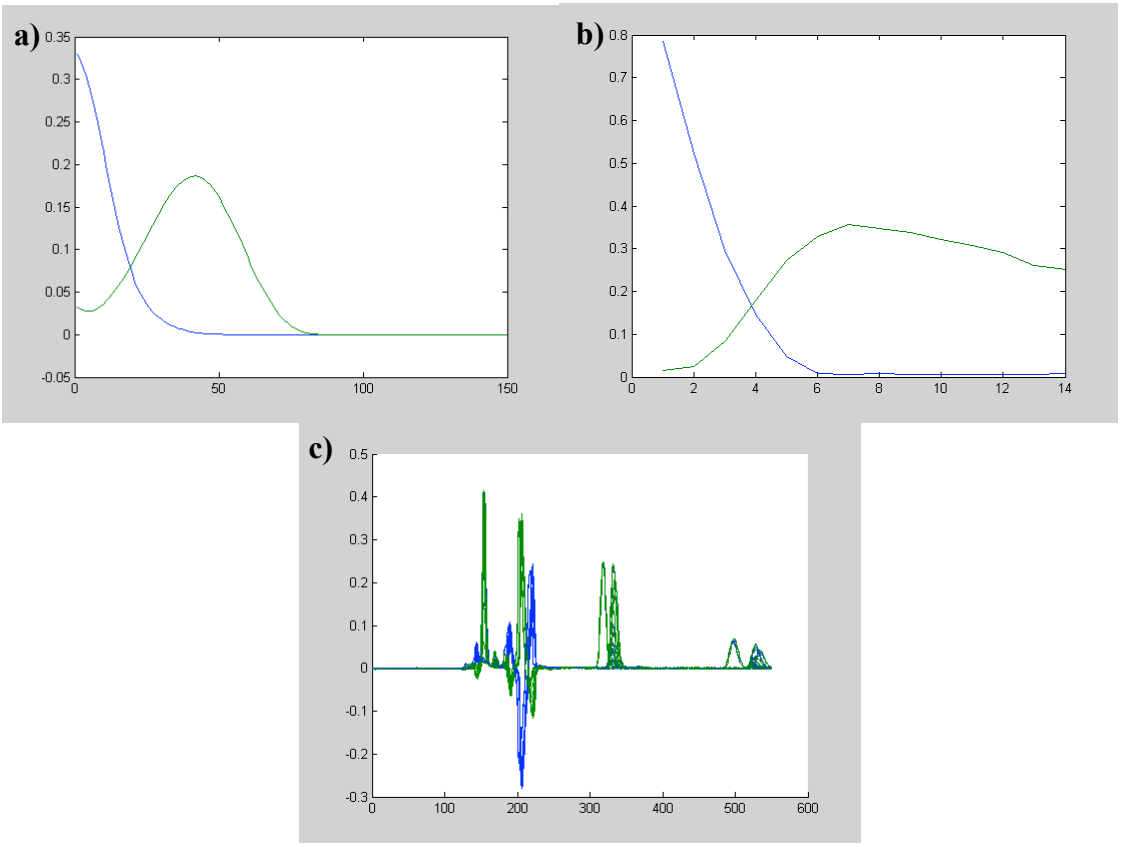
The UV-visible spectrum of factor 3 strongly resembles the ascorbic acid UV-visible spectrum (superimposed in orange on Figure 1.6). This last comparison between factor 3 and ascorbic acid confirms that factor 3 represents the ascorbic acid starting compound. The UV-visible spectrum of factor 1 appears like a copy of, although less intense, factor 3, supporting the argument that this factor is not a fully fledged factor in its own right. The UV-visible spectrum of factor 2 shows two maxima at 216 and 295 nm, which closely resembles the spectrum of one of the degradation products (peak at 5.27 in Figure 1.1 or peak 3 in Figure 1.2).

Although this first multi-way data analysis allowed us to build a model (three-factor model) for our three array data and to determine the factors being the most influential on the data, i.e. to describe the variability of the data during the ascorbic acid degradation, our model was influenced by a problematic injection (sample on day 2). Further, factor 2, that appears to represent the degradation products, is dominated by the major degradation product (peak 3 in Figure 1.2), thereby preventing the other minor peaks to be taken into account in the model.

A subsequent experiment was therefore conducted by injecting a similar 0.5 g/L ascorbic acid solution degrading under the same accelerated ageing conditions from day 0 to day 4 only, as this time period was known to exhibit the major variation in the degradation process. The samples were injected every 4 hours to obtain more accurate data on the minor peaks that were present only in the early stages of the degradation process.

Figure 1.7 shows the three mode (a, wavelength; b, sample and c, retention times) loadings for a two-component PARAFAC model. However, the new model still highlights the same factor contributions, namely ascorbic acid and the main degradation product. A two-factor model showed similar contributions and no extra information. When applying a three-factor model, the third factor only accounted for background noise.

Figure 1.7 Mode loading plot for a two-factor PARAFAC model, a) wavelength, b) sample and c) retention time. x-Axes were not changed to their corresponding abscises, being wavelengths, days and retention times respectively.

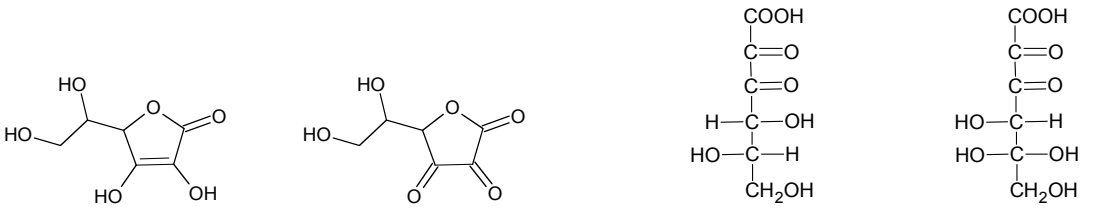


After several other data treatment processes were examined without success, it was concluded that the formation and decay rates of the ascorbic acid degradation products were too similar to be able to be differentiated by such multi-way data analyses described here. In essence, the application of chemometrics to the chromatographic data did not achieve the success that was considered possible when the experiments were planned and so other instrumental approaches were employed.

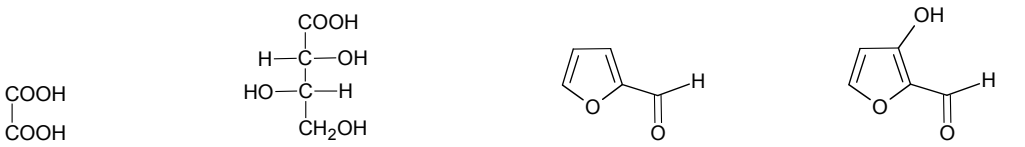
As conducted in the GWRDC CSU01/02 report, a HPLC-MS method was developed based on compounds that have been reported to be ascorbic acid degradation products. The ascorbic acid degradation products are expected to be mainly organic acids such as oxalic acid, L-threonic acid, 4, 5, 5, 6-tetrahydroxy-2, 3-deketohexanoic acid (THDH), 2, 3-diketo-L-gulonic acid and dehydroascorbic acid (intermediate product) and aldehydes such as furfural and β-hydroxyfurfural (Figure 1.8).

Optimum HPLC-MS conditions were investigated for each of these products except for oxalic acid, THDH, 2, 3-diketo-L-gulonic acid, which was replaced by a similar compound that was commercially available, 2-keto-L-gulonic acid (Figure 1.9a) and β-hydroxyfurfural replaced by 5-hydroxymethylfurfural (Figure 1.9b).

Figure 1.8 Major suggested ascorbic acid degradation products at wine pH.

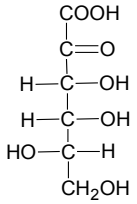
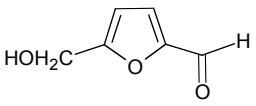


Ascorbic acid | Dehydroascorbic acid | 2, 3-diketo-L-gulonic acid | THDH



Oxalic acid | L-threonic acid | Furfural | β-hydroxyfurfural

Figure 1.9 a) 2-keto-L-gulonic acid and b) 5-hydroxymethylfurfural structures.

a)  ***b)*** 

HPLC analyses were performed using a SpectraSYSTEM LC run by Xcalibur software with a P4000 sample pump and a AS3000 autosampler. The column was a security guard column using two 4×2mm Phenomenex C18 cartridges with 4 μm particle diameter. Elution conditions were 0.15 mL/min flow rate with an injection volume of 20 μL; solvent water/acetonitrile (90:10) at the desired pH.

MS experiments were conducted on a Finnigan AQA quadrapole MS with an electrospray source. The ionisation was achieved by ESI using the desired mode (positive, negative or both). The acquisition rate was set up at 0.5 scans per second when acquiring in both ionisation modes and 3 scans per second when using only one. The mass spectrum range was set up in accordance to the molecular mass of the analyte and was also limited by the acquisition rate allowed.

The LC-MS cone voltage was found to have the biggest impact on mass signal sensitivity during the instrumental MS optimisation investigation. Probe voltage had no influence and probe temperature was set as indicated by the "MS user's guide" in accordance with the LC flow rate adopted.

The molecule ionisation was obtained using an electrospray (ESI) either in positive or negative mode depending on the chemical structure of the analytes. Acids were detected in negative ionisation mode as they are more likely to lose a proton while aldehydes were detected in positive ionisation mode as they are, on the contrary, more likely to gain a proton. Some compounds may be detected in both modes, particularly those that contain multi-functional groups.

The mobile phase pH was also investigated as being a factor for an enhanced loss or gain of proton, *i.e.* a lower pH should be responsible for an enhanced gain of proton during the aldehyde positive ionisation while a higher pH should enhanced a loss of proton during the negative ionisation of acidic compounds.

Figures 1.10 and 1.11 show examples of the optimisation trials for furfural and ascorbic acid, respectively. The obtained results determined the following parameters to be the optimum conditions for both acids and aldehydes using respectively the negative and positive ionisation mode: cone voltage 20 V, probe voltage 3 kV and probe temperature 180 °C. Unexpectedly, no improvement in analyte sensitivity was obtained by increasing or decreasing the mobile phase pH, in either the negative and positive ionisation modes.

Figure 1.10 LC-MS optimisation for furfural.

Mobile phase pH = 3

				Probe voltage (kV)			Cone voltage (V)				Probe temperature (°C)			Method	
Standard	Date	Sample name	Peak area (a. u.)	2	3	4	10	20	25	40	150	180	210	name *	Comments
Furfural	8-Dec	CB030			✓			✓				✓		(-) b	no signal in - ESI
		CB031			✓			✓				✓			
		CB032			✓			✓					✓	(-) h	
		CB033	1,012,870		✓			✓				✓		(+) i	
		CB034	950,026		✓			✓				✓			
	9-Dec	CB037			✓		✓					✓		(+) j	no signal with 10 V
		CB038			✓		✓					✓			
		CB041	140,839		✓					✓		✓		(+) k	better with 20 V
		CB042	145,634		✓					✓		✓			
		CB045	527,521		✓				✓			✓		(+) l	
		CB046	541,129		✓				✓			✓			
		CB049	372,479	✓				✓				✓		(+) m	better with 3 kV
		CB050	389,230	✓				✓				✓			
		CB053	874,359			✓		✓				✓		(+) n	
		CB054	842,641			✓		✓				✓			
		Mobile phase pH = 4													
		CB061			✓			✓				✓		(+) i	no signal at pH 4
		CB062			✓			✓				✓			
	12-Dec	***Mobile phase pH = 2***													
		CB081	353,332		✓			✓				✓		(+) i	better with pH 3
		CB082	409,844		✓			✓				✓			
	13-Dec	***Mobile phase pH = 3***													
		CB100	455,827		✓			✓				✓		(+) i	same vial as previously lower area..., why?
		CB101	514,620		✓			✓				✓			
		Mobile phase pH = 2													
		CB111	555,698		✓			✓				✓		(+) i	better with pH 3
		CB112	603,155		✓			✓				✓			

*: general method names are as follow: Celia_ESI-_ph3_a.met for *instrument method in Xcalibur* and Celia_ph3a.tun in *tune*

red parts of the method names are detailled in the table

: best results

Figure 1.11 LC-MS optimisation for ascorbic acid.

				Probe voltage (kV)			Cone voltage (V)				Probe temperature (°C)			Method	Comments
Standard	Date	Sample name	Peak area (a. u.)	2	3	4	0	20	40	70	150	180	210	name *	
				Mobile phase pH = 3											
Ascorbic acid	7-Dec	CB001	775,498	✓				✓				✓		a	Repetability test OK ?
		CB003	869,109	✓				✓				✓			
		CB004	957,025	✓				✓				✓			
		CB005	987,188	✓				✓				✓			
		CB006	1,005,487	✓				✓				✓			both +ve & -ve ionisation mode
		CB007	1,003,896	✓				✓				✓			
		CB008	1,026,692	✓				✓				✓			
		CB009	1,002,579	✓				✓				✓			-ve mode from this pt
		CB010	1,163,688		✓			✓				✓		(-) b	no big difference
		CB011	1,142,578		✓			✓				✓			
		CB012	1,154,753			✓		✓				✓		(-) c	
		CB013	1,149,001			✓		✓				✓			
		CB014	437,528	✓					✓			✓		(-) d	best response obtained with 20V
		CB015	438,596	✓					✓			✓			
		CB016	177,440	✓						✓		✓		(-) e	
		CB017	186,145	✓						✓		✓			
		CB018	200,219	✓			✓					✓		(-) f	
		CB019	195,023	✓			✓					✓			
	8-Dec	CB020	744,541		✓			✓			✓			(-) g	better with 180 °C
		CB021	730,325		✓			✓			✓				
		CB024	781,491		✓			✓			✓				
		CB022	826,704		✓			✓					✓	(-) h	better with 180 °C
		CB023	965,222		✓			✓					✓		
		CB025	873,076		✓			✓					✓		
	12-Dec	***Mobile phase pH = 4***													
		CB067	208,899		✓			✓				✓		(-) b	better with pH 3
		CB068	198,294		✓			✓				✓			
		CB069	192,469		✓			✓				✓			
		Mobile phase pH = 5													
		CB074	90,723		✓			✓				✓		(-) b	getting worse
		CB075	88,467		✓			✓				✓			
		CB076	92,352		✓			✓				✓			
	13-Dec	***Mobile phase pH = 2***													
		CB087	120,114		✓			✓				✓		(+) i	(+) ESI, not good
		CB088	128,514		✓			✓				✓			
		Mobile phase pH = 3													
		CB090	441,524		✓			✓				✓		(-) b	same vial as previously lower area..., why?
		CB091	450,079		✓			✓				✓			
		CB096	654,648		✓			✓				✓		(-) b	same flask as previously lower area..., why?
		CB097	647,419		✓			✓				✓			
		CB104	1,017,669		✓			✓				✓		(-) b	fresh solution, ~ area degradation confirmed
		CB105	979,317		✓			✓				✓			
	14-Dec	***Mobile phase pH = 4***													
		CB106	314,528		✓			✓				✓		(-) b	fresh solution better with pH 3
		CB107	299,943		✓			✓				✓			

*: general method names are as follow: Celia_ESI-_ph3_a.met for *instrument method in Xcalibur* and Celia_ph3a.tun in *tune*
red parts of the method names are detailled in the table
: best results

However, when using the optimised HPLC-MS method to analyse collected peaks or fractions, it was proven that the products could not be identified by this technique due to poor stability of the compounds and dilution during the collection step (concentration of the sample with various methods, under nitrogen or freeze-drying, did not improve the analyses).

The next approach to allow the identification of the ascorbic acid degradation products was to use an in-line method as collection of the products for off-line analysis is prevented by their poor stability and low concentration. HPLC-NMR is a technique which combines the separation of a mixture by HPLC and their unequivocal identification by Nuclear Magnetic Resonance. Furthermore, the technique allows the use of "stop-flow" which is required when compounds are not concentrated enough. Basically, the elution of compounds from the HPLC is stopped so the selected compound in the NMR can undergo accumulation of data, for a better sensitivity.

The HPLC-NMR analyses were performed thanks to a collaboration with Dr. Daniel Dias and Dr. Julie Niere from the Royal Melbourne Institute of Technology.

A highly concentrated solution of ascorbic acid (5.0 g/L) was degraded under accelerated ageing conditions and injected into the HPLC-NMR. Figure 1.12 and 1.13 show the chromatogram in HPLC-NMR/DAD and the NMR spectrum of the peak at 5.31 minutes, being the major peak observed in typical chromatograms (peak 3 in Figure 1.2).

Figure 1.12 Chromatogram by HPLC-NMR/DAD of a 5.0 g/L ascorbic acid degradation solution.

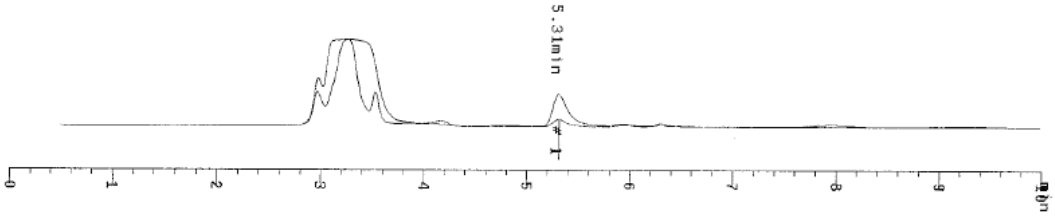
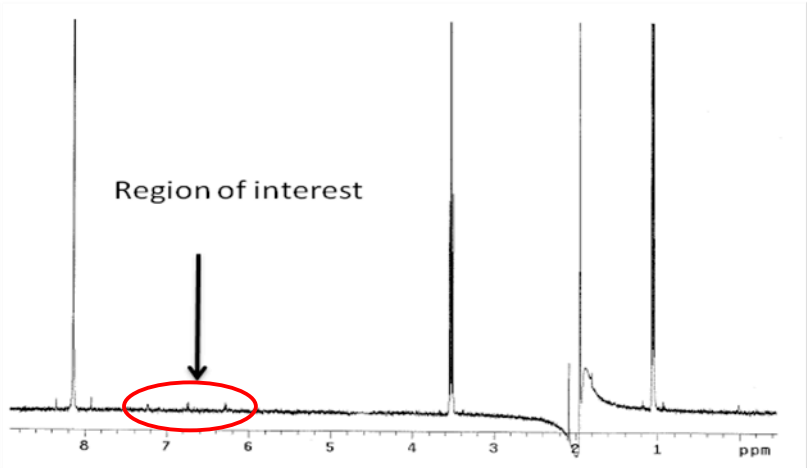


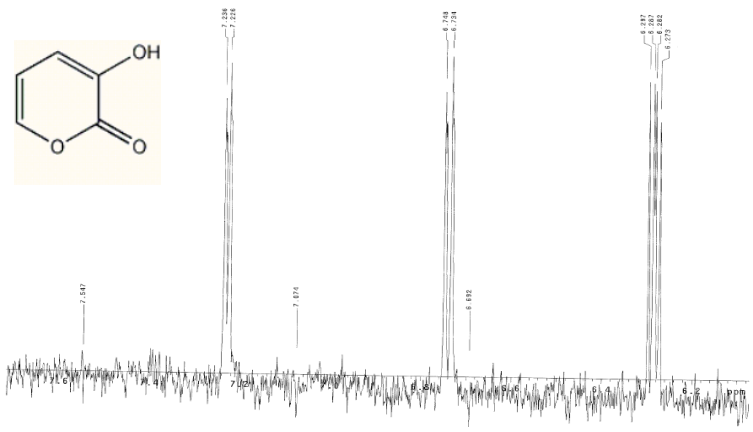
Figure 1.13 NMR spectrum of the peak at 5.31 minutes.



The same NMR spectrum was recorded after 20 hours in stop-flow conditions, in order to increase the sensitivity of the signal. Figure 1.14 shows the NMR chemical shifts associated with the compound corresponding to peak 3 in Figure 1.2. Based on the chemical shifts, peak 3 in Figure 1.2 could be assigned to 3-hydroxy-2-pyrone. Unfortunately, the sensitivity for peaks 1 and 2 (as observed in Figure 1.2) was insufficient to generate meaningful NMR spectra. Similarly, the peaks observed at the beginning of the chromatogram are too low in concentration to even be detected by NMR.

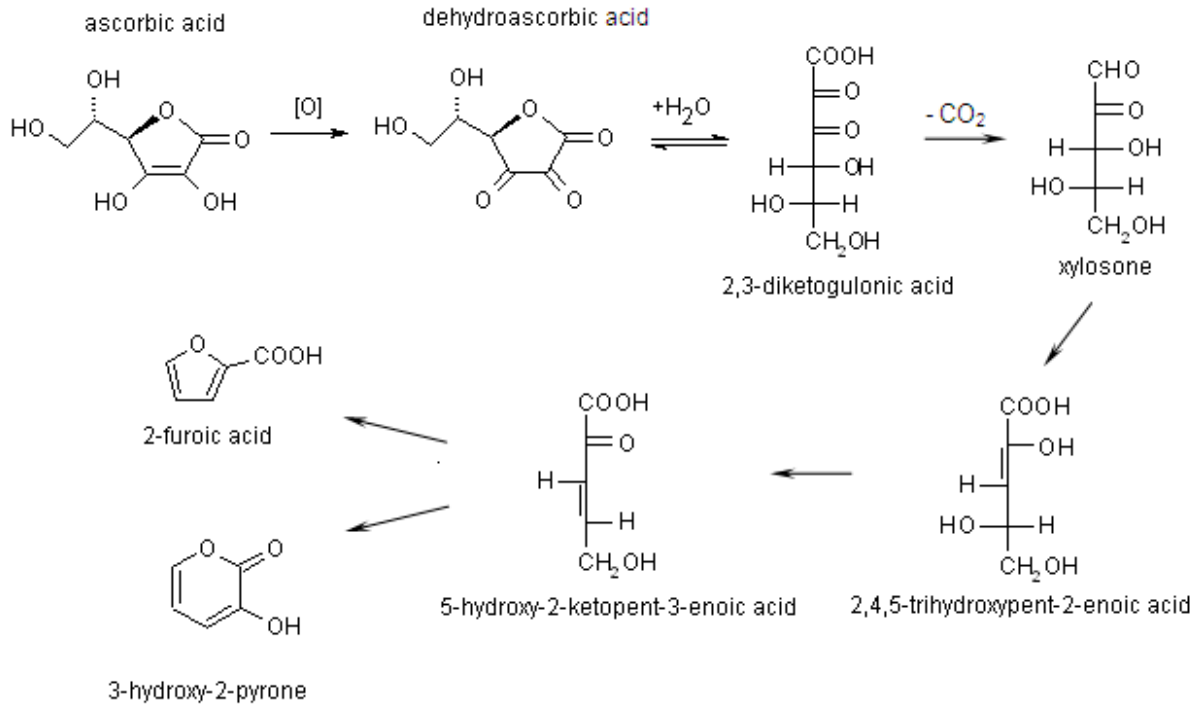
Yuan and Chen (Yuan and Chen, 1998a & 1998b) have published a HPLC-DAD methods for the identification of furan-type compounds as well as the 3-hydroxy-2-pyrone identified above, found in fruit juice and emanating from sugars and/or ascorbic acid degradation. The method uses an organic acid column that had not been used yet in this project for the degradation of ascorbic acid.

Figure 1.14 NMR spectrum of the peak at 5.31 minutes after 20 hours acquisition in stop-flow conditions and structure of the corresponding compound.



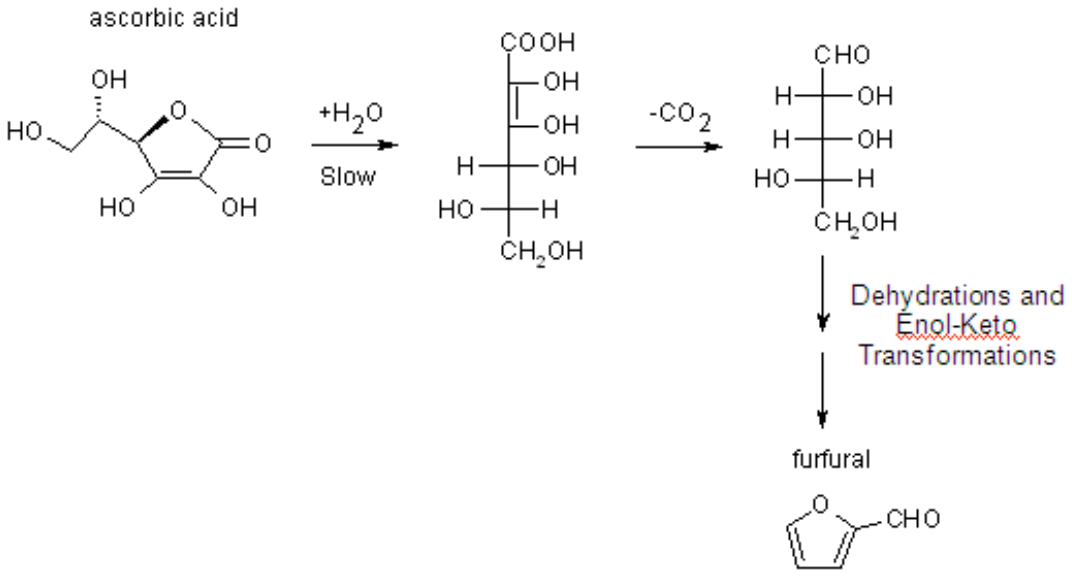
The literature reporting 3-hydroxy-2-pyrone as one of the main ascorbic acid degradation products under conditions close to wine also reports 2-furoic acid as another major degradation product arising from the same degradation pathway (Figure 1.15). This pathway is known as one of the aerobic degradation routes and occurs via the oxidation of ascorbic acid into dehydroascorbic acid.

Figure 1.15 Aerobic degradation pathway leading to the formation of 3-hydoxy-2-pyrone and furoic acid. Adapted from Yuan and Chen 1998b.



Another pathway for ascorbic acid to degrade is via a non-oxidative mechanism, whose major degradation product is furfural (Figure 1.16), or furaldehyde, better known as the oak-extracted flavour compound. The non-oxidative pathway does not involve dehydroascorbic acid or hydrogen peroxide, and in aqueous solutions is known to be accelerated at higher temperatures (Anmo et al. 1971). Although minor under ideal wine storage conditions (i.e. less than 20°C), it could become significant for wines that are aged for long periods (i.e. 5 to 10 years) and/or at higher temperatures.

Figure 1.16 Anaerobic degradation pathway leading to the formation of furfural. Adapted from Yuan and Chen 1998b.



Due to the different pathways of formation of these compounds and the clear impact of the amount of oxygen on their production, aerobic and anaerobic degradations of ascorbic acid and dehydroascorbic acid were performed. Both ascorbic acid and dehydroascorbic acid were dissolved in 12% ethanol tartaric acid-buffered model wine systems, and oxygen concentration was reduced by N_2 bubbling for anaerobic reaction media. The 'anaerobic' set up consisted of Schlenk reaction vessels linked to N_2-filled balloons for a constant O_2-diminished atmosphere (Figure 1.17), while the aerobic samples were exposed to an O_2-saturated atmosphere by aerating the samples on a daily basis. All solutions were kept in the dark at 45°C.

Figure 1.17 Anaerobic degradation set up.



All samples were monitored using the HPLC method published by Yuan and Chen (Yuan and Chen, 1998a & 1998b) and the ascorbic acid degradation and the formation of 3-hydroxy-2-pyrone, 2-furoic acid and furfural were monitored over time.

The aerobic and anaerobic degradations showed little difference in terms of ascorbic acid consumption rate and product formation, highlighting the difficulty of achieving an absolute O_2-excluded atmosphere. Future experiments will be conducted under more defined

anaerobic conditions, with measurement of trace oxygen concentrations, as well as assessing the impact of sulfur dioxide on the decay of ascorbic acid at low oxygen concentrations.

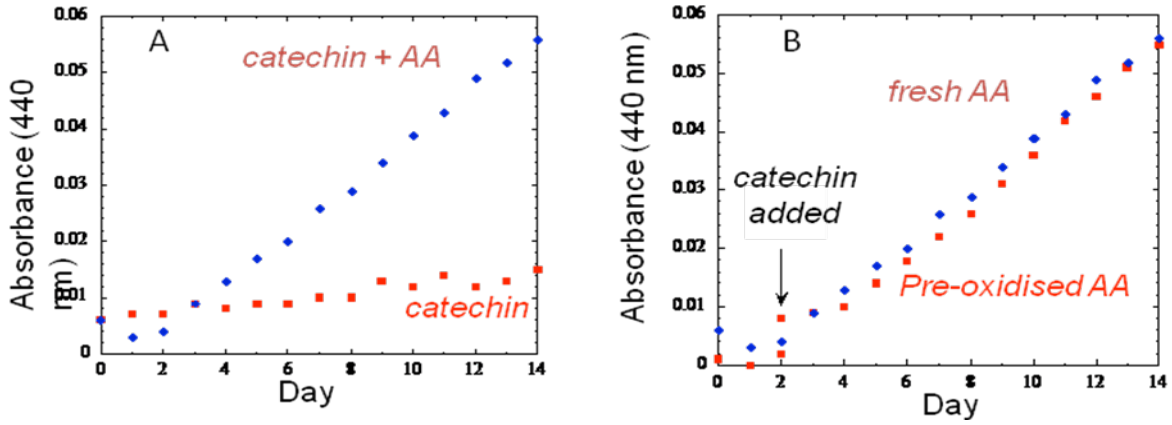
However, the comparison between dehydroascorbic acid and ascorbic acid confirmed that the formation of furfural arises from the non-oxidative degradation of ascorbic acid as no furfural was produced during the degradation of dehydroascorbic acid, the intermediate of aerobic ascorbic acid degradation.

Despite using a range of analytical approaches described here, identification of the ascorbic acid degradation products remains a challenge to be resolved. Rather than focus on the direct identification of the compounds that are formed from ascorbic acid, the remainder of this report describes the research undertaken to determine why ascorbic acid, or its degradation compounds, enhances the rate of pigment formation in the presence of catechin-type phenolic compounds. The focus therefore was directed towards identifying the winemaking conditions where ascorbic acid is safe to use and when its degradation is likely to lead to enhanced colour development of white wine.

2. The contribution of ascorbic acid to pigment development

In a previous GWRDC project (UM96/1), we were able to demonstrate that the degradation of ascorbic acid leads to an enhanced absorbance at 440 nm. This observation was made in solutions containing both (+)-catechin and ascorbic acid in the standard model white wine system used in our research. The effect is clearly shown in Figure 2.1. Figure 2.1A shows that, following a lag period of about two days when the absorbance at 440 nm (A_{440}) actually dropped, a rapid rise in A_{440} occurred with the final value after 14 days, being significantly higher than that of a solution of (+)-catechin alone. If the ascorbic acid was allowed to pre-oxidise before the addition of (+)-catechin (Figure 2.1B), there was no lag period, but the same final A_{440} value was reached after 14 days.

Figure 2.1. Effect of ascorbic acid on (+)-catechin pigment development.
(+)-Catechin = 100 mg/L (3.4×10^{-4} M); ascorbic acid = 200 mg/L (1.1×10^{-3} M).

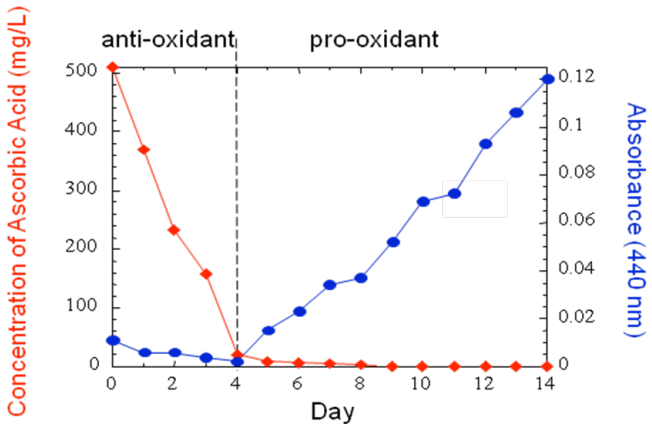


The significant outcomes of this experiment are

- A degradation product of ascorbic acid is influencing the colour development
- Colour development only occurs after the ascorbic acid is degraded

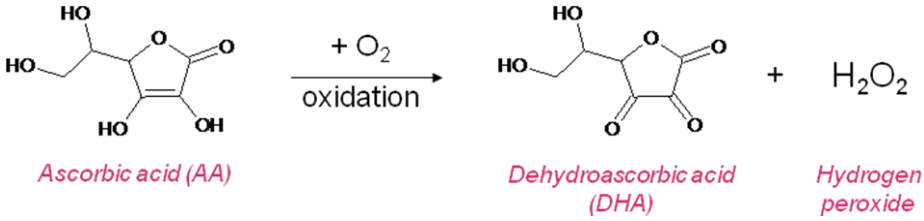
The link between the onset of colour development and the loss of ascorbic acid can be seen more clearly in Figure 2.2. The 'crossover' from antioxidant to pro-oxidant is obvious as soon as more than 95% of the ascorbic acid is lost.

Figure 2.2. Crossover effect from anti- to pro-oxidant effect.
(+)-Catechin = 250 mg/L; ascorbic acid = 500 mg/L.



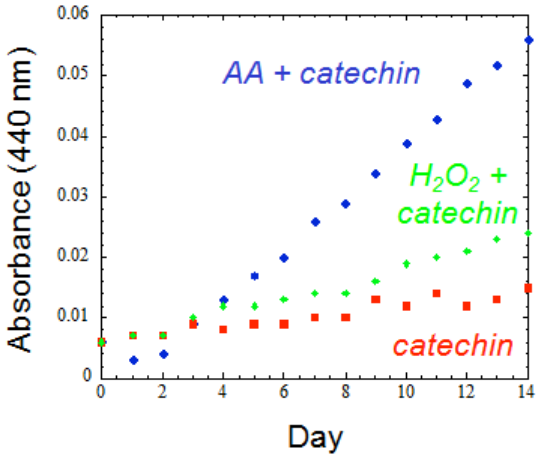
The oxidation of ascorbic acid leads to the production of hydrogen peroxide, a well-known oxidant, which can be represented as in Figure 2.3.

Figure 2.3. Mechanism of ascorbic acid oxidation.



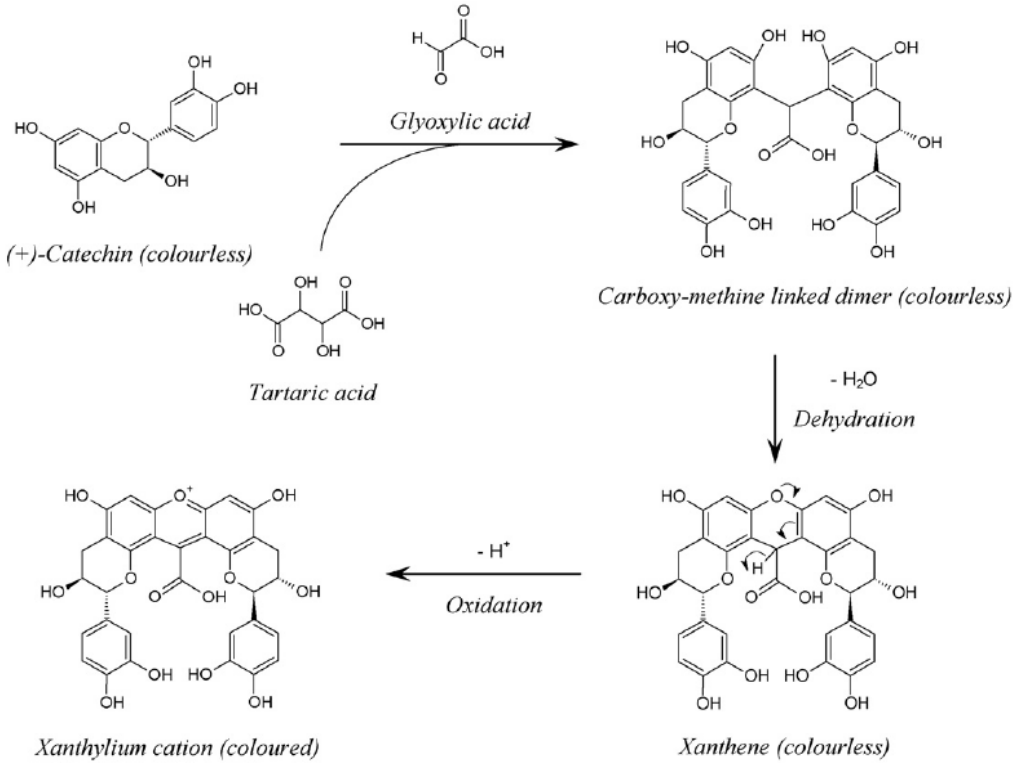
The impact of hydrogen peroxide on the increased colouration of the model wine system was investigated by adding a certain amount of the oxidising agent. The added amount had previously been determined by amperometry as the free hydrogen peroxide (i.e. 9.7 x 10^{-5} M) measured in a degrading 200 mg/L ascorbic acid solution, that is, the maximum residual amount not scavenged by ascorbic acid. When hydrogen peroxide was added to the model wine system containing (+)-catechin, only a moderate increase in the A_{440} value was observed (Figure 2.4) that could not account for the total increased colouration induced by ascorbic acid.

Figure 2.4. Impact of ascorbic acid and hydrogen peroxide on the colouration of (+)-catechin solution.
(+)-Catechin = 100 mg/L (3.4 x 10^{-4} M); ascorbic acid = 200 mg/L (1.1 x 10^{-3} M); hydrogen peroxide = 9.7 x 10^{-5} M.



(+)-Catechin, under oxidising conditions, is known to form xanthylium pigments, as shown in Figure 2.5. Critical to the formation of xanthyliums is the requirement for glyoxylic acid, a two-carbon molecule formed through cleavage of tartaric acid that may be light-induced (see GWRDC report CSU01/02) or via Fenton chemistry (i.e. hydrogen peroxide and metal ions). Glyoxylic acid contains both aldehyde and carboxylic acid functional groups: the aldehyde carbon can bridge the (+)-catechin molecules which, through subsequent reactions, lead to the formation of xanthylium pigments.

Figure 2.5. Mechanism of xanthylium pigment production.



In understanding why ascorbic acid makes a significant contribution to colour development, the critical questions that needed to be addressed were:

- Can the degradation product(s) of ascorbic acid responsible for enhanced colour development be identified?
- Is the pigment(s) responsible for colour development the same xanthylium pigment described above (derived from glyoxylic acid) or is there one or more different pigments formed?
- Are free radicals involved in the process?

To address these questions, solutions of

- ascorbic acid (AA) – 500 mg/L
- (+)-catechin (cat) – 250 mg/L
- combination of ascorbic acid and (+)-catechin

were prepared in

- tartaric acid wine base, pH 3.2
- ethanol(EtOH)-free tartaric acid wine base, pH 3.2
- formic acid wine base, pH 3.2
- ethanol(EtOH)-free formic acid wine base, pH 3.2

Formic acid was selected so that the contribution of hydrogen peroxide (inducing tartaric acid cleavage into glyoxylic acid) to xanthylium pigment production alone could be assessed. The formic acid-buffered solution was prepared so that in contained similar amounts of potassium and acid as the tartaric acid-buffered model system (1.72 g of potassium hydroxide (0.026 M) dissolved in 1 L of 12% (v/v) aqueous ethanol acidified with 423 μL of formic acid (0.011 M)). Ethanol is an effective radical scavenger and its absence should lead to a higher colour enhancement, if free radicals are involved. Figure 2.6 presents an overview of the reaction systems used.

Figure 2.6. Overview of the reaction solutions used.

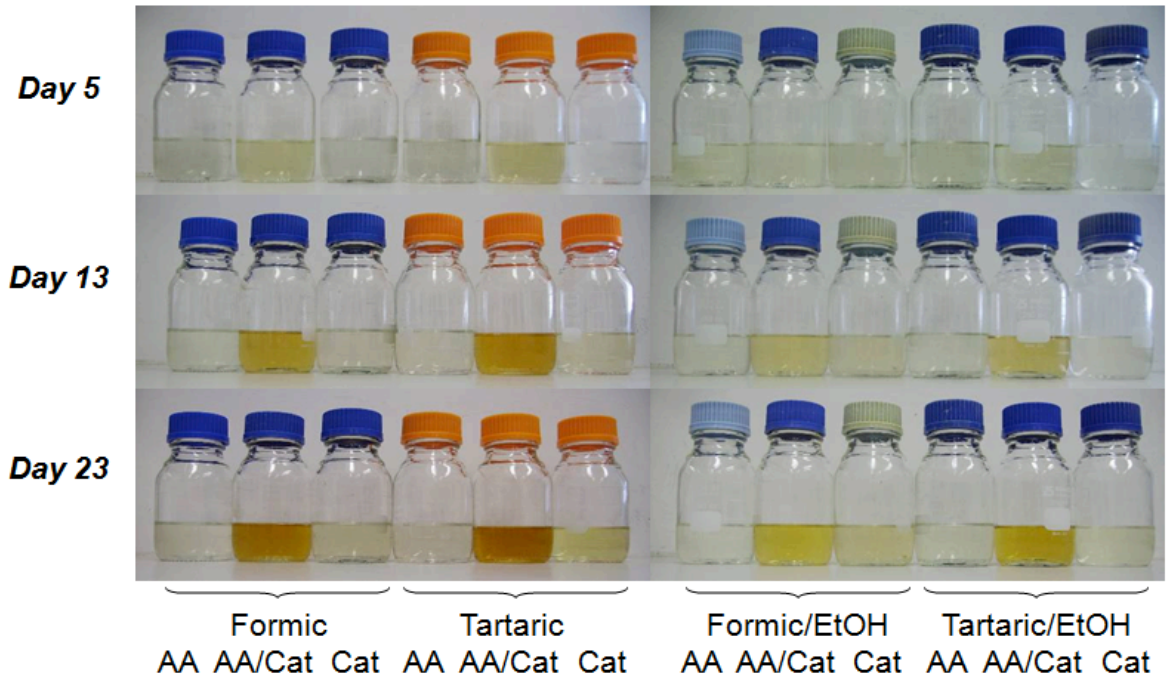


The samples were stored at 45°C in darkness and aerated daily. A_{440} measurements were made every second day and HPLC-DAD chromatograms recorded every four days. HPLC-MS chromatograms were only recorded at the end of the experiment, i.e. on Day 24.

Figure 2.7 presents images of the reaction bottles at Day 5, Day 13 and Day 23. These images show:

- Ethanol inhibits the production of colour
- Ascorbic acid and (+)-catechin combinations significantly enhances the colour as observed from day 13
- A higher colour is observed in the tartaric acid solutions compared to the formic acid solutions

Figure 2.7. Colour development of the model solutions over 3 weeks.



The capacity of ethanol to reduce the production of colour confirms that a free radical process is involved in the pigmentation process. The ethanol-derived radicals have recently been identified by Elias et al. (2009) in model wine systems by using radical trapping agents in combination with Electron Paramagnetic Resonance (EPR). Knowledge of the causal factors for oxidative degradation is an area of importance for parameters that affect shelf life and storage of wine and will form the basis for a new project submission.

Figure 2.8 presents plots of the A_{440} values as a function of the reaction period (24 days). Absorbance values were recorded at 440 nm as this is the maximum absorbance for the samples when measured in the visible spectrum. These plots confirm the observations noted above and underscore the significant inhibitory role played by ethanol. It is also important to

note that some colour development occurs in the (+)-catechin/tartaric acid ethanol-free wine base (line Cat/tartaric on the left hand side plot). This colour development was shown previously in our work to be a consequence of xanthylium pigment production arising from cleavage of tartaric acid under the conditions used in these experiments. That is the formation of glyoxylic acid initiates the stepwise formation of the xanthylium pigments, as shown in Figure 2.5.

Figure 2.8. Absorbance at 440 nm of the model solutions over 3 weeks.

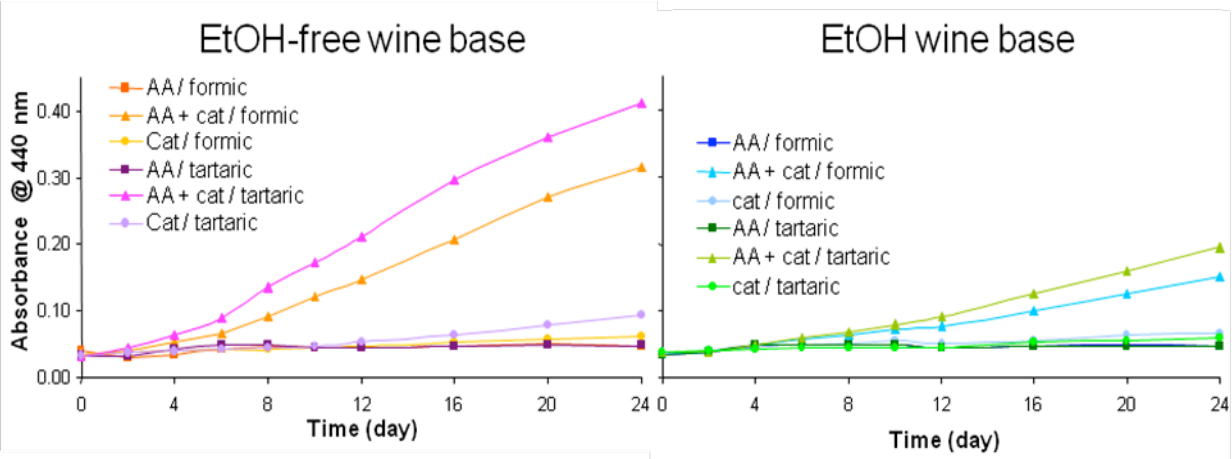
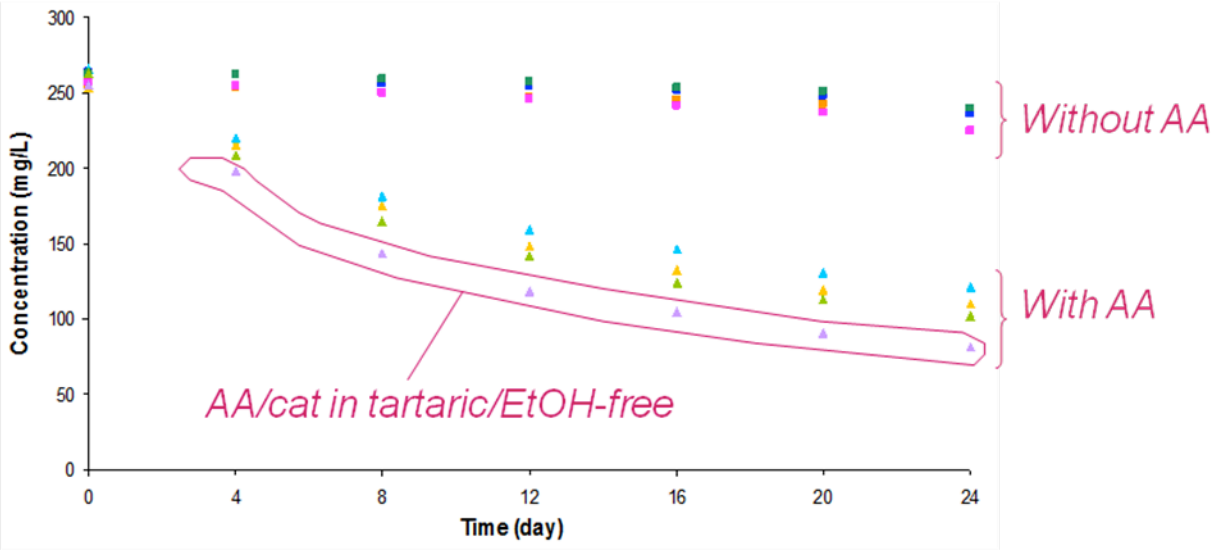


Figure 2.9. (+)-Catechin decay over 3 weeks.

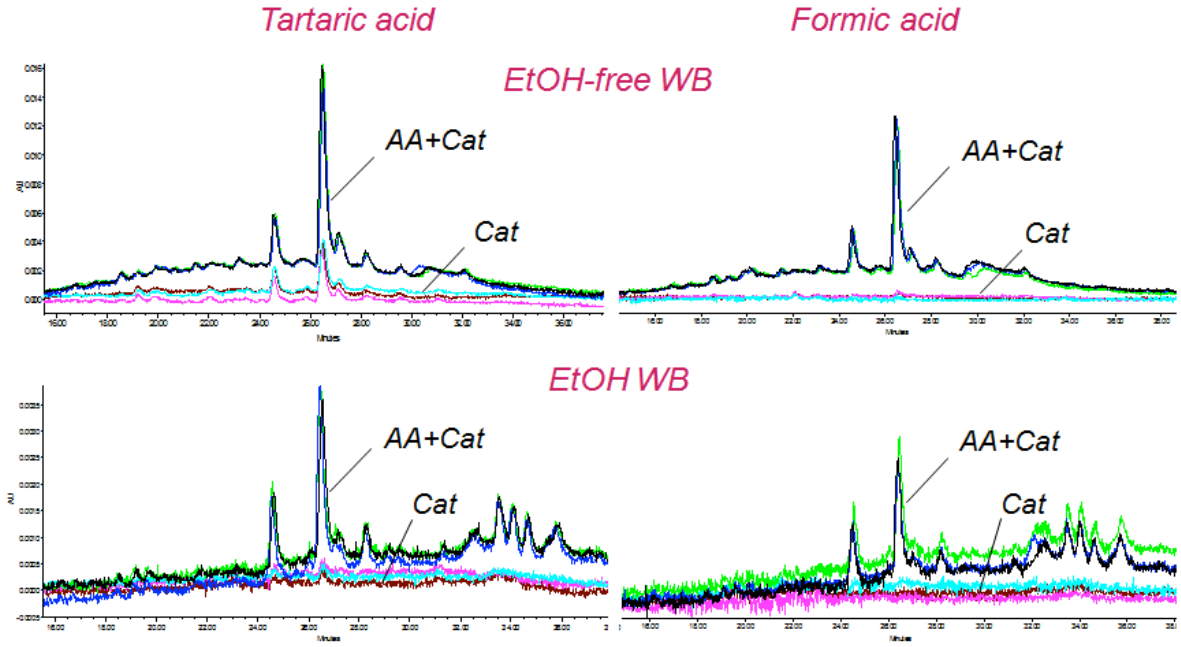


The (+)-catechin concentration was monitored by HPLC-DAD during the 24 day reaction period. The decrease in (+)-catechin concentration over time is shown in Figure 2.9. Clearly, ascorbic acid significantly enhances the loss of (+)-catechin in the reaction system, with the major loss being in the ascorbic acid/catechin mixture in the ethanol-free tartaric acid wine base. These data in Figure 2.9 again underscore the inhibitory role of ethanol and also indicate that (+)-catechin is involved in the compounds that produce the colour at 440 nm.

HPLC chromatograms with detection at 440 nm are shown in Figure 2.10; that is, the wavelength corresponding to the maximum absorbance for the pigments generated in these reactions. There is an obvious similarity in the pattern of peaks for the tartaric acid and formic acid model wine systems, with the peaks in the chromatogram for the tartaric acid system being slightly higher in intensity, in agreement with the colours of the reaction solutions shown above in Figure 2.7. The inhibitory effect of ethanol on pigment production is again apparent, with lower peak intensities for the solutions containing ethanol.

These chromatograms imply that the same pigments are being formed in both tartaric acid and formic acid wine systems, with a somewhat higher pigment production in the tartaric acid model system.

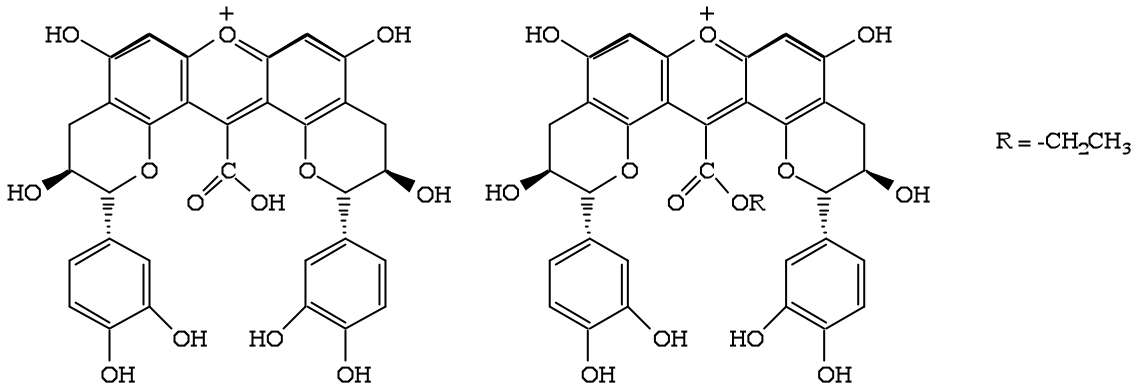
Figure 2.10. The 440 nm chromatograms from the analysis of samples by HPLC-DAD.



We have already established that xanthylium pigments are formed in a tartaric acid model wine system containing (+)-catechin. The mechanism for this was described earlier in this report (Figure 2.5). It is important to note that there are two types of xanthylium pigments that can be formed: one contains the free carboxyl group on the glyoxylic acid carbon and the other form is the ethyl ester. The structures of these two pigments are shown in Figure 2.11. Of course, the esterified form will only be present in the wine systems containing ethanol.

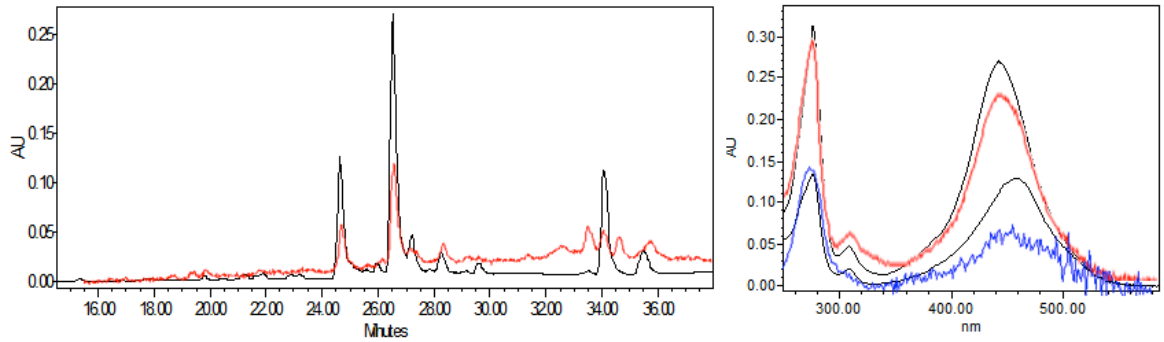
To determine if the colour generated in the experiments was actually due to xanthylium pigments, chromatograms at 440 nm of a 'standard' solution known to contain xanthylium pigments were compared with those of the samples used in this experiment containing ascorbic acid and (+)-catechin. The 'standard' solution was prepared by the procedure that we have described previously (Clark and Scollary, 2002). Figure 2.12 shows a distinct similarity between the chromatogram of the standard (black line) and of ascorbic acid/(+)-catechin solution in tartaric acid-bufferd ethanol wine system (red line). The same UV-visible spectra were observed for the samples in formic acid-buffered wine systems.

Figure 2.11. The xanthylium cation in acid (left-hand side) and ethyl ester (right-hand side) forms



Further, the UV-Visible spectra for the standard (Figure 2.12, right-hand side, black line) and ascorbic acid/(+)-catechin in tartaric acid and formic acid wine systems confirm the existence of xanthylium pigments generated in the presence of ascorbic acid (Figure 2.12, right-hand side, red line). The esterified pigments were also identified in the ethanol-containing wine systems (Figure 2.12, right-hand side, blue line).

Figure 2.12. Left-hand side: The 440 nm chromatogram of (+)-catechin/ascorbic acid (in red) and (+)-catechin/glyoxylic acid (in black) samples. Right-hand side: The UV/Visible spectra of peaks from the chromatogram (see text for assignment).



Mass spectral detection is an effective method for determining the existence of compounds in solution, particularly when a standard is available. Figure 2.13 shows the LC-DAD (440 nm) and LC-MS chromatograms for the 'xanthylium pigment standard' solution prepared from (+)-catechin and glyoxylic acid. In the LC-DAD (440 nm) chromatogram, the group of peaks at 35-40 minutes retention time correspond to the xanthylium cation pigments, while the peak at 74 minutes is the xanthylium ester pigment. In the LC-MS single ion monitoring chromatograms, the xanthylium cations can be detected at m/z 617 in the positive ion mode and the xanthylium ester pigments at m/z 645, also in the positive ion mode.

Figure 2.13. The 440 nm (upper), 617 m/z ion (middle) and 645 m/z ion (lower), chromatograms of xanthylium cations.

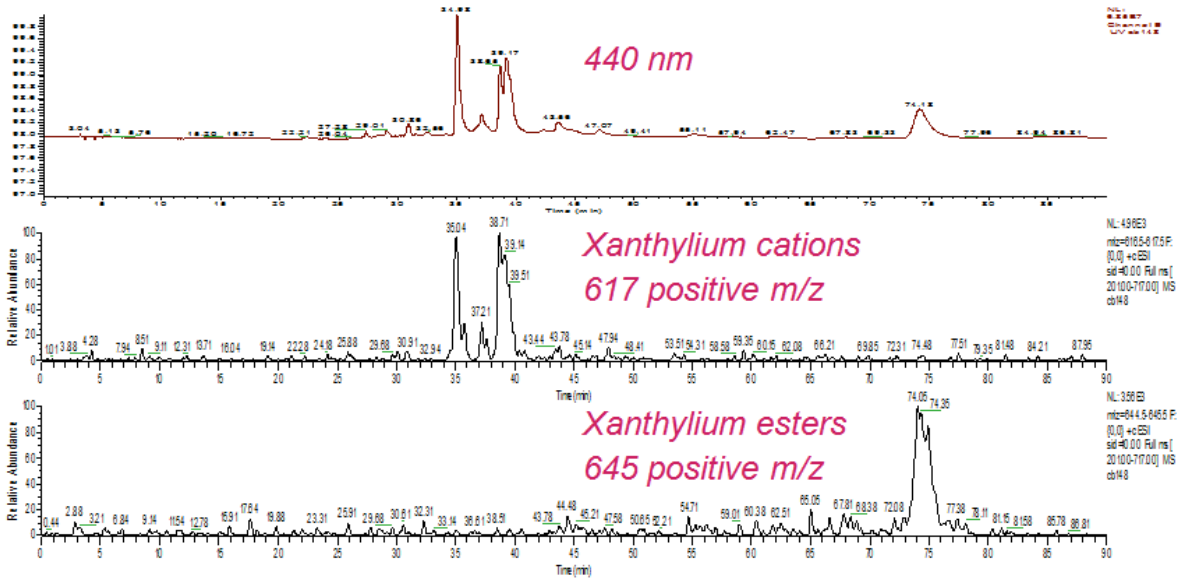
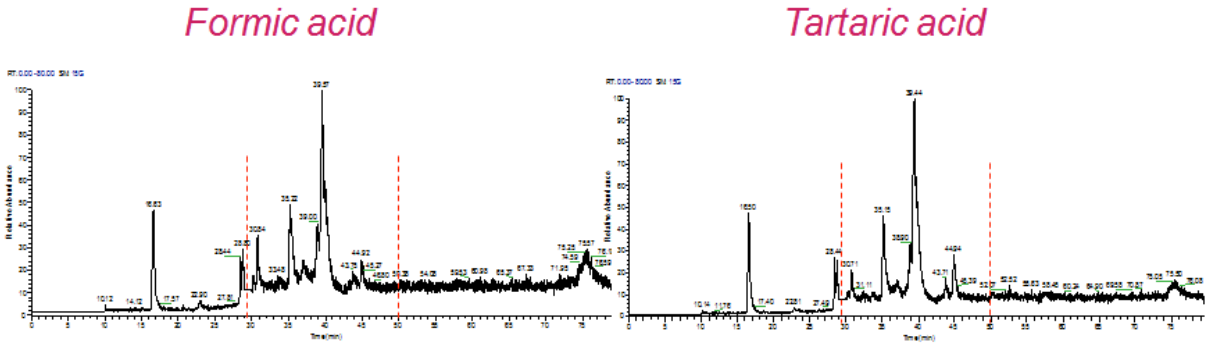


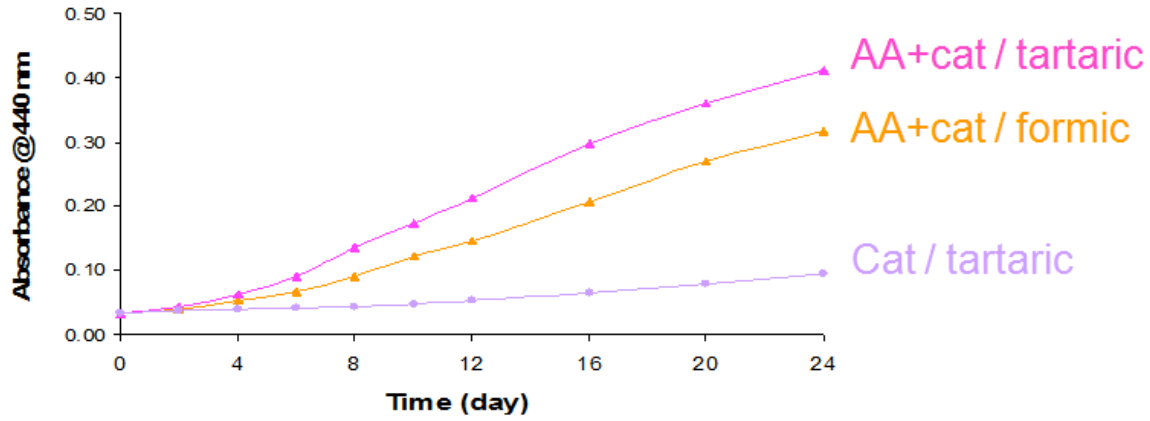
Figure 2.14 shows the single ion monitoring (m/z 617) HPLC-MS chromatograms for the formic acid and tartaric acid ethanol-free wine systems. From these chromatograms, it can be concluded that xanthylium pigments are formed in both wine systems. That is, these analyses demonstrated that the main pigments produced in the (+)-catechin/ascorbic acid samples, regardless of the supporting buffer, were identical to the glyoxylic acid-derived xanthylium cations and corresponding ethyl esters.

Figure 2.14. The 617 m/z ion chromatograms for the (+)-catechin/ascorbic acid samples in formic and tartaric acid-buffered solutions.



The commonality between the end-products in the two supporting buffers is further demonstrated in Figure 2.15. The absorbance change over time for the (+)-catechin/ascorbic acid systems follows a similar trend for the two supporting buffers. In fact, if the A_{440} value of the solution containing only (+)-catechin in tartaric acid is added to that of the (+)-catechin/ascorbic acid system in formic acid, the value approximates that of the mixed system in tartaric acid. This in turn implies that the amount of pigment production generated by ascorbic acid, or one of its degradation products, may be the same in the two supporting buffers.

Figure 2.15. The absorbance at 440 nm of (+)-catechin alone and (+)-catechin/ascorbic acid in tartaric acid-buffered wine system and (+)-catechin/ascorbic acid in formic acid-buffered wine system.



These results imply that ascorbic acid degrades into glyoxylic acid or, alternatively, some other compounds capable of reacting with (+)-catechin to form glyoxylic acid-derived xanthylium pigments. For example compounds such as glyoxal and dihydroxyfumaric acid are known to produce the same glyoxylic acid-derived xanthylium cation pigments when incubated in model wine solutions with (+)-catechin (Clark 2008, Es-Safi et al. 2003).

These observations also argue against the role of hydrogen peroxide as the sole causal agent for the production of the glyoxylic acid-derived xanthylium pigments. The generation of hydrogen peroxide from the degradation of ascorbic acid and its subsequent conversion into the hydroxyl radical allows the production of glyoxylic acid in the case of the tartaric acid wine system. However, the interaction of formic acid with hydroxyl radicals would only lead to the production of carbon dioxide. As the same glyoxylic acid-derived xanthylium pigments are formed in both the formic acid and tartaric acid wine systems, a compound derived from ascorbic acid itself is critical to pigment production.

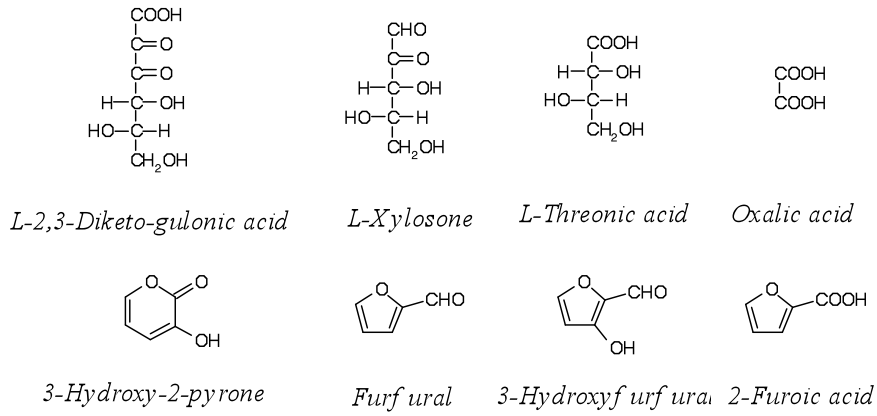
3. Identification of the pigment precursor formed from the degradation of ascorbic acid

The previous section demonstrated that a degradation product of ascorbic acid, in the model wine system, was contributing to the production of coloured xanthylium cation compounds when incubated with (+)-catechin. Furthermore, it was shown that the hydrogen peroxide generated by ascorbic acid, and subsequent Fenton chemistry, was not the major product contributing to the production of the xanthylium cation pigments. This section investigates the carbon-based ascorbic acid degradation product, as opposed to hydrogen peroxide, which is acting as a precursor to the yellow xanthylium cation pigments.

Prior to this study, the only known precursors to xanthylium cations in model wine systems were compounds containing aldehyde groups, such as glyoxylyic acid and glyoxal (Es-Safi et al. 1999a, 2003). Solutions of dihydroxyfumaric acid and (+)-catechin are also known to generate xanthylium cations (Clark 2008). It is possible that the dihydroxyfumaric acid first degraded and its subsequent aldehyde products reacted with (+)-catechin to afford the xanthylium cations.

As already discussed, on reaction with oxygen, ascorbic acid generates hydrogen peroxide and dehydroascorbic acid (Figure 2.3). The dehydroascorbic acid then undergoes rapid degradation into a variety of species including numerous carboxylic acid, ketone and aldehyde compounds (Yuan and Chen 1998, Whiting and Coggins 1960, Shinoda et al. 2004, Kimoto et al. 1993) (Figure 3.1). In Section 1, it was shown that several identifiable terminal products in the degradation of ascorbic acid in the model wine system were 3-hydroxy-2-pyrone, 2-furoic acid and furfural. Of the products in Figure 3.1, it is furfural and xylosone that contain aldehyde groups, thereby making them likely candidates to react with (+)-catechin and consequently generate pigments. In fact, furfural derived-xanthylium cations have already been reported (Es-Safi et al. 2000) but their production has only been achieved using much higher concentrations of furfural than would be found in the degradation of ascorbic acid.

Figure 3.1. Some reported degradation products of ascorbic acid.



To identify the degradation product of ascorbic acid that appeared most reactive with (+)-catechin, both these species were incubated in a model wine system and the products monitored. As described in section 2, the presence of ascorbic acid led to the accelerated consumption of (+)-catechin. Concomitant with this rapid decrease in (+)-catechin was the increase of two peaks in the 280 nm chromatogram, as followed by either Ultra Performance Liquid Chromatography (UPLC, Figure 3.2) or HPLC (Figure 3.3). These two peaks were only present in samples that containing both (+)-catechin and ascorbic acid, and were produced regardless of the identity of the supporting buffer in the model wine, or the

presence of ethanol and/or metal ions (i.e. copper/iron). The intensities of the peaks rise to a maximum at the near depletion of ascorbic acid (i.e. >90% depleted) and then undergo a rapid decrease in concentration (Figure 3.4), during which (+)-catechin undergoes its rapid decrease in concentration. For simplicity, these peaks 1 and 2 will be referred to as compounds **1** and **2**, respectively.

The comparison of the two chromatographic techniques shows that the retention times for the UPLC method were less than 6-times that of the HPLC technique. This rapid analysis time of UPLC was an advantage in the measurement of ascorbic acid samples that tended to degrade over time despite storage at around 8 °C in the chromatographic autosampler. Lower storage temperatures could not be utilised due to the risk of inducing potassium hydrogen tartrate precipitation.

Figure 3.2. UPLC chromatogram at 280 nm of ascorbic acid/(+)-catechin solution after 4 days at 45 °C.

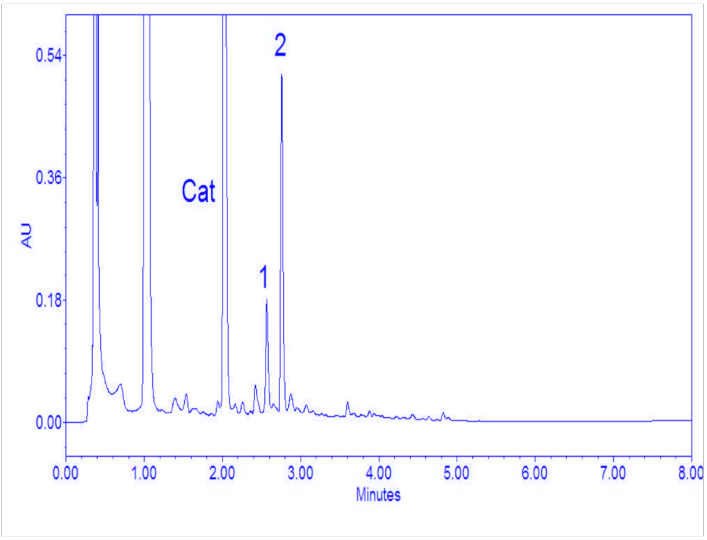
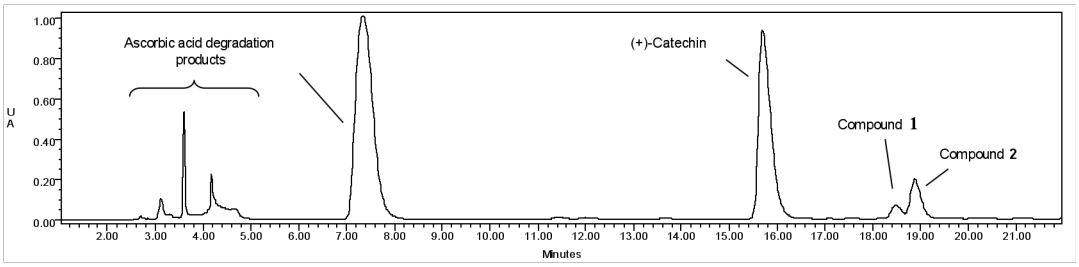


Figure 3.3. HPLC chromatogram at 280 nm of ascorbic acid/(+)-catechin solution after 4 days at 45 °C.



Compounds **1** and **2** yielded identical ESI-MS spectra with peaks at m/z 401 and 403 in the negative and positive ion modes respectively, and high resolution MS data established a molecular formula of $C_{20}H_{18}O_9$ (±3 ppm) for both compounds. These facts coupled with identical UV/Visible spectra (Figure 3.5) suggested that the compounds were isomers and contained the (+)-catechin chromophore (λ_{max} 278 nm), albeit slightly bathochromically shifted to 284 nm. Loss of 113 amu from the compounds gave ions with the same mass as (+)-catechin-H^+ (m/z 289 (negative ion mode), $C_{15}H_{13}O_6$), suggesting that the two compounds were both substituted with a $C_5H_5O_3$ fragment.

Figure 3.4. Formation and decay of compounds 1 and 2 in model wine system.

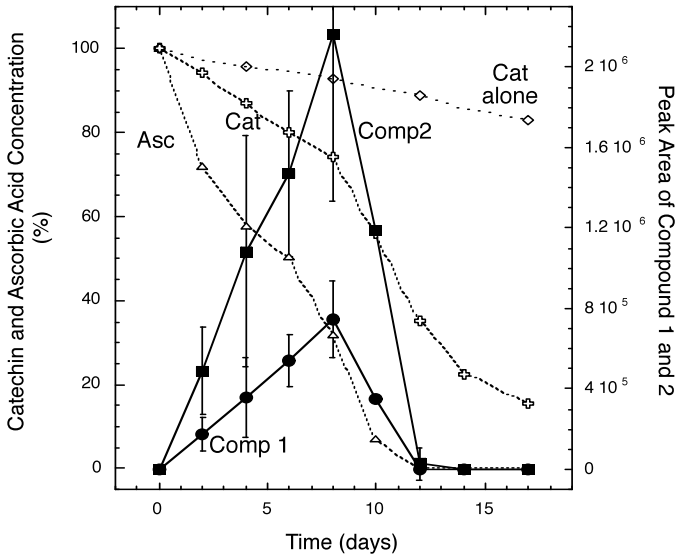
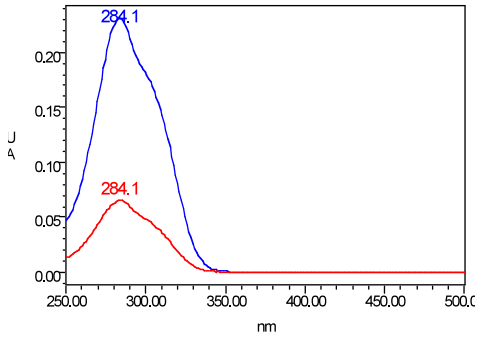


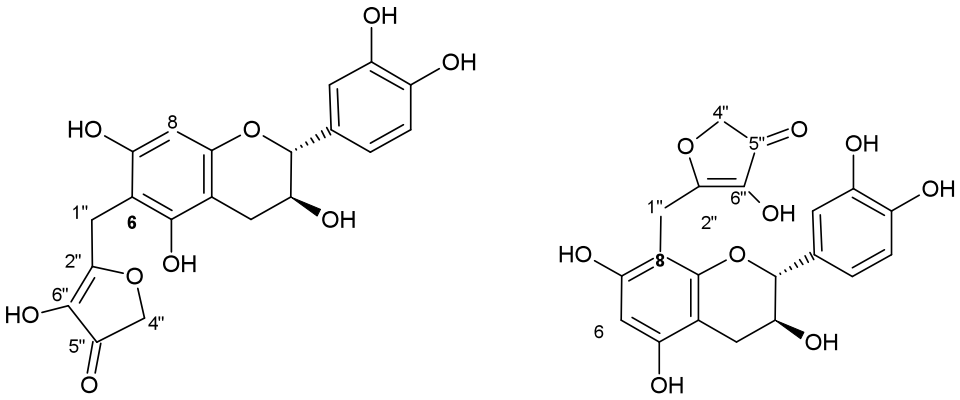
Figure 3.5. UV/Visible spectra of compounds 1 and 2.



The compounds were first isolated by a crude extraction and then semi-preparative chromatography in order to fully characterise their identity. This isolation method is outlined in detail in Barril et al. (2009) but several aspects of the isolation warrant comment. An ethanol free sample containing ascorbic acid (1 g/L) and (+)-catechin (0.5 g/L) was prepared and incubated at 45 °C until compound **1** and **2** were at maximum intensities (i.e. 8 days). At this time ascorbic acid (1 g/L) was added, to slow the otherwise rapid degradation of the accumulated colourless products, and the sample was stored at 4 °C. The solution was then later passed through a solid-phase extraction cartridge (Strata C18-E, 70g/150mL, Phenomenex, USA) and compounds **1** and **2** eluted with 80% aqueous methanol with 2% formic acid. The eluent was concentrated and then injected directly onto preparative scale HPLC (Barril et al. 2009) where compounds **1** and **2** were collected. NMR analyses were then conducted on compounds **1** and **2** as described by Barril et al. 2009.

Based on the NMR data (Barril et al. 2009), and the data already mentioned above, compounds **1** and **2** were identified as (+)-1”-methylene-6”-hydroxy-*2H*-furan-5”-one-6-catechin (Figure 3.6 left-hand side, MHF-6-catechin) and (+)-1”-methylene-6”-hydroxy-*2H*-furan-5”-one-8-catechin (Figure 3.6 right-hand side, MHF-8-catechin), respectively.

Figure 3.6. Identity of compound 1 (left hand side) and 2 (right hand side).



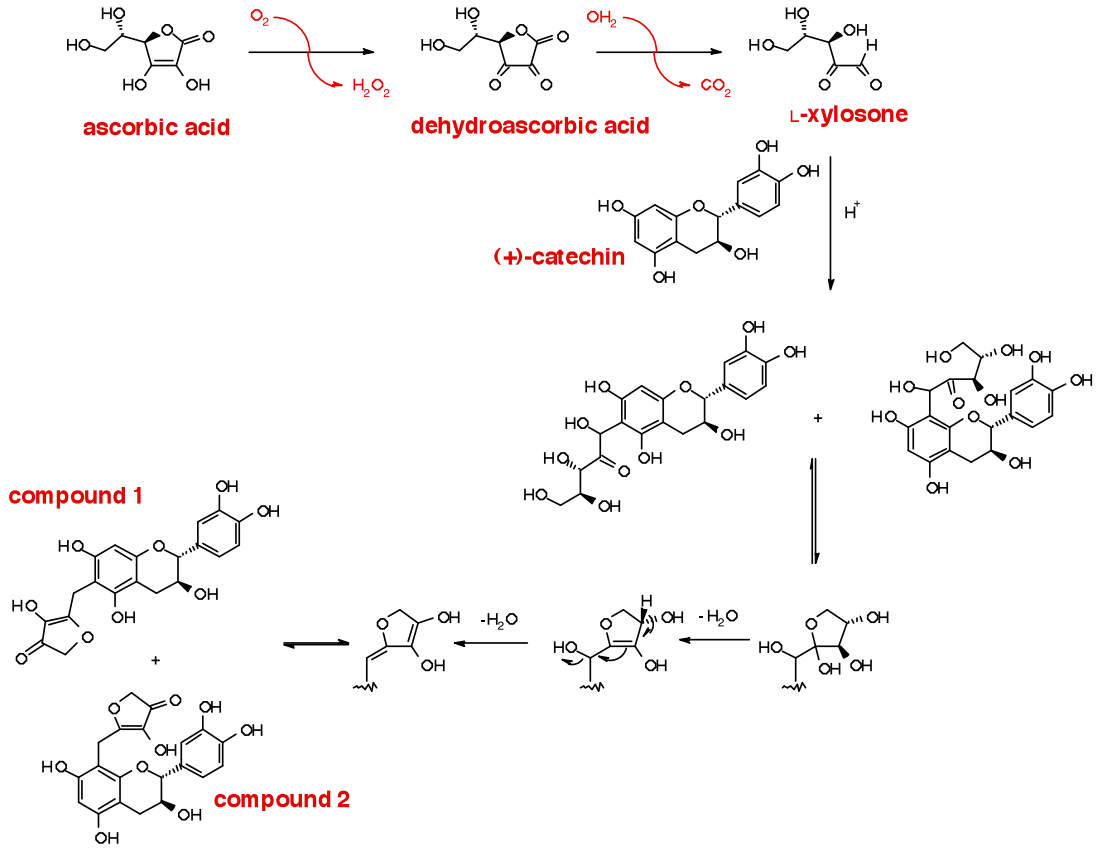
Compounds **1** and **2** are the first reported products to form between a carbon-based degradation product of ascorbic acid and (+)-catechin. Their formation explains the accelerated degradation of (+)-catechin in the model wine solutions containing ascorbic acid. The formation of compounds **1** and **2** also confirms that (+)-catechin is not being directly oxidised in the presence of ascorbic acid but is rather forming an addition product with a degradation compound of ascorbic acid. These results highlight the multifaceted role that ascorbic acid may have in wine or other food systems. While on the one hand, ascorbic acid undoubtedly acts as an antioxidant, on the other, breakdown products of ascorbic acid are capable of further reactions. In wines, those reactions may be with flavanols (e.g. (+)-catechin), which are the compounds most correlated to the browning of white wine.

Compounds **1** and **2** are stable substituted (+)-catechin monomers provided some ascorbic acid remains in the model wine system. Other examples of stable (+)-catechin-adduct monomers are formaldehyde-substituted (+)-catechins generated as minor products in the reaction between glyoxylic acid and (+)-catechin (Es-Safi et al. 2000), and certain oak-derived aldehydes that also form pyrylium-chromophores on addition to single (+)-catechin units (De Freitas et al. 2004). This is in contrast to many other (+)-catechin addition products that readily undergo further addition and form bridged-(+)-catechin dimers. The reliance on ascorbic acid for the stability of compounds **1** and **2** suggests that an oxidation step is required for their conversion to some other species.

Of the many degradation products of ascorbic acid that are reported in the literature, L-xylosone (Figure 3.1) appeared to be a reasonable candidate as a precursor to compounds **1** and **2**. Indeed, a retro-synthetic analysis (Figure 3.7) suggested that xylosone could be involved in the formation of **1** and **2**. Indeed, no other known degradation product of ascorbic acid allowed such feasible formation.

L-xylosone, an aldehyde, is formed from the hydrolysis and subsequent decarboxylation of dehydroascorbic acid (Whiting and Coggins 1960), but rapidly degrades to other aldehydes and ketones (Yuan and Chen 1998, Shinoda et al. 2004). Its lack of an appropriate chromophore to allow detection above 250 nm, and its relatively polar character and expected elution near the injection front under the chromatographic conditions used here means that its detection by the system utilised in this experiment was not possible.

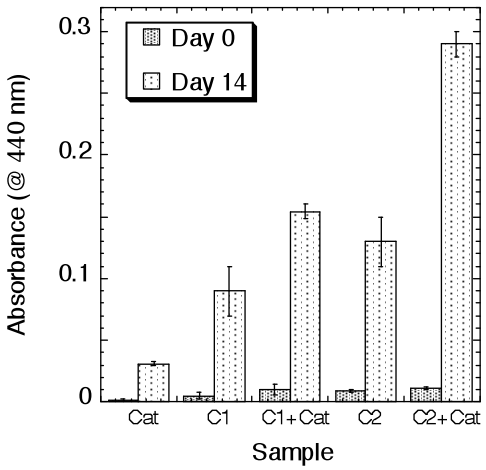
Figure 3.7. Proposed mechanism for the formation of compounds 1 and 2.



As adapted from Barril et al. (2009)

Consequently, L-xylosone was synthesised as per the method described by Salomon et al. (1952), and reacted with (+)-catechin in a model wine system consisting of a tartaric acid-buffered 12% ethanol solution. Colourless compounds were subsequently generated that had identical retention times, UV-visible spectra and MS data as compounds **1** and **2** (as reported above). This confirmed that L-xylosone was the precursor to compounds **1** and **2** in model wine solutions of (+)-catechin and ascorbic acid.

Figure 3.8. 440 nm absorbance of samples during storage at 45 °C.
Cat = 0.25 mM (+)-catechin, C1 = 0.02 mM compound 1, and C2 = 0.1 mM compound 2.

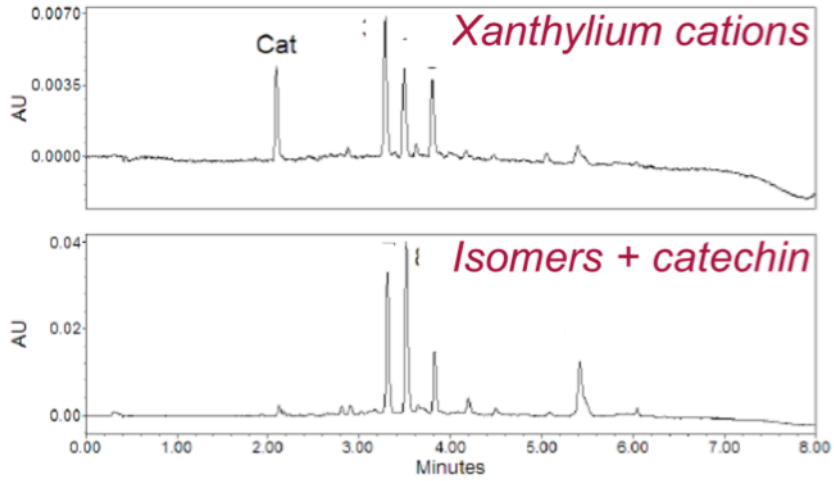


Given that a link existed between the degradation of compounds **1** and **2** and the accelerated production of colour in wine-like solutions (Section 2), experiments were conducted to confirm the ability of compounds **1** and **2** to form coloured compounds. When 0.02 mM of compound **1** and 0.1 mM of compound **2**, were prepared (n = 3) at (+)-catechin

concentrations of 0 and 0.25 mM in the model wine solution (formic-buffer) they initially afforded 440 nm absorbances (a measure of yellow colour) of less than 0.012 absorbance units. However after incubation at 45 °C in darkness, the samples all increased in 440 nm absorbance to reach the values shown in Figure 3.8. It is evident that both compound **1** and **2** were precursors to coloured compounds and that that the presence of 0.25 mM (+)-catechin enhanced the formation of coloured compounds by compounds **1** and **2**.

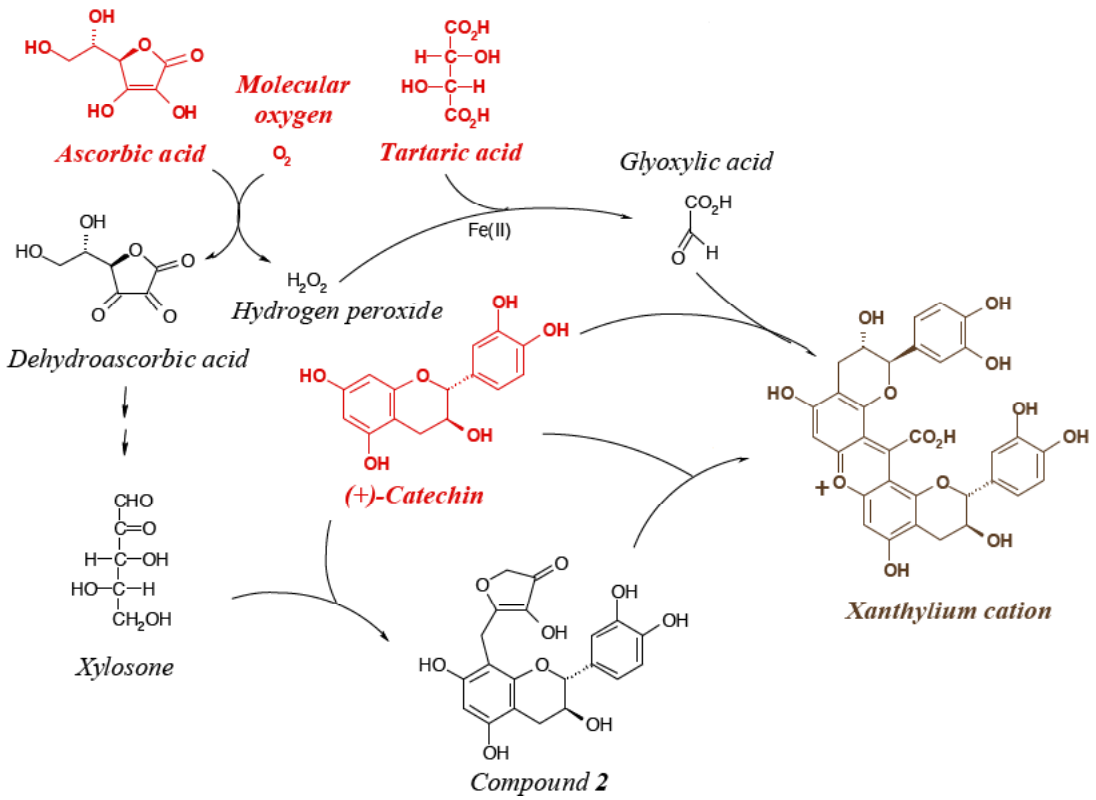
Analysis by UPLC at day 14 revealed that all samples with added compound **1** or **2** had three major peaks (Figure 3.9) in the 440 nm chromatograms. The UV-Visible spectra extracted from the UPLC-DAD demonstrated that these major peaks, in all the samples with compound **1** or **2** present, showed identical absorbance maxima at 280 and 440 nm with a shoulder at 310 nm. LC-MS data associated with these peaks all showed m/z signals at 617 in the positive ionisation mode and the major fragmentation ion at 465 m/z. Such data was identical to the xanthylium cations, discussed in detail in Section 2 (Figure 2.5), generated from the reaction of (+)-catechin with glyoxylic acid. Consequently, the results demonstrate that compounds **1** and **2** form the same xanthylium cations as those generated by (+)-catechin and glyoxylic acid. Intriguingly, this was the case whether compound **1** or **2** was incubated with (+)-catechin or not, but more xanthylium cations were generated if (+)-catechin was present (Figure 3.9).

Figure 3.9. 440 nm chromatograms of xanthylium cations and pigments generated from compound 2 and (+)-catechin.



The actual mechanism for the conversion of compounds **1** and **2** to xanthylium cations is not certain and is the subject of on-going investigations. However, the results do establish a mechanistic link between (+)-catechin, a degradation product of ascorbic acid and the production of xanthylium cations (Figure 3.10) that has previously been postulated (Section 3, Bradshaw et al. 2003). The results also add xylosone to the list of aldehyde compounds (i.e. glyoxylic acid and glyoxal) that can react with (+)-catechin to generate xanthylium cation pigments. Such a mechanism will be relevant to all beverages that contain flavanols and ascorbic acid, such as fruit juices.

Scheme 3.10. Formation of xanthylium cations from (+)-catechin and ascorbic acid in a tartaric acid-buffered model wine system



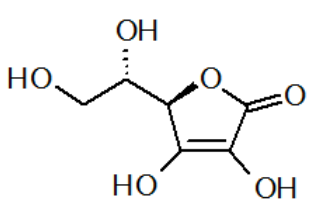
In conclusion, the results of this study show a unique outcome from the reactions of two 'antioxidants' in wine-like conditions, ascorbic acid and (+)-catechin. The process is far more complex than the anticipated and simple transfer of two electrons and two protons, and demonstrates that ascorbic acid is a highly unpredictable molecule capable of inducing a variety of reaction processes. From a model white-wine system, two new "natural" products derived from (+)-catechin and ascorbic acid were isolated. The two compounds were unequivocally identified based on spectral data, primarily NMR spectroscopy. A retro-synthetic study indicated that the compounds could be formed from L-xylosone, a known degradation product of ascorbic acid, and (+)-catechin. Both isomers were independently synthesised from L-xylosone to establish this compound as a key precursor to compounds **1** and **2**. The accumulation of the isomers (**1** and **2**) depends on the presence of ascorbic acid and they eventually react further to give coloured xanthylium cation pigments.

4. Comparison of ascorbic acid and erythorbic acid in terms of extent of pigment production

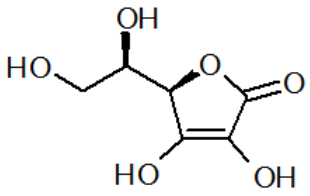
When this project was being initiated, anecdotal advice was received from some winemakers that the project should also investigate erythorbic acid as well as ascorbic acid. One winemaker stressed that 'in my experience, erythorbic acid always gives less colour that ascorbic acid'.

Erythorbic acid and ascorbic acid are diastereoisomers (see Figure 4.1). Diastereoisomerism means that the orientation in space around one carbon atom differs: this is shown by the dotted line and solid line in Figure 4.1. Given the similarity in structures, it is reasonable to expect that they should both have antioxidant activities. Erythorbic acid, in fact, is commonly described as having identical antioxidant capability to ascorbic acid in white wine (Ribèreau-Gayon et al. 2006, Fessler 1961), while in other foods or biological systems, their comparative antioxidant capability is variable (Mancini et al. 2007, Sapers and Ziolkowski 1987, Kröger-Ohlsen and Skibsted 1997) but in some instances erythorbic acid shows superior activity to ascorbic acid (Reyes and Luh 1962, Yourga et al. 1944, Fidler et al. 2004). There are also several anecdotal observations in the Australian wine industry that erythorbic acid may have some improved abilities over ascorbic acid to prevent the production of dominant red tints, or pinking, in white wine. However, despite this, ascorbic acid is still the main complementary antioxidant to sulfur dioxide used in white wine.

Figure 4.1. Structures of the diastereoisomers, ascorbic acid and erythorbic acid



Ascorbic acid



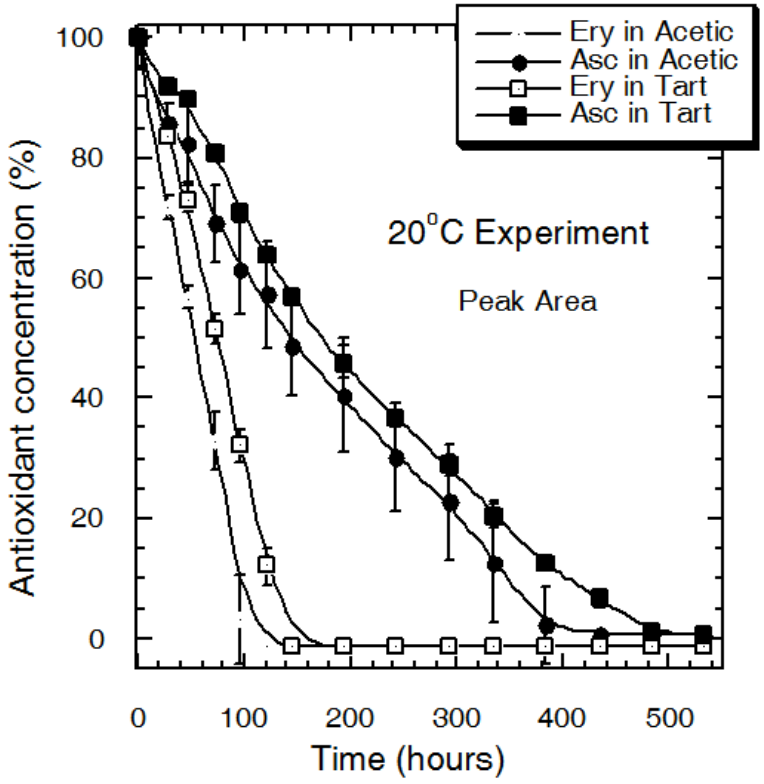
Erythorbic acid

The first step was to compare the relative rates of degradation of both anti-oxidant compounds in the model wine system. The degradation rates were determined by monitoring the concentrations of both compounds over time by UPLC. As discussed in Section 3, UPLC measurement times are much faster than traditional HPLC and this minimises the amount of sample degradation that can occur during the analysis itself. After trace metal analysis of the reagents, the ascorbic acid used was found to contain trace metal contamination which contributed ~10 µg/L in the final model wine system. The oxidation rate of ascorbic acid (and presumably erythorbic acid) is known to be critically dependent on trace metal concentrations (Buettner and Jurkiewicz 1996). Consequently, 100 µg/L iron was added to all samples in order to eliminate the impact of the trace iron contamination in the ascorbic acid. The experiments were conducted in such a fashion to maintain oxygen saturation (or as near as possible) in the solution throughout the experiment.

Figure 4.2 compares the degradation rates. Intriguingly, erythorbic acid decays much faster than ascorbic acid in these wine-like conditions. In the model wine system adopted here, iron ions at a concentration of 100 µg/L are most probably chelated by the chiral pH buffer L-tartrate. An explanation as to the different rate of antioxidant degradation may be that it was due to the chirality of the metal-buffer complex. To test this hypothesis, L-tartaric acid was substituted by acetic acid, an achiral organic acid, and the antioxidant decay monitored

during storage at 20 °C. Despite the change in supporting buffer, the decay rates for ascorbic acid and erythorbic acid were still significantly different from each other at 1.6 mg/hour and 3.9 mg/hour respectively from 0 to 96 hours, and only slightly different to those with the tartrate buffer (Figure 4.2). Based on these results, it can be concluded that the rates of decay of the antioxidants are not related to the chirality of the supporting buffer.

Figure 4.2. Decay in erythorbic acid and ascorbic acid in model wines buffered to pH 3.2 with either tartaric acid or acetic acid.



Consideration also must be given to the ability of both ascorbic acid and erythorbic acid to undergo non-oxidative degradation. This degradation is often termed 'anaerobic degradation' despite still occurring during the oxidative degradation of the antioxidants (Kurata and Sakurai 1967). However, based on the kinetic and activation energy data of Anmo et al. (1971), the first order non-oxidative decay constants (20 °C) for ascorbic acid and erythorbic acid were calculated to be 0.28×10^{-4} hours^{-1} and 0.60×10^{-4} hours^{-1}, respectively. This corresponds to a loss of less than 1.5 % the initial concentration of the antioxidants in the time scale of Figure 4.2. Based on these low rates of anaerobic degradation, it was apparent that non-oxidative decay mechanisms were not responsible for the different rates of antioxidant decay between antioxidants in Figure 4.2.

Consequently, it seems that erythorbic acid is intrinsically capable of reacting more rapidly, compared to ascorbic acid, with either molecular oxygen, hydrogen peroxide and/or some other degradation product of the antioxidant. Therefore, although ascorbic acid may protect the model wine for a longer period of time, due to its slower reaction in the presence of oxygen, erythorbic acid may offer a greater capacity for protection, and less possibility of oxidative side-reactions, due to its faster reaction in the presence of oxygen.

We have previously reported (GWRDC Report CSU01/02) that the increased colouration that is observed in the presence of (+)-catechin-type flavanols is due to the formation of

xanthylium pigments. In this earlier work, we also demonstrated that the extent of colour production, as measured by the absorbance at 440 nm, was much higher with (-)-epicatechin compared to (+)-catechin. This, we demonstrated, was a consequence of higher absorptivity coefficient for the (-)-epicatechin derived xanthylium pigment, rather than the production of a higher concentration of the pigment.

The two flavanols, (+)-catechin and (-)-epicatechin are diastereoisomers (Figure 4.3) and this diasteoisomerism is carried forward to the xanthylium pigments (Figure 4.4). The important outcome is that (-)-epicatechin will always generate a higher colour than (+)-catechin in an oxidative environment, an observation first made by Simpson in his 1982 study of the link between browning propensity of white wine and phenolic composition.

Figure 4.3. Structures of the diasteroisomers, (+)-catechin and (-)-epicatechin

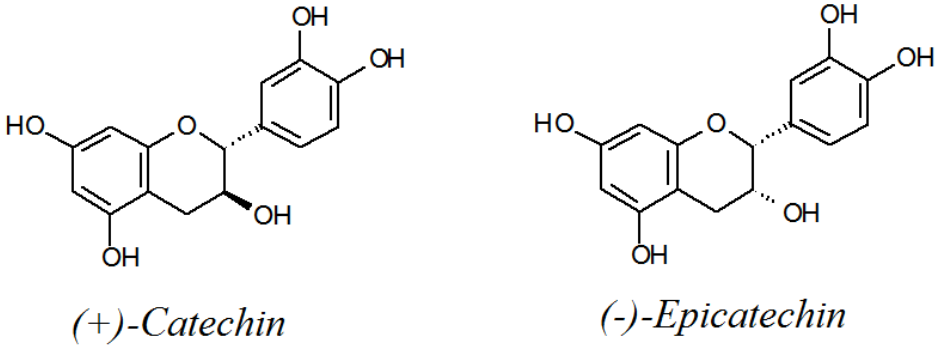
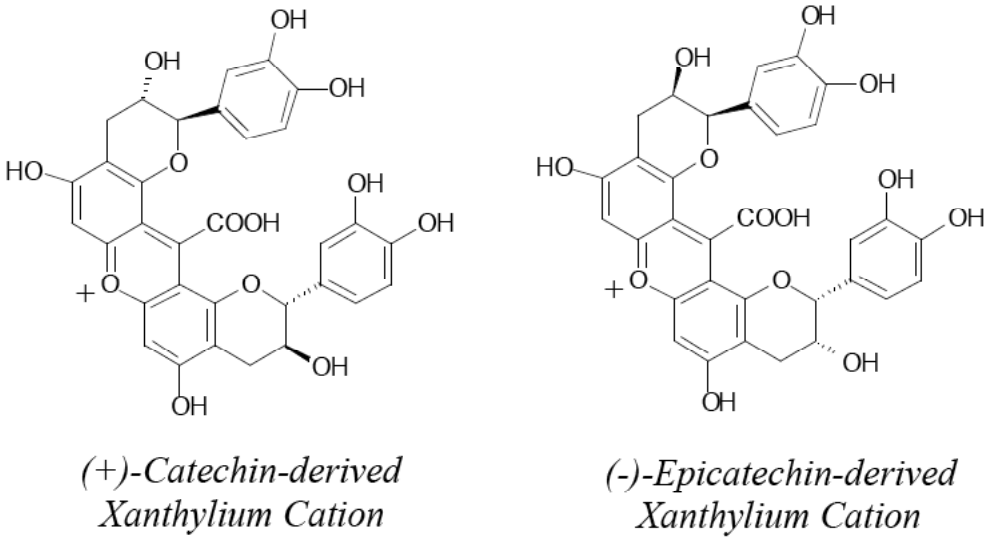


Figure 4.4. Structures of the xanthylium pigments derived from (+)-catechin and (-)-epicatechin. Note that the free carboxyl (-COOH) group can form the ethyl ester in a wine matrix.



In this project, as described above in Section 2, we have demonstrated that ascorbic acid enhances the production of xanthylium pigments. It was therefore of interest to examine the various combinations of ascorbic acid, erythorbic acid, (+)-catechin and (-)-epicatechin to ascertain whether erythorbic acid does in fact reduce the amount of colour development in comparison to ascorbic acid.

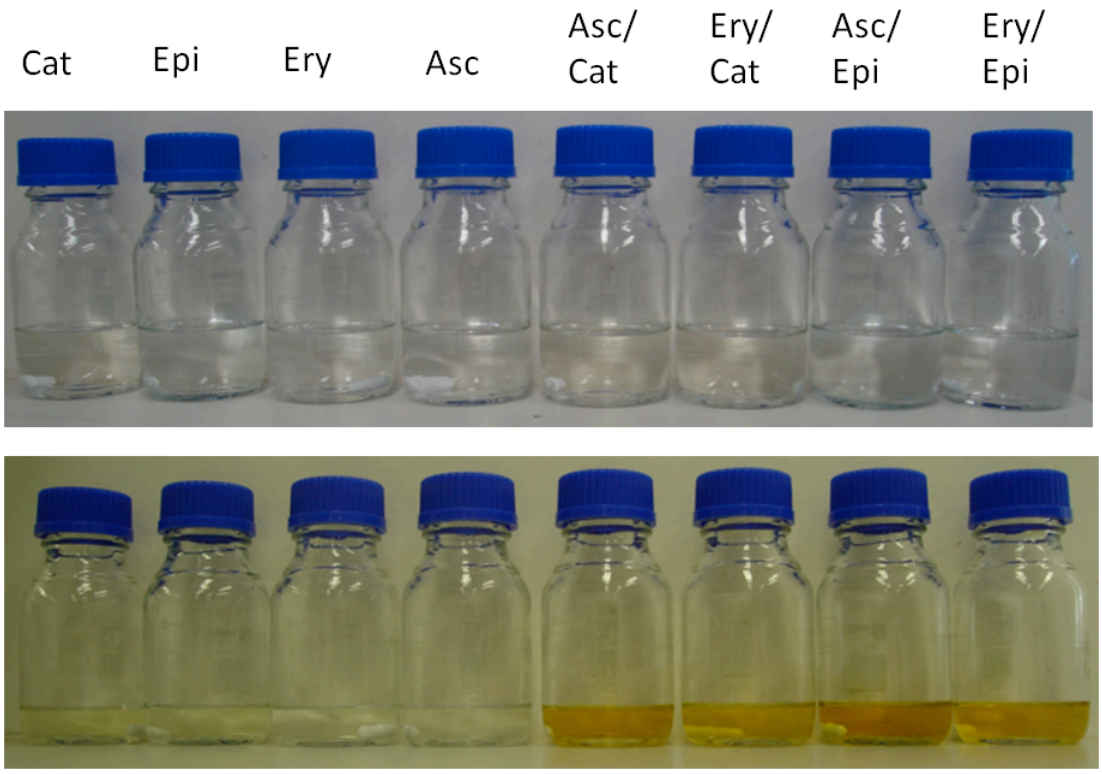
The following solutions were prepared, in triplicate to allow for statistical analysis, in the tartaric acid wine base:

- (+)-catechin (Cat)
- (-)-epicatechin (Epi)
- ascorbic acid (Asc)
- erythorbic acid (Ery)
- (+)-catechin and ascorbic acid (Asc/Cat)
- (+)-catechin and erythorbic acid (Ery/Cat)
- (-)-epicatechin and ascorbic acid (Asc/Epi)
- (-)-epicatechin and erythorbic acid (Ery/Epi)

The standard reaction protocol (see Method section above) was followed for the 14 days reaction time.

The importance of ascorbic acid and erythorbic acid in enhancing the colour development is apparent in the images in Figure 4.5. These images also show that the colour development is higher with (-)-epicatechin compared with (+)-catechin, in agreement with previous observations. The images also show that ascorbic acid produces more colour than does erythorbic acid: compare Asc/Cat with Ery/Cat and Asc/Epi with Ery/Epi.

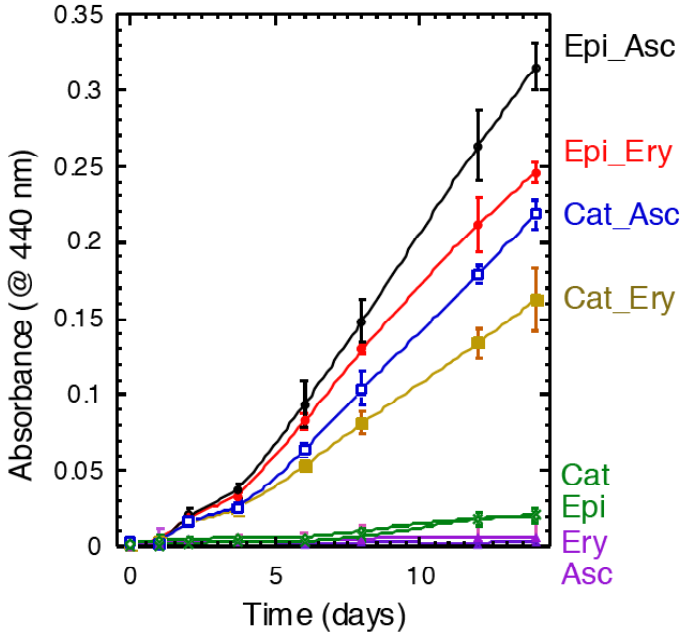
Figure 4.5. Images of the reaction systems at Day 0 (upper set) and Day 14 (lower set).



All the samples were initially a similar pale yellow colour and consequently of low 440 nm absorbance (day 0, Figure XX). After 14 days of incubation in darkness at 45 °C the binary combinations of flavanol and antioxidant constituents increased in 440 nm absorbance intensity, much more rapidly than for the samples with a single component (Figure 4.6). This was consistent with past reports for the ascorbic acid and (+)-catechin combination in model wine systems (Section 2 and 3) but demonstrated that erythorbic acid also had the potential to induce colouration. Of the binary systems, those with ascorbic acid as the antioxidant were of higher 440 nm absorbance, as were those with the flavanol compound, (-)-epicatechin. Consequently, the ascorbic acid/(-)-epicatechin pair resulted in the highest 440 nm absorbance after 14 days while the erythorbic acid/(+)-catechin combination gave the lowest

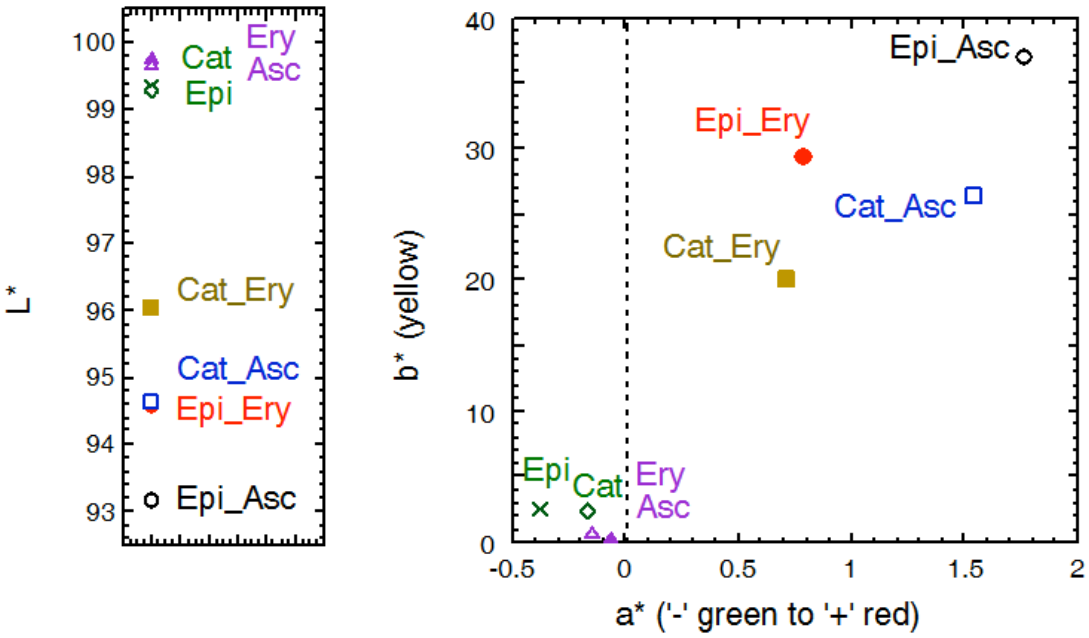
440 nm absorbance. The presence of a lag period before the accelerated colouration was evident in all the binary samples (Figure 4.6), and duration of the lag period appeared similar in length regardless of the antioxidant present.

Figure 4.6. The 440 nm absorbance of samples over time. Error bars indicate the 95 % confidence limits.



Although the data in Figure 4.6 provide an insight into the 440 nm absorbance change in the samples, it does not provide a measure of the overall colour of the samples. To achieve this, the samples were also analysed by CIELab, which provides an analytical description of sample colour with respect to three parameters: a* indicating green to red colouration, b* indicating blue to yellow colouration, and L* indicating transparent (100) to opaque (0) colouration. At day 0, the samples were either of little, or no, difference to each other as described by the CIELab parameters (data not shown). However, by day 14 a divergence in the CIELab data for the samples was apparent (Figure 4.7). The description of the samples by the b* and L* parameters provided an ordering of the samples consistent with the 440 nm data (Figure 4.6): Epicatechin/Ascorbic > Epicatechin/Erythorbic > Catechin/Ascorbic > Catechin/Erythorbic. Intriguingly, the CIELab analysis also showed that the samples with ascorbic acid had higher a* values than those with erythorbic acid. Therefore, these CIELab data demonstrate that not only can erythorbic acid provide lower intensity of yellow colour in the oxidising samples compared to ascorbic acid, but it also can limit the amount of red colouration. Such an outcome is consistent with the anecdotal observations by winemakers that erythorbic acid is more effective than ascorbic acid against pinking, or development of red tints, in white wines. In the samples with ascorbic acid, the increased production of the primary yellow and red colours meant that the samples appeared more orange than the samples with erythorbic acid, which was in agreement with the visual assessment of the samples (Figure 4.5).

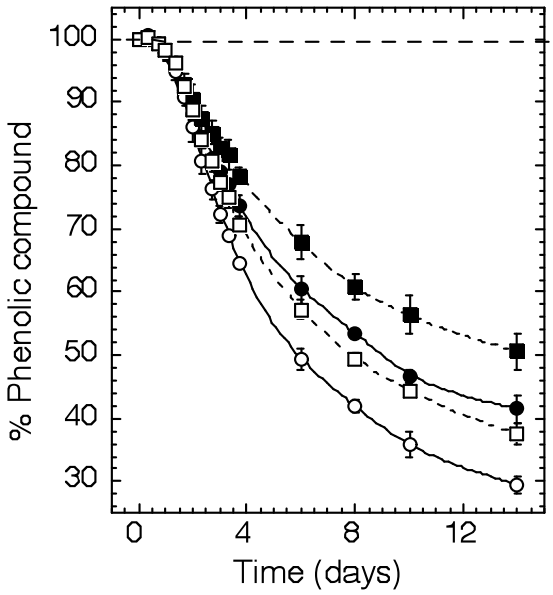
Figure 4.7. CIELab analysis of samples after 14 days of storage at 45 °C in darkness. The results are significantly different (P = 0.05) for any samples whose symbols are not in contact for a given parameter. The samples are: (●) (-)-epicatechin/erythorbic acid, (○) (-)-epicatechin/ascorbic acid, (■) (+)-catechin/erythorbic acid, (□) (+)-catechin/ascorbic acid, (X) (-)-epicatechin, (◊) (+)-catechin, (▲) erythorbic acid, and (Δ) ascorbic acid. Error bars indicate the 95% confidence limits (n = 3).



Pigment production in these model systems requires flavanol consumption and so it was of value to measure the change in the concentration of (+)-catechin and (-)-epicatechin. A comparison of the rate of loss of each flavanol compound (Figure 4.8) demonstrates that erythorbic acid, like ascorbic acid (Section 2), accelerates the consumption of the accompanying flavanol. However, at day 14, the loss of the flavanol compound is significantly less (P = 0.05) in the samples with erythorbic acid compared to those with ascorbic acid, and the difference is evident well after the depletion of both antioxidants in the samples (i.e. 48 hours). Such results support the above proposition that erythorbic may offer a greater capacity for protection of wine components, such as flavanols, against oxygen than ascorbic acid. As far as the flavanol compounds are concerned, the loss of (-)-epicatechin is significantly faster than (+)-catechin, and this is consistent with the respective reaction rates of these compounds with aldehydes such as acetaldehyde and glyoxylic acid (Labrouche et al. 2005, Es-Safi et al. 1999c).

In Section 3 of this report, we described the identification of a colourless precursor formed during the degradation of ascorbic acid. Tracking the possible role of this precursor in explaining the difference in reactivity between ascorbic acid and erythorbic acid was undertaken in this component of the project.

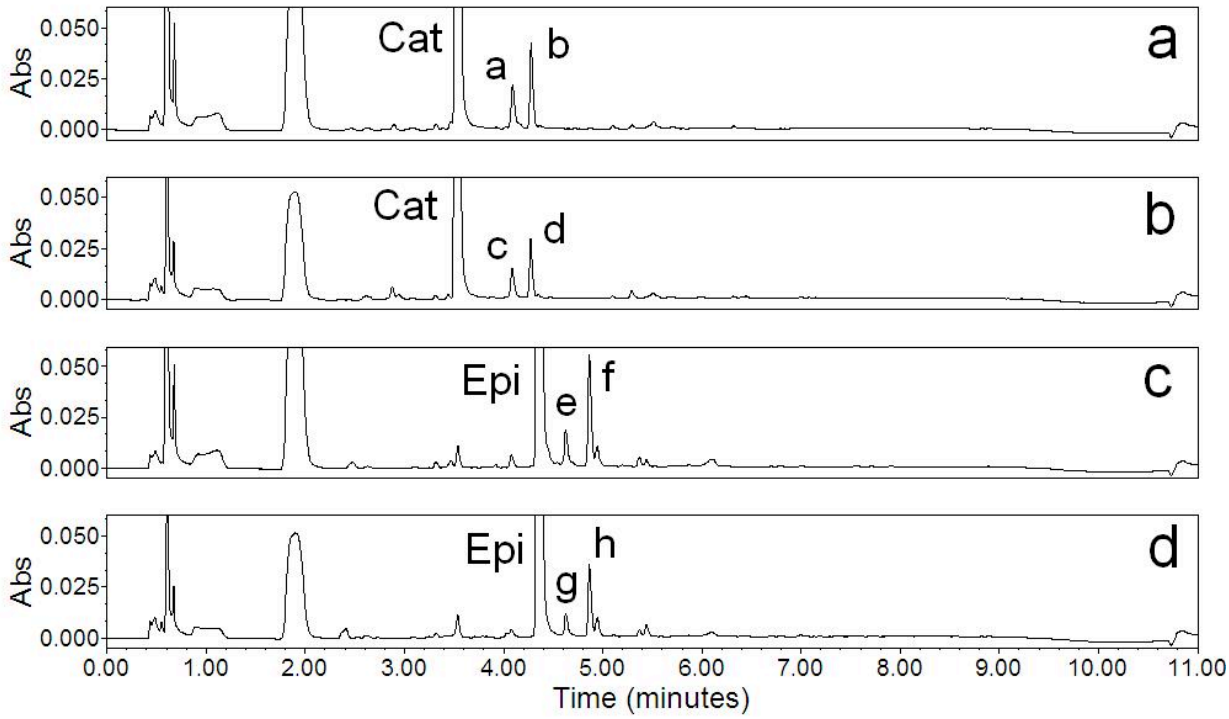
Figure 4.8. Decay in flavanol compound concentration during the storage of samples at 45 °C. The samples are: (●) (-)-epicatechin/erythorbic acid, (○) (-)-epicatechin/ascorbic acid, (■) (+)-catechin/erythorbic acid, (□) (+)-catechin/ascorbic acid. The concentrations for (+)-catechin or (-)-epicatechin alone did not significantly (P = 0.05) deviate from their starting concentration. Error bars indicate the 95% confidence limits (n = 3).



During the incubation of a flavanol with an antioxidant, the major products generated in the first two days were peaks **a** to **h** (Figure 4.9) in the 280 nm chromatograms. Samples containing (+)-catechin with either ascorbic acid or erythorbic acid showed colourless products with the same retention times (peaks **a** with **c**, and **b** with **d**, Figure 4.9), UV/visible spectra (λ_{max} 284nm), and LC-MS data (403 m/z, positive ion mode), and hence were identical products. As mentioned in section 3, these colourless products have already been identified in the (+)-catechin and ascorbic acid system (peaks **a** and **b**, Figure 4.9; **1** and **2** in Figure 4.10) as MHF-6-catechin (Figure 3.6) and (+)-MHF-8-catechin (Figure 3.6) isomers respectively, and both have the ability to evolve into yellow xanthylium cation pigments. Their formation involves the degradation of ascorbic acid to form the aldehyde, L-xylosone, which then undergoes an electrophilic addition to the phloroglucinol ring of (+)-catechin at either carbons-8 or -6, followed by rearrangement to form the (+)-MHF-catechin (Figure 3.8). Given that the MHF moiety has no chiral carbons, and that erythorbic acid presumably can form either identical degradation products to ascorbic acid or related stereoisomers (Nishikawa and Kurata 2000, Miyake and Kurata 1998), it is understandable that erythorbic acid and ascorbic acid provide the same substituted-(+)-catechin compounds.

Equivalent products (peaks **e** to **h** in Figure 4.9, **3** and **4** in Figure 4.10) were also identified for (-)-epicatechin during its incubation with either ascorbic acid or erythorbic acid. This identification was based on the identical UV/visible spectra (λ_{max} 284nm), LC-MS parent ion data (403 m/z, positive ion mode) and LC-MS fragmentation data as for the (+)-MHF-catechin isomers. The fragmentation ions included the retro Dies-Alder cleavage of the dihydropyran moiety in the isomers to provide an ion at 251 m/z, the loss of the (+)-catechin or (-)-epicatechin unit to leave the methylenehydroxyfuranone unit at 113 m/z, or a combination of both fragmentation mechanisms to yield an ion with m/z of 139. Another fragment ion at 151 m/z appeared to be the 139 m/z ion with a $-CH_2^+$ group attached, hence the cleavage of the hydroxyfuranone unit with the methylene group remaining behind.

Figure 4.9. 280 nm chromatograms of the (a) (+)-catechin/ascorbic acid, (b) (+)-catechin/erythorbic acid, (c) (-)epicatechin/ascorbic acid, and (d) (-)-epicatechin/erythorbic acid samples after 32 hours of storage at 45 °C. The peaks eluting before the flavanol are due to the antioxidant and its degradation products.



During the reaction of (+)-catechin and ascorbic acid, the concentration of MHF-addition isomers increases to a maximum and then begins to decrease upon depletion of ascorbic acid in solution (Section 3). The results in Figure 4.11 confirm that this is also the case for the (-)-MHF-epicatechin isomers. In fact, the decrease in the erythorbic acid-derived MHF-addition products (Figure 4.11) occurs earlier than for the equivalent ascorbic acid products, coinciding with the different decay rates for the respective antioxidants (i.e. demonstrated at 20 °C in Figure 4.2).

The 280 nm chromatogram peak areas for the MHF-addition products generated by erythorbic acid are substantially lower in intensity than those generated by ascorbic acid. In order to convert chromatographic peak areas into concentrations of the MHF-addition products, it is necessary to know the molar aborptivity coefficients of these species. Although the molar absorptivity coefficients for these compounds are yet to be reported, relative concentrations of the various MHF-addition products (**1**-**4**, Figure 4.10) can be calculated based on the ratio of their 280 nm molar aborptivity coefficients. Using the method reported by Labrouche *et al.* (2005), the ratio of 280 nm molar aborptivity coefficients for MHF-8-catechin (**1**) to MHF-8 -epicatechin (**3**) was 1.08 ± 0.09. For the 6-substituted isomers **2** and **4** the ratio was 1.1 ± 0.2. Therefore, the molar aborptivity coefficient between the different MHF-flavanols are not significantly different (P = 0.05) and hence the peak areas shown in Figure 4.11 allow the relative concentrations of the different species to be compared. That is, erythorbic acid produces a maximum concentration for the MHF-flavanols that is around half that of those generated from ascorbic acid. Furthermore, the type of flavanol appeared to have little influence on the maximum MHF-flavanol concentration reached.

Figure 4.10. MHF-flavanol compounds identified in the model wine systems.

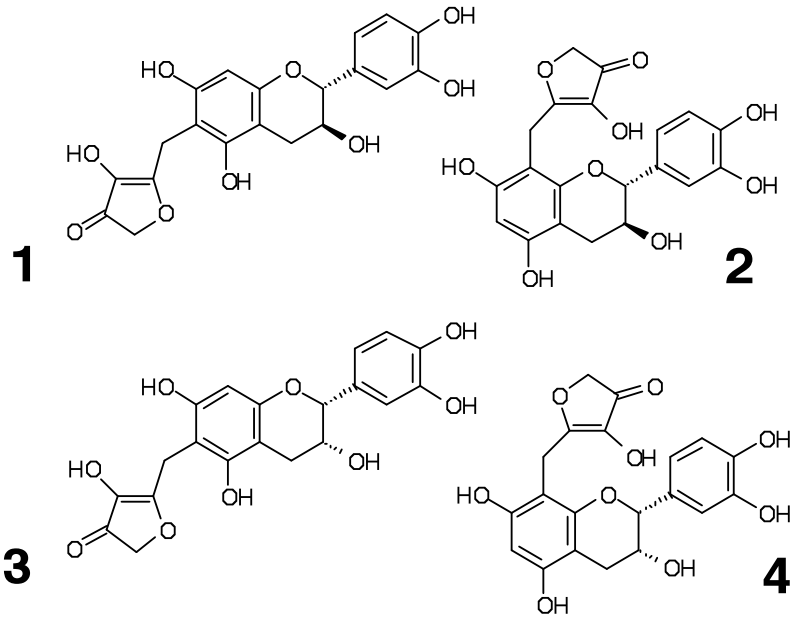
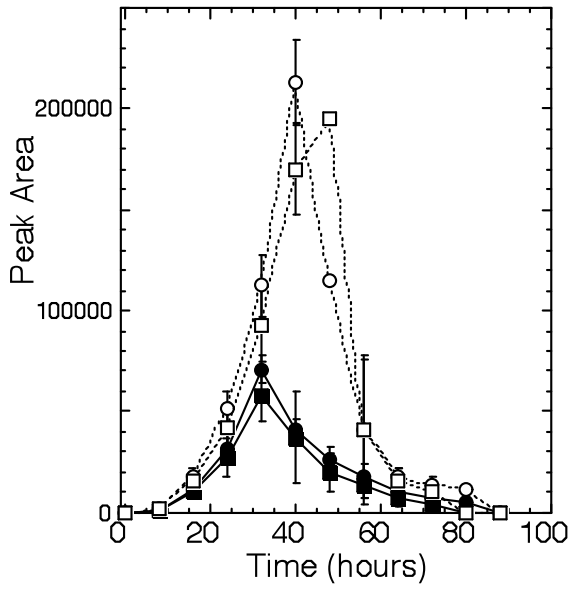


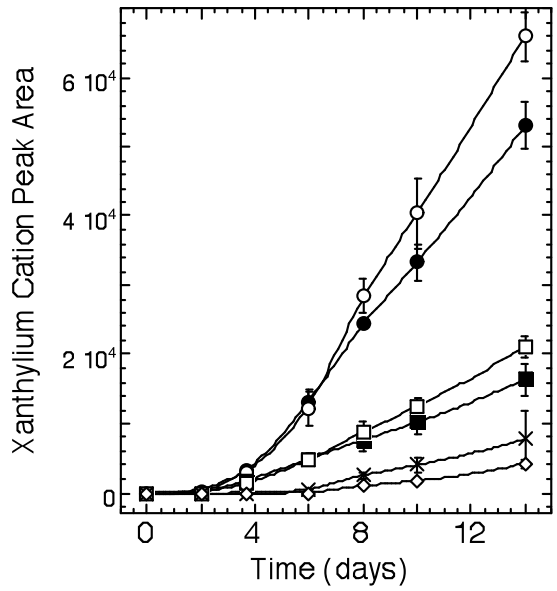
Figure 4.11. Peak area of the MHF-8-flavanols during the storage of samples at 45 °C. The samples are: (●) (-)-epicatechin/erythorbic acid, (○) (-)-epicatechin/ascorbic acid, (■) (+)-catechin/erythorbic acid, (□) (+)-catechin/ascorbic acid. Error bars indicate the 95% confidence limits (n = 3).



In all model wines, the main coloured compounds detected in the solutions were the xanthylium cations, in both their acid and ester forms, as reported by Labrouche *et al*. (2005). The xanthylium cations were identified by matching their UPLC retention times with xanthylium cations synthesised by published methods (Es-Safi et al. 1999a), UV/visible spectra (λ_{max} 280, 440 or 460 nm, with shoulder at 310 nm), and LC-MS parent ion data (617 m/z acid form, 645 m/z ester form, positive ion mode). Therefore, regardless of whether ascorbic or erythorbic acid is present in solution, identical xanthylium cations were formed for a given flavanol. However, peak areas from the 440 nm chromatograms (Figure 4.12) show that the (-)-epicatechin-derived xanthylium cations provide over twice the intensity of the corresponding (+)-catechin-derived pigments. This is consistent with the known extinction coefficient data for the corresponding xanthylium cations (Labrouche et al. 2005),

as well as the increased rate of loss of (-)-epicatechin compared to (+)-catechin (Figure 4.8). Furthermore, there is good consistency between the 440 nm absorbance data in Figure 4.6 and the xanthylium peak area data in Figure 4.12, indicating that xanthylium cations make the predominant contribution to absorbance at 440 nm. In the samples with ascorbic acid, the higher concentration of the yellow xanthylium cations led to increased tailing of their absorbance into the 500 nm region of the spectrum and this accounted for the increased yellow and red colouration in the samples with ascorbic acid. This is consistent with the CIELab data reported above. Given that the xanthylium cations were the main pigments generated in all samples, it is apparent that the (-)-MHF-epicatechin isomers were also precursors to xanthylium cation pigments, as is known to be the case for (+)-MHF-catechin (*12*).

Figure 4.12. Comparison of the xanthylium cation peak areas, in the 440 nm chromatograms, formed during the storage of samples at 45 °C. The samples are: (●) (-)-epicatechin/erythorbic acid, (○) (-)-epicatechin/ascorbic acid, (■) (+)-catechin/erythorbic acid, (□) (+)-catechin/ascorbic acid, (X) (-)-epicatechin, (◊) (+)-catechin, (▲) erythorbic acid, and (Δ) ascorbic acid. Error bars indicate the 95% confidence limits (n = 3).



After day 7, it is evident that the rate of xanthylium cation production for the erythorbic acid-derived xanthylium cations has slowed compared to that of the ascorbic-acid derived xanthylium cations (Figure 4.12). This is consistent with the lower maximum concentrations reached for the MHF-flavanols in the case of erythorbic acid compared to ascorbic acid (Figure 4.11). It must be noted that the MHF-flavanols are not the only precursors for the xanthylium cation production as these pigments can also form via the oxidative degradation of tartaric acid in the presence of hydrogen peroxide and metal ions (Section 2), or via exposure to light (Maury et al. 2010). The source of hydrogen peroxide in the model wine system may be from the oxidation of flavanol compounds and/or the oxidation of the antioxidant compounds (Danilewicz 2003). However, based on the results in Section 3, the MHF-flavanol pathway would appear to make the more significant contribution to xanthylium cation production in samples containing ascorbic acid, and therefore by implication, also samples containing erythorbic acid.

The results described in this section of the report have shown that on deliberate oxidation of a model wine system the stereochemical impacts of antioxidants and flavanols are significant.

In the presence of either of the flavanols (+)-catechin or (-)-epicatechin, erythorbic acid produces less oxidative colouration, at wavelengths corresponding to yellow and red, than the equivalent solutions containing ascorbic acid. Stereochemistry was also important in the oxidative degradation of both antioxidants and flavanols. Erythorbic acid was oxidised faster than ascorbic acid and (-)-epicatechin was oxidised faster than (+)-catechin. This difference in reaction rates has significant implications in binary mixtures. First, the decomposition of flavanols did not occur as rapidly in the presence of erythorbic acid as it did in the presence of ascorbic acid. Second, this translated into lower production of the colourless MHF-flavanol adducts, which are precursors to coloured xanthylium cations. Hence solutions containing erythorbic acid develop less colour than those of ascorbic acid for a given flavanol. Based on these results, erythorbic acid has certain advantages over ascorbic acid as an antioxidant and further studies are required to assess whether such advantages still exist during the complementary role of erythorbic acid to sulfur dioxide in wine-media and under less oxidative storage conditions.

In summary regarding the roles of ascorbic acid and erythorbic acid in winemaking, erythorbic acid:

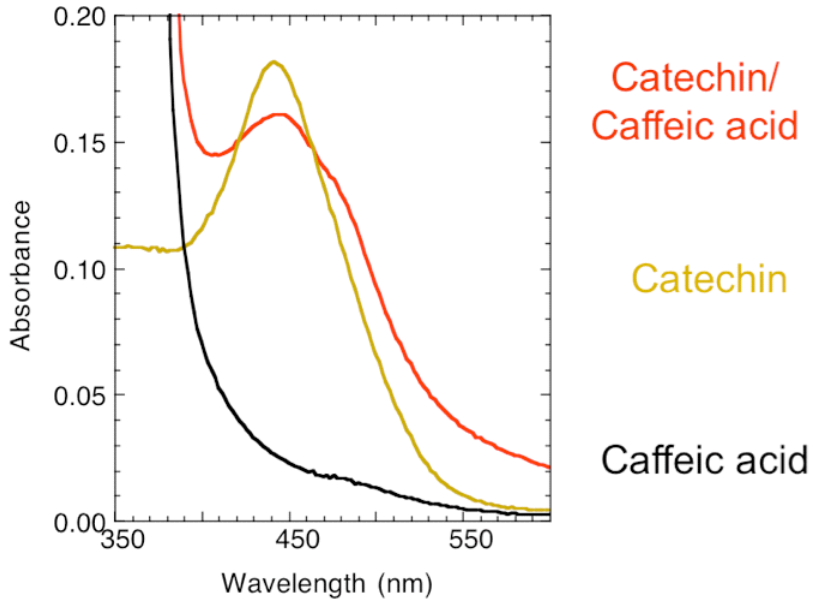
- Produces less colour when oxidised than ascorbic acid
- Produces less red colour
- Less expensive that ascorbic acid

However, there remains a perception in the marketing divisions of wine companies than ascorbic acid is ‘natural’ while erythorbic acid is not. This view is likely to continue to carry weight in any determination regarding the advantages of replacing ascorbic acid with erythorbic acid.

5. The impact of caffeic acid on ascorbic acid and oxidative colouration

The complexity of the model wine system was increased with the addition of caffeic acid. Although the concentration of flavanols, such as (+)-catechin, are well correlated with the potential of white wine to undergo an increase in colour as measured by 440 nm absorbance (Simpson 1982), white wine also contains around 200-300 mg/L of non-flavonoid phenolic compounds (Ough and Amerine 1988). These are typically hydroxycinnamic acids, such as caffeic and coumaric acids, which are esterified with tartaric acid units. Previous studies (George et al. 2006) had shown that the inclusion of caffeic acid in a model wine system with (+)-catechin, and subsequent accelerated oxidation (i.e. 45 °C, constant oxygen), led to a sample with a broader absorbance profile in the visible region (Figure 5.1) than with (+)-catechin alone. Therefore, despite reduced 440 nm absorbance in (+)-catechin/caffeic acid samples, the higher absorbance at wavelengths over 470 nm meant that the (+)-catechin/caffeic acid sample appeared orange/brown in colour whilst the (+)-catechin sample appeared yellow. This was also consistent with the work of Skouroumounis et al. (2005) who showed that absorbance increase at 500 nm was often a better indication of 'brown' colouration of wine rather than 420-440 nm increase.

Figure 5.1. Comparison of spectra of model wine system after 14 days of storage at 45° C. The samples contained either 150 mg/L (+)-catechin, 200 mg/L caffeic acid or both phenolic compounds.



Previous work (Lutter et al. 2007) also demonstrated the complexity of caffeic acid in a model wine system. After incubation at 45 °C in the model wine system with 1.5 mg/L iron ions, caffeic acid provided numerous products as demonstrated by each peak in the 280 nm chromatogram of Figure 5.2. This study showed that even under these mild conditions, caffeic acid could be cleaved, in a Fenton chemistry mechanism, and generate dihydroxybenzaldhyde (Figure 5.2). When dihydroxybenzaldehyde was separately incubated with (+)-catechin it generated an orange/red solution with the broad UV/Visible spectrum as shown in Figure 5.3.

Figure 5.2. The 280 nm chromatogram of 200 mg/L caffeic acid and 1.5 mg/L iron ions after storage at 45° C.

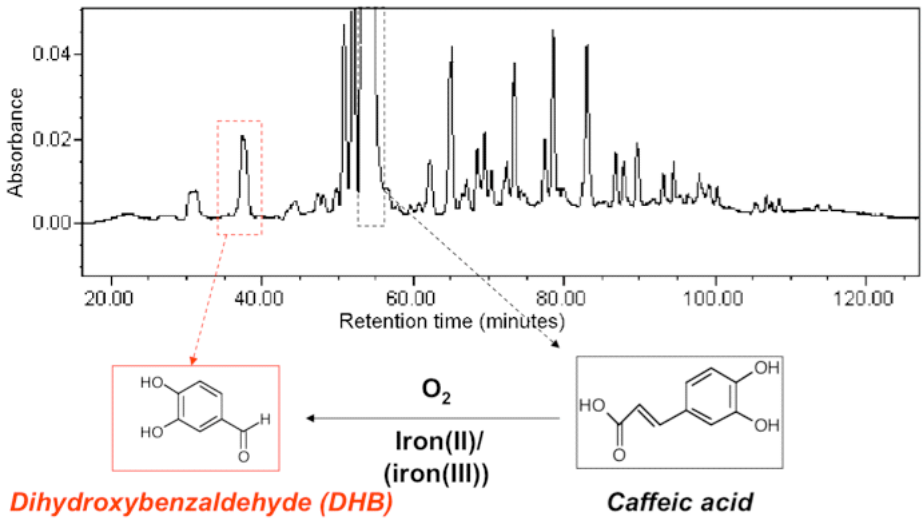
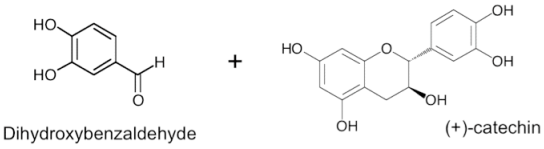
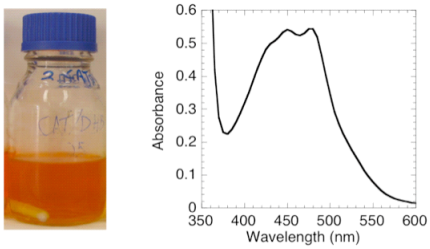


Figure 5.3. Production of orange pigments from dihydroxybenzaldehyde and catechin.





This section of experiments was conducted in order to assess the role of ascorbic acid in inhibiting the oxidative colour formation in the model wine system containing caffeic acid, particularly the inhibition of the orange/brown colouration as opposed to yellow. Importantly, the colour was to be monitored by CIELab analysis to account for the impact of ascorbic acid on the different colour coordinates over time. The CIELab analysis measures L* that relates to colour intensity (i.e. 100 is low, to 0 which is high/opaque), a* that gives an indication of green (-a*) to red (+a*), and b* that gives an indication of blue (-b*) to yellow (+b*).

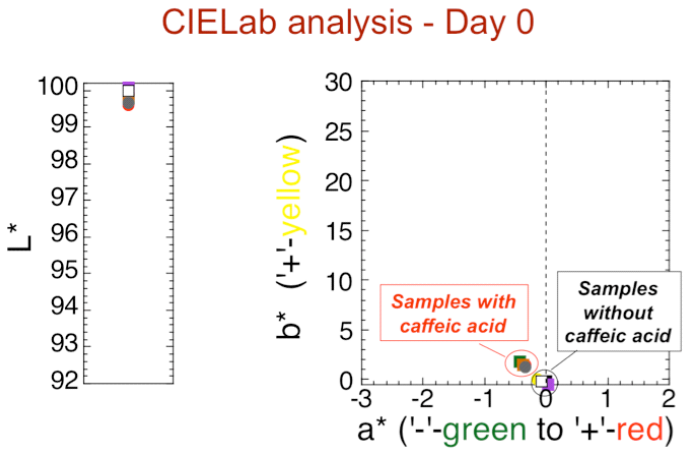
The following samples were prepared in the model wine system (i.e. 12 % aqueous ethanol buffered to pH 3.2 with potassium hydrogen tartrate/tartaric acid and 1.5 mg/L iron ions):

1. Control
2. Ascorbic acid (1000 mg/L)
3. Caffeic acid (200 mg/L)
4. Catechin (150 mg/L)
5. Caffeic acid + ascorbic acid
6. Catechin + ascorbic acid
7. Catechin + caffeic acid
8. Catechin + caffeic acid + ascorbic acid

Iron was included in the model wine system to induce the degradation of caffeic acid, as described above, and this level of iron was relevant to the conditions found in white wine (Cacho et al. 1995, Riganakos and Veltsistas 2003). The relatively high level of ascorbic acid adopted (i.e. 1000 mg/L) was required to ensure sufficient time for the depletion of ascorbic acid for detailed spectroscopic and chromatographic analyses.

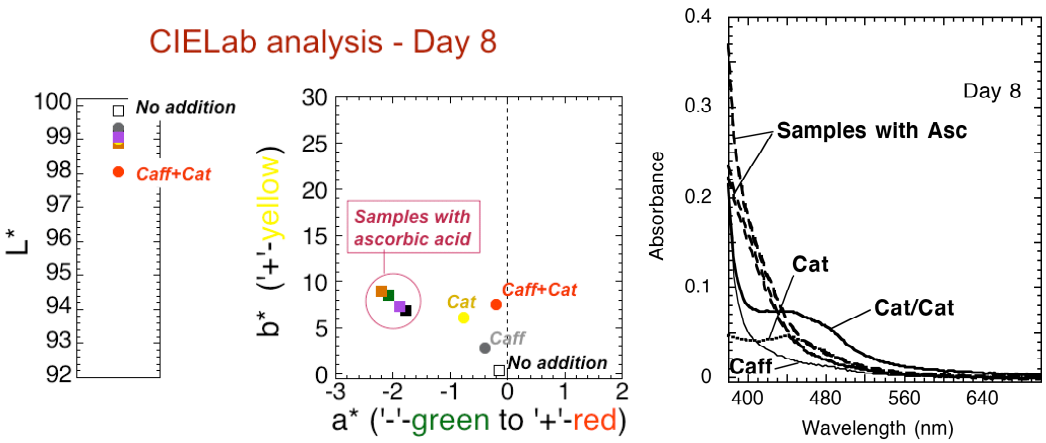
At day 0 (Figure 5.4), the CIELab data showed that all samples had low levels of colour, however, the tailing of absorbance for caffeic acid into the visible region meant that these samples had a slight yellow/green tint compared to the remaining samples.

Figure 5.4. CIELab data and UV/Visible spectra of samples at 0 day of storage at 45 °C.



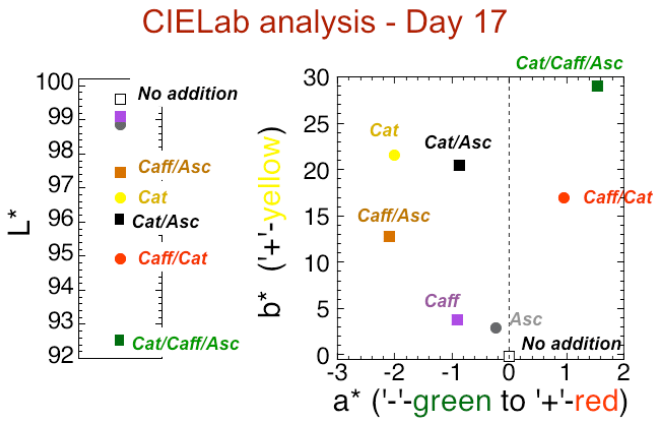
The CIELab data at day 8 (Figure 5.5) showed the clustering of all samples with ascorbic acid that had more of a green/yellow tint compared to the other samples. The most intensely coloured sample was the caffeic acid/catechin sample by day 8 and, excluding the 'no addition' sample with negligible colour, it was also nearest to having red colouration (i.e. +a*) according to CIELab. Such results were also consistent with the UV/Visible spectra for these samples (Figure 5.5, right-hand side) that showed the (+)-catechin/caffeic acid sample had the most intense absorbance at wavelengths greater than 450 nm, including the highest absorbance in the region associated with red colour (i.e. 500 nm).

Figure 5.5. CIELab data and UV/Visible spectra of samples after 8 days of storage at 45 °C



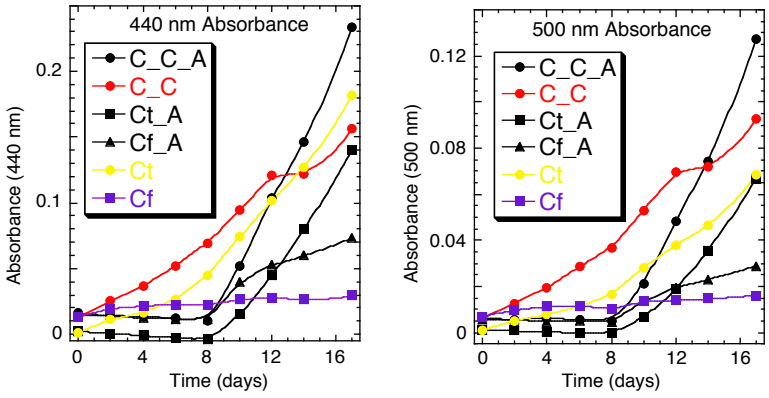
By day 17, the CIELab data (Figure 5.6) for the samples showed a clear divergence in all samples particularly those containing ascorbic acid. The ternary sample, (+)-catechin/caffeic acid/ascorbic acid, had the most colouration followed by (+)-catechin/caffeic acid, and both of these samples also had the most orange (or yellow/red) colouration. Comparing the data at day 8 to day 17, it is interesting to note that only (+)-catechin had an appreciable change in colour away from red and towards more green colouration.

Figure 5.6. CIELab data and UV/Vis spectra of samples at 17 days of storage at 45 °C.



The 440 nm and 500 nm absorbance/time course plots (Figure 5.7) show these changes clearly over time. In these time course data, the absorbances for all the samples containing ascorbic acid have been corrected for the absorbance provided by ascorbic acid alone. The notation '*A' will be used to denote this corrected absorbance. The data in Figures 5.7 clearly demonstrate that from days 0 to 8, the samples with ascorbic acid have little $*A_{440}$ or $*A_{500}$ intensity. Hence, the main absorbance from days 0 to 8 in the samples with ascorbic acid would be that explained by ascorbic acid alone. Alternatively, during the same period, the samples without ascorbic acid are all steadily increasing in $*A_{440}$ or $*A_{500}$ absorbance. After day 8, the increase in absorbance in the ascorbic acid-binary and ternary mixtures is more rapid than in the corresponding samples without ascorbic acid. Importantly, in the first 8 days of the experiment, the $*A_{500}$ of the ternary mixture is prevented by the presence of ascorbic acid and this leads to the sample appearing yellow but not brown.

Figure 5.7. Time course absorbance plots at 440 nm (left-hand side) and 500 nm (right-hand side). As described in the text, the data for samples with ascorbic acid (i.e. in black) have been corrected using the absorbance provided by ascorbic acid alone.



No significant difference (P = 0.05) was observed for the rate of ascorbic acid decay in any of the samples (i.e. 84 mg/(L.day)), which suggested that the decay of ascorbic acid in the model wine system was independent of the presence of (+)-catechin and caffeic acid. The independence of ascorbic acid decay is consistent with its lower redox potential compared to phenolic compounds such as (+)-catechin and caffeic acid (Danilewicz 2003). At day 10, around 90% of the ascorbic acid is depleted in the samples and it is at this time that binary and ternary mixtures containing ascorbic acid had a rapid increase in colour (Figure 5.7). This observation is also similar to model wine systems without caffeic acid, as discussed previously in this report (Section 2).

In the first 8 days of the experiment, all samples containing ascorbic acid had the identical LC-DAD peak eluting early in the 440 nm chromatogram (Figure 5.8), which was the main

coloured species in these solutions. This peak was a direct consequence of the degradation of ascorbic acid and not dependent upon either (+)-catechin or caffeic acid. Alternatively, the (+)-catechin and (+)-catechin/caffeic acid samples had much later eluting phenolic-derived pigments as their main pigments during this same time period. The pigments were identified as being either xanthylium cation pigments and/or a broad unidentified peak similar to those in Figure 5.9. Intriguingly, the broad peak was observable on HPLC but less evident on UPLC where it had the appearance of increased baseline intensity rather than being a peak. Given the absence of this broad peak in the experiments of the previous sections of this report (Sections 2 and 3), its dependence on the higher iron concentrations in this experiment (i.e. 1.5 mg/L) was likely. Regardless, ascorbic acid was clearly preventing the production of the later eluting phenolic-pigments as long as its concentrations remained sufficiently high, and in this manner maintaining the subtle green/yellow colour of its degradation products (Figure 5.5) compared to the more intense yellow to orange colour of the (+)-catechin and (+)-catechin/caffeic acid samples.

Figure 5.8. The 440 nm chromatogram of model wine containing both ascorbic acid and (+)-catechin after 8 days of storage at 45 °C.

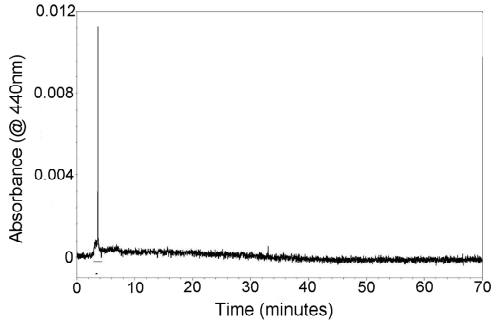
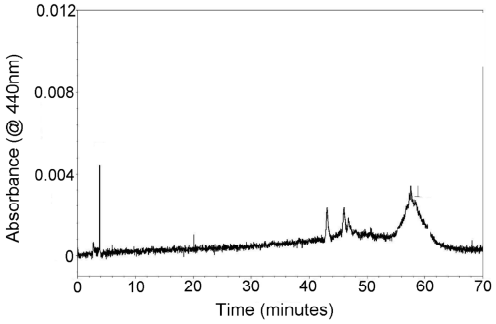
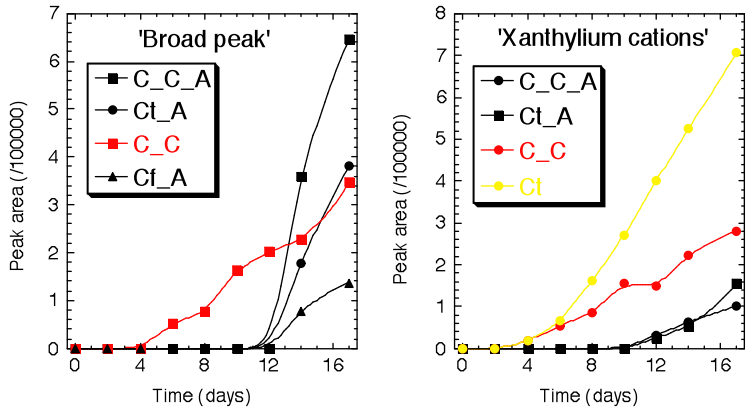


Figure 5.9. The 440 nm chromatogram of model wine containing both ascorbic acid and (+)-catechin after 17 days of storage at 45 °C.



After the depletion of the ascorbic acid in the samples, the same phenolic-derived pigments developed in both the ascorbic acid samples and the non-ascorbic acid samples, albeit at an accelerated rate for the broad peak (Figure 5.9 and 5.10). Again, this is consistent with both the CIELab and single wavelength time course data, and explains the predominance of the yellow/red colour in the ascorbic acid/catechin caffeic acid sample.

Figure 5.10. Time course profiles for the formation of the main phenolic pigments in the model wine systems.



The products from the ascorbic acid/catechin reaction have already been discussed in Section 3, and the main precursors to xanthylium cations from this reaction (i.e. MHF-catechin) were observed to reach a maximum concentration at day 8 of the experiment and then rapidly decay before the formation of the xanthylium cations. These intermediates were also observed in the ascorbic acid/catechin/caffeic acid sample.

The HPLC analysis (data not shown), however, reveals that dihydroxybenzaldehyde (Figure 5.2) production is enhanced when ascorbic acid is combined with caffeic acid. The dihydroxybenzaldehyde concentration increased in a linear manner ($r^2 = 0.979$) throughout the experiment, and was found 14 times more concentrated than in the equivalent sample without ascorbic acid at day 17 of the experiment (i.e. 34.8 µM versus 2.5 µM). Intriguingly, the rate of production does not change after the ascorbic acid is depleted from solution at day 12. Given that dihydroxybenzaldehyde is a known precursor to orange/red pigments in reaction with (+)-catechin (Lutter et al. 2007), the enhancement of dihydroxybenzaldehyde by ascorbic acid in the presence of caffeic acid may contribute to the increased red colouration of the ternary ascorbic acid/catechin/caffeic acid sample. Further work is required to assess if the broad peak observed in the 440 nm chromatograms is related to the dihydroxybenzaldehyde emanating from the degradation of caffeic acid.

The results to date have demonstrated two important mechanisms for the oxidative production of coloured pigments in the model wine systems and both mechanisms involved the reaction of aldehyde compounds with flavanols. The first involves the production of the aldehyde compound xylosone from ascorbic acid, and the second involves the production of aldehyde compounds via Fenton chemistry (see Section 3). For example, the latter mechanism can result in the production of glyoxylic acid and dihydroxybenzaldehyde from tartaric acid and caffeic acid, respectively. Consequently, it was decided to assess how another major wine component, glycerol, would impact the oxidative colouration in the caffeic acid/catechin model wine system. Glycerol was originally not included in the model wine system as it had negigible impact on pH and was considered fairly inert in the presence of 2.0 M (or 12% (v/v) ethanol. However, given the demonstrated degradation of tartaric acid in the presence of 12% (v/v) aqueous ethanol, and the reports of glycerol degradation via Fenton chemistry (Laurie and Waterhouse 2006), it was decided to assess its importance in the model wine system utilised for this study.

Glycerol (0.2 M) was added to the original model wine system as well as caffeic acid (200 mg/L), (+)-catechin (50 mg/L), 0.2 mg/L copper ions and 5 mg/L iron ions. These levels were chosen to represent typical wine-like concentrations. Ascorbic acid was not included in this study in order to ensure that the pigments generated would indeed be via a Fention-style mechanism and not via xylosone. The result in Table 5.1 shows that glycerol inhibited the 440 nm absorbance of the model wine system, and analysis by UPLC (Figure 5.11) demonstrated that while the pigments that were formed were identical in both cases, the

concentration was lower in the presence of glycerol. That is, the compounds responsible for the 440 nm absorbance were glyoxylic acid-derived xanthylium cations and an increase in the baseline intensity. Given that the hydroxyl radicals are suggested to react in a near-diffusion controlled manner, it was expected that glycerol may inhibit the production of the tartaric acid-derived xanthylium cations by a considerable extent given that its concentration was 10 times that of tartaric acid (i.e. 0.2 M vs 0.02M). The actual result, showing only mild inhibition by glycerol, implies that either glycerol can contribute to the production of xanthylium cations or that tartaric acid is particularly susceptable to cleavage when in the presence of hydrogen peroxide/metal ions, regardless of the presence of high concentrations of competing substrates (i.e. 2M ethanol, 0.2M glycerol). Further work is required to ascertain which of the above outcomes is more important and also to ascertain if there is any impact of glycerol on the ascorbic acid-xylosone-induced formation of pigmented compounds.

Figure 5.11. The 440 nm UPLC chromatograms of the model wine system with 0 M (upper trace) and 0.2 M glycerol (lower trace) after 14 days of storage at 45 °C. The first major eluting peak (3.5 min) is due to tailing from caffeic acid, the remaining four major peaks after 4.0 mins are due to xanthylium cations.

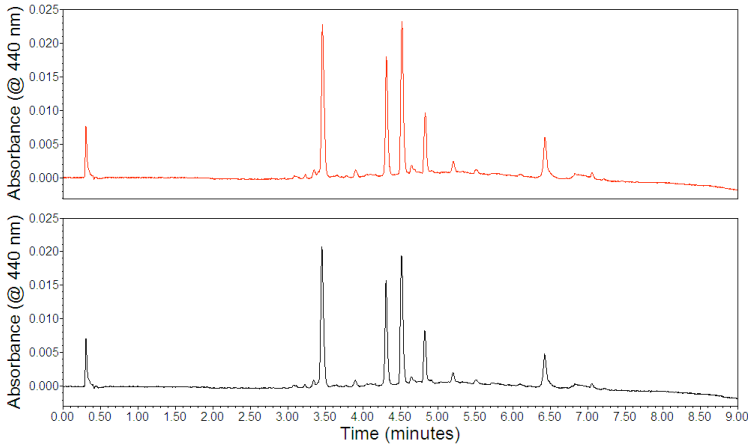


Table 5.1. The 440 nm absorbance of model wine systems during storage at 45 °C. The model wine system consisted of 12% aqueous ethanol, potassium hydrogen tartrate/tartaric acid (0.02M) at pH 3.2, 200 mg/L caffeic acid, 50 mg/L (+)-catechin, 0.2 mg/L copper ions, 5 mg/L iron ions. '*' represents a significant difference (P = 0.05).

	Day 0	Day 2.5	Day 7	Day 14*
0.20 M glycerol	0.003 ± 0.006	0.028 ± 0.002	0.15 ± 0.02	0.29 ± 0.03
0 M glycerol	0.004 ± 0.005	0.03 ± 0.02	0.20 ± 0.05	0.36 ± 0.03

6. Ascorbic acid and sulfur dioxide chemistry

As already mentioned, ascorbic acid is able to react with molecular oxygen to generate dehydroascorbic acid and hydrogen peroxide (Figure 6.). The presence of sulfur dioxide is critical to allow hydrogen peroxide scavenging (Figure 6.2), which results in a drop of both 'free' sulfur dioxide and 'total' sulfur dioxide concentrations. Sulfur dioxide can also interact with dehydroascorbic acid, and its degradation products (Wedzicha 1984), but it is not known whether such interactions are sufficiently strong in wine-conditions to result in a decrease in 'free' and/or 'total' sulfur dioxide. Bradshaw et al. (2004) showed a 1.7 : 1 ratio for free sulfur dioxide consumption to ascorbic acid consumption in a model wine system, free of added metal ions, suggesting that dehydroascorbic acid or its degradation products may have been capable of binding sulfur dioxide in this system.

Figure 6.1. The reaction of ascorbic acid with molecular oxygen.

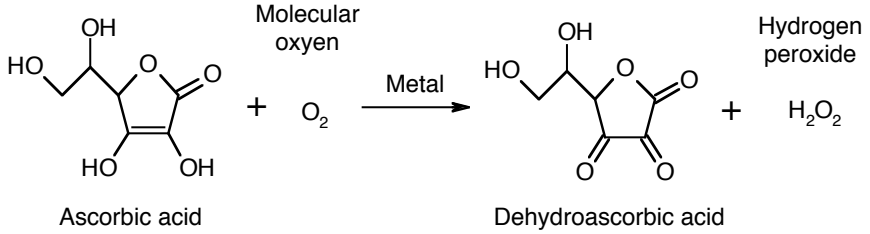


Figure 6.2. The reaction of sulfur dioxide with hydrogen peroxide.

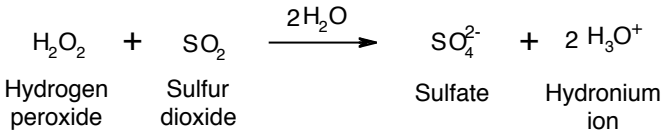
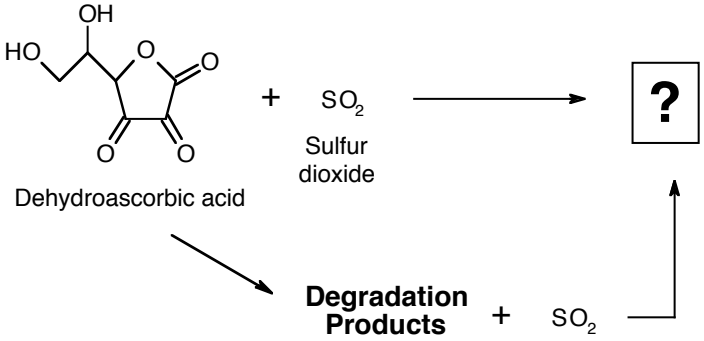
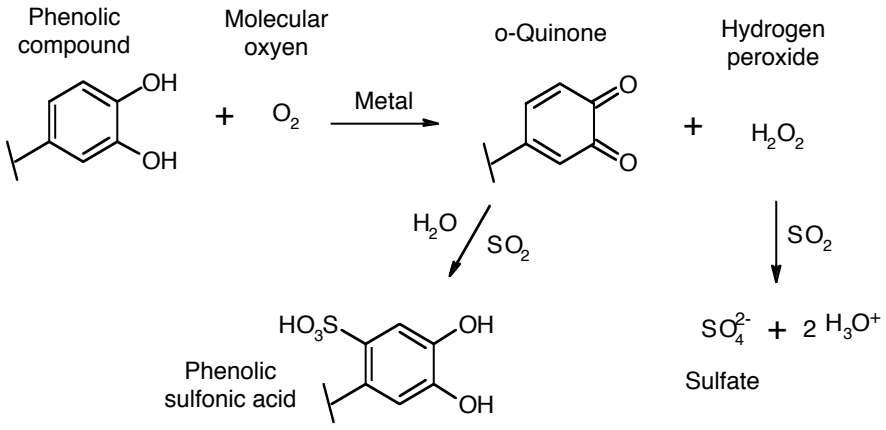


Figure 6.3. The interaction of sulfur dioxide with dehydroascorbic acid and its degradation products.



In the absence of ascorbic acid, it is suggested that sulfur dioxide normally operates as depicted in Figure 6.4 (Danielwicz et al. 2008). In this case, oxygen undergoes a metal-mediated reaction with phenolic compounds, of *ortho*-dihydroxybenzene functionality, and generates hydrogen peroxide and *ortho*-quinone compounds. The sulfur dioxide can then scavenge hydrogen peroxide and the otherwise reactive *ortho*-quinone compound. Figure 6.4 shows the addition of sulfur dioxide onto the *ortho*-quinone compound, however, sulfur dioxide can also react with the *ortho*-quinone in an alternate mechanism. Sulfur dioxide can reduce the *ortho*-quinone compound back to its original phenolic structure and produce sulfate in the process. As a consequence of the reactions depicted in Figure 6.4, the ratio of oxygen consumption to sulfur dioxide consumption in wine is around 1:2, which has been confirmed experimentally by Danielwicz et al. (2008).

Figure 6.4. The consumption of sulfur dioxide in the absence of ascorbic acid



The earlier sections of this report (i.e. 1-5), have concentrated on the chemistry of ascorbic acid and its oxidative degradation in wine-like media. This section will investigate the impact of sulfur dioxide on this reaction system as well as the importance of metal ions and the molar ratio of the initial sulfur dioxide to ascorbic acid concentrations in the model wine media.

The model wine system consisted of the 12% aqueous ethanol solution at pH 3.2 (i.e. 0.011 M tartaric acid-buffer) with 100 mg/L (+)-catechin, the latter representative of a heavily pressed white wine. The non-flavonoid content of white wine (typically 200-300 mg/L) was omitted from this study, as when analysed by the adopted UPLC method, caffeic acid co-eluted with the MHF-catechin isomers discussed in Section 3, and it was considered critical to assess the impact of sulfur dioxide on these pigment precursors. Despite several attempts at resolving this co-elution, it could only be achieved at the cost of poor resolution for other target compound peaks.

To this model wine system, the following samples were prepared (concentrations and acronyms shown in parentheses):

1) sulfur dioxide (S),
2) sulfur dioxide, metal (SM),
3) 82.5 mg/L ascorbic acid (80A),
4) 82.5 mg/L ascorbic acid, sulfur dioxide (80AS),
5) 82.5 mg/L ascorbic acid, metal (80AM),
6) 82.5 mg/L ascorbic acid, metal, sulfur dioxide (80AMS),
7) 41.3 mg/L ascorbic acid, metal (40AM),
8) 41.3 mg/L ascorbic acid, metal, sulfur (40AMS),
9) 165 mg/L ascorbic acid, metal (165AM), and
10) 165 mg/L ascorbic acid, metal, sulfur dioxide (165AMS).

The concentration of sulfur dioxide used was 30 mg/L, which is a typical free level adopted in white wine. The concentrations of ascorbic acid at 40, 80 and 165 mg/L provide sulfur dioxide to ascorbic acid molar ratios of 2:1, 1:1 and 1:2, respectively, and cover the concentration range at which ascorbic acid is usually used in white wine. The metal compositions and concentrations were 0.2 mg/L copper(II) and 5 mg/L iron(II), which also fall within the range encountered in white wine.

Each sample (150 mL) was prepared in triplicate and placed in a 500 mL Schott reagent bottle with a head space of 470 mL. Samples were aerated daily and the oxygen content in the samples monitored by a Presens luminescence oxygen detector to ensure the solution was aerated to near saturation (7-8 mg/L dissolved oxygen). The only significant drop in dissolved oxygen before aeration was evident in the first few days of the experiment for the

samples containing 165 mg/L ascorbic acid and in this case the level only dropped to ~4mg/L. The samples were stored in the darkness at 20 °C and analysed by UPLC-DAD, FIAstar wine analyser (i.e. for sulfur dioxide), and spectrophotometric methods (i.e. CIELab colour analysis and UV/Vis Spectra). This particular storage temperature was adopted as it is relevant to wine storage conditions and the reactions were known to occur within a reasonable time frame at this temperature and model wine composition.

The results of the decay in both free and total sulfur dioxide are shown in Figures 6.5 and 6.6. The data demonstrates that metal ions and ascorbic acid accelerate the rate of sulfur dioxide consumption, both as a function of free and total sulfur dioxide. Metal ions are well known to accelerate oxidative processes (Figure 6.1 and 6.4, Danielwicz 2003, Buettner and Jurkiewicz 1996a), thereby increasing the oxidative production of hydrogen peroxide and subsequent consumption of sulfur dioxide (Figure 6.2). The impact of ascorbic acid on sulfur dioxide consumption is most likely its ability to accelerate consumption of oxygen, and thereby hydrogen peroxide production (Figure 6.1), which in turn leads to sulfur dioxide decay (Figure 6.2). This was further confirmed by dissolved oxygen measurements that showed that after one day of reaction, the dissolved oxygen concentrations in the samples was lowest in the samples containing 165 mg/L ascorbic acid (data not shown). The presence of both ascorbic acid and metal ions had a synergistic effect that led to an accelerated rate of sulfur dioxide consumption.

Figue 6.6 shows the decay in sulfur dioxide at different concentrations of ascorbic acid with metal present. As mentioned above, the ascorbic acid concentrations adopted related to initial sulfur dioxide to ascorbic acid ratios of 2:1 (41mg/L ascorbic acid), 1:1 (82 mg/L ascorbic acid) and 1:2 (165 mg/L ascorbic acid). The results show increased free and total sulfur dioxide consumption at higher initial ascorbic acid concentrations. Again this is consistent with increased oxygen consumption at high ascorbic acid levels.

The results in Figure 6.5 and Figure 6.6 show little difference in the free and total sulfur dioxide levels, especially at the earlier stages of the experiment. Given that the mechanism in Figure 6.2 is known to lower both free and total sulfur dioxide, this suggests that dehydroascorbic acid and its degradation products are either weak binders of sulfur dioxide that dissociate during the measurement of free sulfur dioxide, or are strong binders of sulfur dioxide and do not dissociate during the measurement of total sulfur dioxide. Further comment on the binding of sulfur dioxide to ascorbic acid degradation products is made below after the rate of degradation of ascorbic acid is described.

Figure 6.5 Decay in sulfur dioxide at day 1 (LHS) and 6 (RHS) as influenced by ascorbic acid concentration (0 and 80 mg/L) and metal ions.
S = sulfur dioxide, M = metal, 80A = 82 mg/L ascorbic acid. Dashed line indicates the initial sulfur dioxide concentration. The error bars represent the 95% confidence limits.

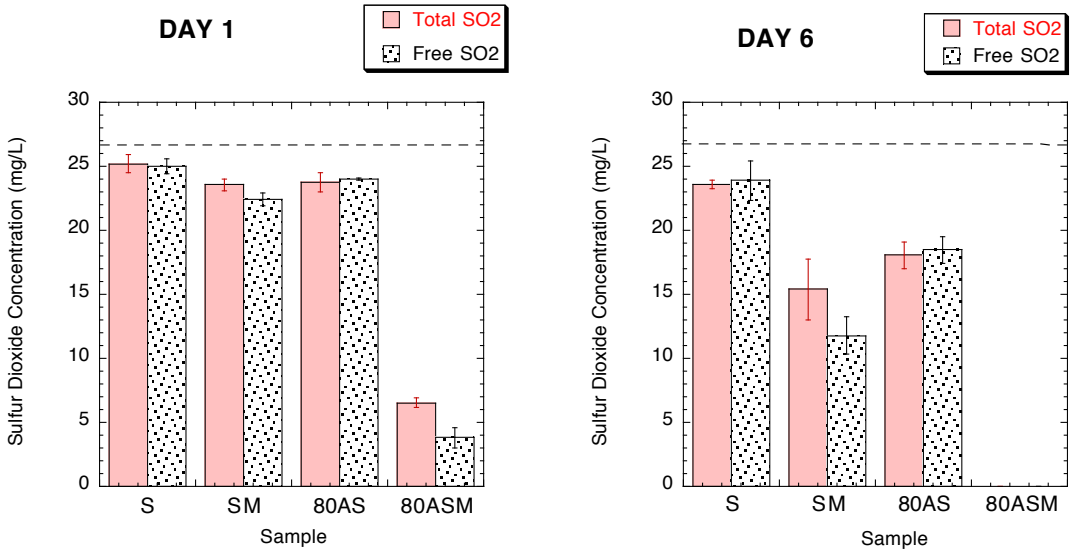


Figure 6.6: Decay of sulfur dioxide at 3.5 hours (LHS) and 1 day (RHS) as influenced by ascorbic acid concentration (41, 83 and 165 mg/L) in the presence of metal ions.

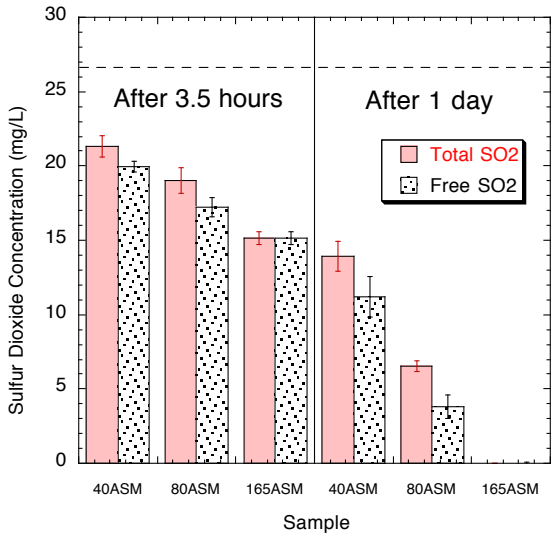
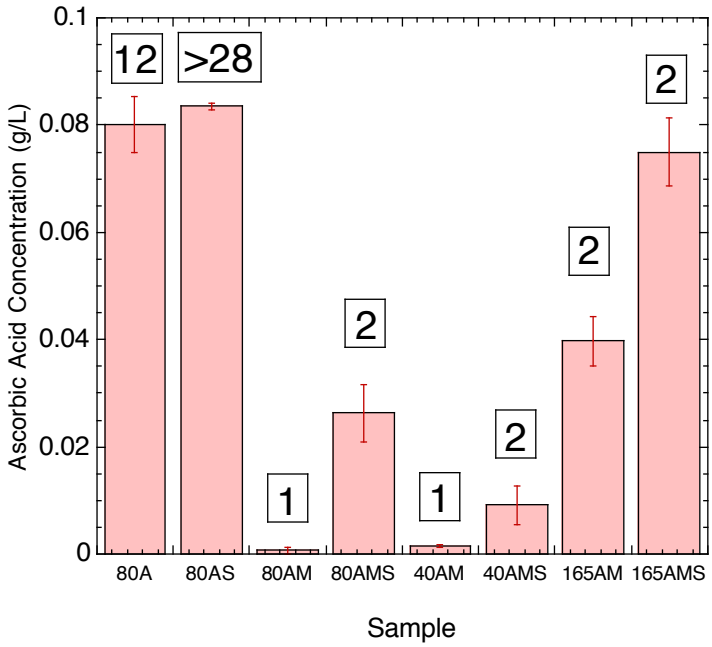


Figure 6.7 shows that sulfur dioxide slows the degradation of ascorbic acid both in the presence and absence of metal ions. Although the sulfur dioxide decay at day 1 appears similar for the samples without metal ions (i.e. 80A and 80AS), sulfur dioxide extends the time before 90% depletion of ascorbic acid (i.e. >28 days vs 12 days). Given the ability of ascorbic acid to react with hydrogen peroxide (Deutsch 1998), it is likely that sulfur dioxide can scavenge the hydrogen peroxide before it is able to degrade ascorbic acid. At this stage, the relative rates of reaction (SO2/H2O2 and H2O2/ascorbic acid) have not been determined. The ability of metal ions to accelerate the decay of ascorbic acid is also reinforced by the data in Figure 6.7 (i.e. compare 80A vs 80AM, 80AS vs 80AMS).

Figure 6.7 Concentration of ascorbic acid at day 1 of experiment and number of days (in boxes) before depletion of ascorbic acid in samples. Analysis days were day 0, 1 2, 3, 4, 6, 12, 19, and 28. The starting concentrations of ascorbic acid were either 0.041 g/L (i.e. 40A), 0.083 g/L (i.e. 80A) or 0.165 g/L (i.e. 165A). Error bars represent the 95% confidence limits.



The data in Figure 6.7 also allows calculation of the oxygen consumed in the samples after 1 day by assessing the amount of ascorbic acid consumed and utilising the 1:1 oxygen to ascorbic acid stoichiometry evident in Figure 6.1. Consequently, after 1 day the total oxygen consumed would have been around 16, 11 and 6 mg/L for the 165, 80 and 40 mg/L ascorbic acid samples (i.e. 165AMS, 80AMS, 40AMS), respectively. Therefore, although higher concentrations of ascorbic acid consume oxygen more rapidly, this means that in an experiment where oxygen concentration is essentially in excess, the total oxygen consumed at the end of the experiment is also likely to increase with initial ascorbic acid concentration. This is in contrast to bottled wine where the oxygen supply is usually low, and limited to the total packaged oxygen at bottling and low ingress rates through the closure during storage. Therefore in the bottle, the higher concentrations of ascorbic acid would still consume the set amount of oxygen more quickly but not increase the total amount of oxygen consumed.

The molar ratio of 'consumed sulfur dioxide' to 'consumed ascorbic acid' was calculated and is shown in Table 6.1. Unfortunately, the rapid sulfur dioxide depletion in the samples with 165 mg/L ascorbic acid meant that the ratios were not particularly precise. The other complication was the change in the ratio over time that was evident for the sample without added metal ion (Table 6.1, 80AS). The overall average ratio for this 80AS sample (i.e. (2.4 ± 0.5) : 1, n = 15, ± 95% confidence limit) was somewhat similar to the reported value 1.7:1 by Bradshaw et al. (2004), whom conducted the experiment at 45 °C. However, it is clear that the use of the average when the experiment is conducted at 20 °C (as in Table 6.1) is not accurate of the overall process.

Table 6.1. The ratio of consumed sulfur dioxide to ascorbic acid. Also indicated is the time at which the data was calculated (i.e. before depletion of either ascorbic acid or sulfur dioxide). Where not specified n = 3.

Sample	Consumed Ratio Free SO_2 : Ascorbic acid	Consumed Ratio Total SO_2 : Ascorbic acid
80AS		

	(3 ± 1) : 1 (day 3) **(3 ± 1) : 1** (day 4) **(2.8 ± 0.9) : 1** (day 6) **(2.5 ± 0.2) : 1** (day 12) **(1.5 ± 0.1) : 1** (day 19)	**(3.2 ± 0.4) : 1** (day 3) **(3 ± 2) : 1** (day 4) **(2.7 ± 0.7) : 1** (day 6) **(1.8 ± 0.1) : 1** (day 12) **(1.2 ± 0.1) : 1** (day 19)
40AMS	**(1.29 ± 0.04) : 1** (n = 6, day 1 and 2)	**(1.08 ± 0.02) : 1** (n = 6, day 1 and 2)
80AMS	**(1.06 ± 0.06) : 1** (day 1)	**(0.92 ± 0.03) : 1** (n = 6, day 1 and 2)
165AMS	**(0.6 ± 0.4) : 1** (3 hours)	**(0.5 ± 0.3) : 1** (3 hours)

The remaining samples (i.e. 40AMS and 80AMS) had ratios closer to 1:1 for both free and total sulfur dioxide, albeit with the free sulfur dioxide ratios significantly higher (P = 0.05) than the corresponding total sulfur dioxide ratios. With regard to Figure 6.2 and 6.3, this suggests that as ascorbic acid is oxidised by oxygen, the sulfur dioxide is only consumed by hydrogen peroxide. If dehydroascorbic acid, or its degradation products, bind sulfur dioxide (Figure 6.3) then it is sufficiently weak for the sulfur dioxide to be measured as 'free sulfur dioxide'. In the FIAstar measurement of sulfur dioxide, like the other more traditional measures (i.e. aspiration-oxidation, iodiometric), the sample is acidified and such acidification can liberate sulfur dioxide that is in weakly bound complexes. As discussed in Section 3, xylosone is an important degradation product of ascorbic acid due to its ability to act as a pigment precursor, but it is known that xylosone is 100 times a weaker binder of sulfur dioxide compared to acetaldehyde or glyoxylic acid (Bradshaw et al. 2010).

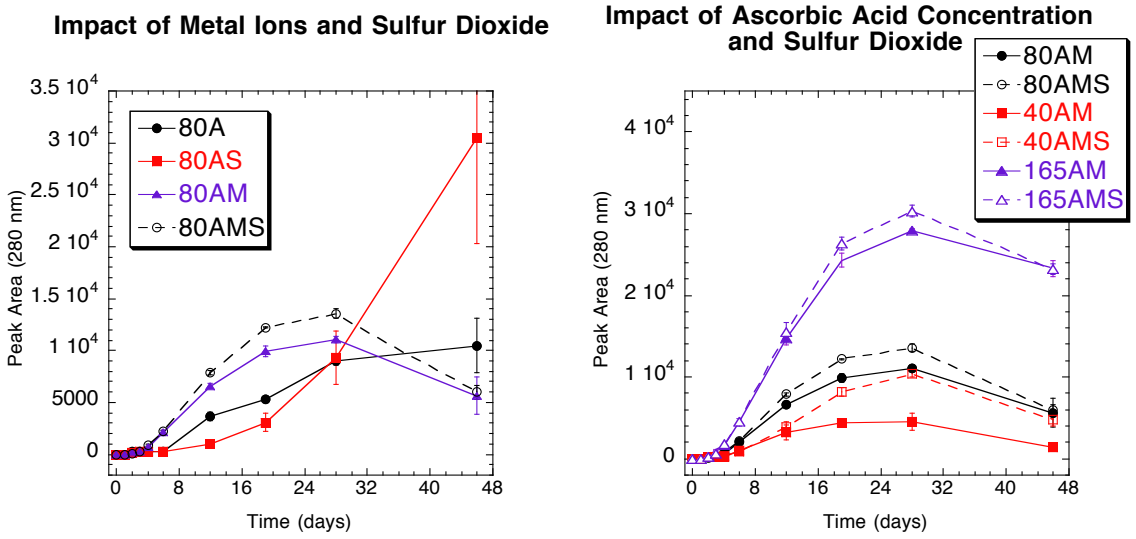
In the case of the samples without added metal ions the higher ratios are most likely due to a competing mechanism also consuming sulfur dioxide and providing an apparent high ratio. For instance, this may due to the mechanism highlighted in Figure 6.4 that may become important due to the slowing of the reaction between oxygen and ascorbic acid (Figure 6.1) at low metal ion concentration. The combined reactions in Figures 6.1, 6.2 and 6.4 would provide a sulfur dioxide to ascorbic acid consumption ratio of 3:1 (i.e. $3\times SO_2$ in Figures 6.1/6.2/6.4, 1×Ascorbic acid in Figure 6.1). As the sulfur dioxide concentration becomes depleted, then ascorbic acid may start to scavenge hydrogen peroxide (Deutsch 1998) and/or the *ortho*-quinone (Isaak and Eldik 1997) itself and thereby lower the 3:1 ratio to 1.5:1 over time.

The concentration of (+)-catechin in the samples was impacted most by the presence of metal ions (data not shown). The presence of sulfur dioxide only had a significant (P=0.05) impact on (+)-catechin decay in the absence of metal ions (i.e. reduced decay by 7 %) or in the presence of metal ions but at low ascorbic acid concentration (i.e. reduced decay by 16 % in 40 mg/L ascorbic acid sample). The samples with metal ions and the higher ascorbic acid concentrations had a trend towards lower (+)-catechin decay with sulfur dioxide but it was not a significant impact (P=0.05). Intriguingly, apart from the 40 mg/L ascorbic acid sample with sulfur dioxide, little impact of increasing ascorbic acid concentration was observed on (+)-catechin loss.

Figure 6.8 shows that the pigment precursor, (+)-MHF-8-catechin (see Section 3), accumulates in the sample without metal ions present. In fact, the accumulation occurs despite the presence of sulfur dioxide in the 80AS sample up to 28 days. This demonstrates that the sulfur dioxide binding to xylosone is not sufficiently strong to prevent the reaction of xylosone with (+)-catechin.

Ascorbic acid concentration increased the production of (+)-MHF-8-catechin irrespective of whether sulfur dioxide was present in the samples or not. The impact of sulfur dioxide was minimal on (+)-MHF-8-catechin production in the samples with metal ions and either 165 mg/L and 80 mg/L ascorbic acid, but in contrast to the (+)-catechin decay, there was a trend towards slightly more (+)-MHF-8-catechin. The sample with both metal ions and 40 mg/L ascorbic acid had significantly higher concentrations of MHF-8-catechin in the presence of sulfur dioxide.

Figure 6.8. The production of the pigment precursor (+)-MHF-8-catechin and the impact of metal ions, sulfur dioxide and ascorbic acid concentration. Error bars represent the 95% confidence limits.



In this experiment, the decay of (+)-MHF-8-catechin (Figure 6.8) in the samples with metal ions occurs well after the depletion of ascorbic acid (i.e. days 1-2 in Figure 6.7), which is in contrast to the results in Section 3 (Figure 3.4). It is likely that the storage temperature (i.e. 20°C versus 45°C) is important in the rate of degradation of these compounds.

Figure 6.9. The production of yellow xanthylium cation pigments and the impact of metal ions, sulfur dioxide and ascorbic acid concentration. Error bars represent the 95% confidence limits.

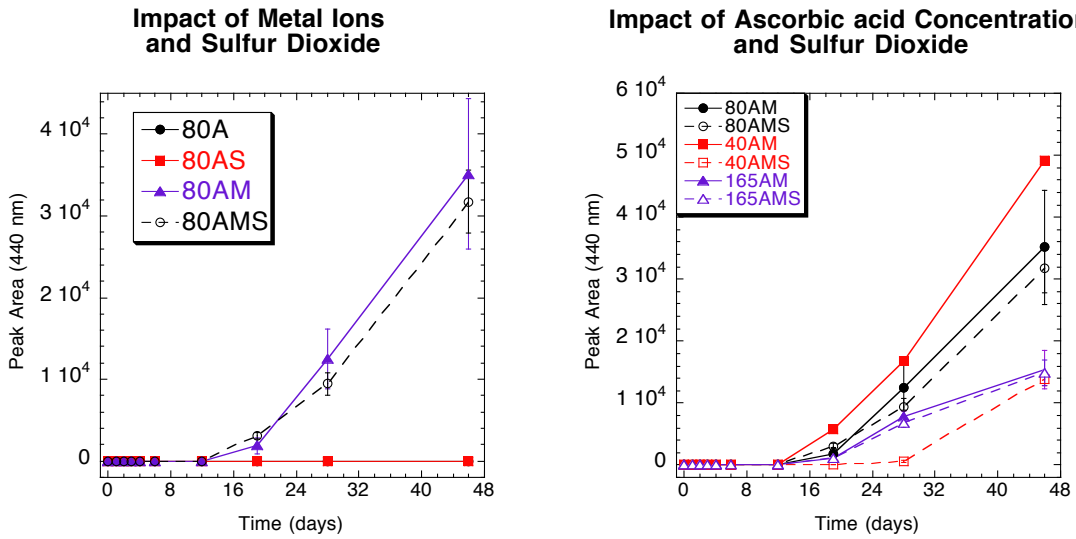
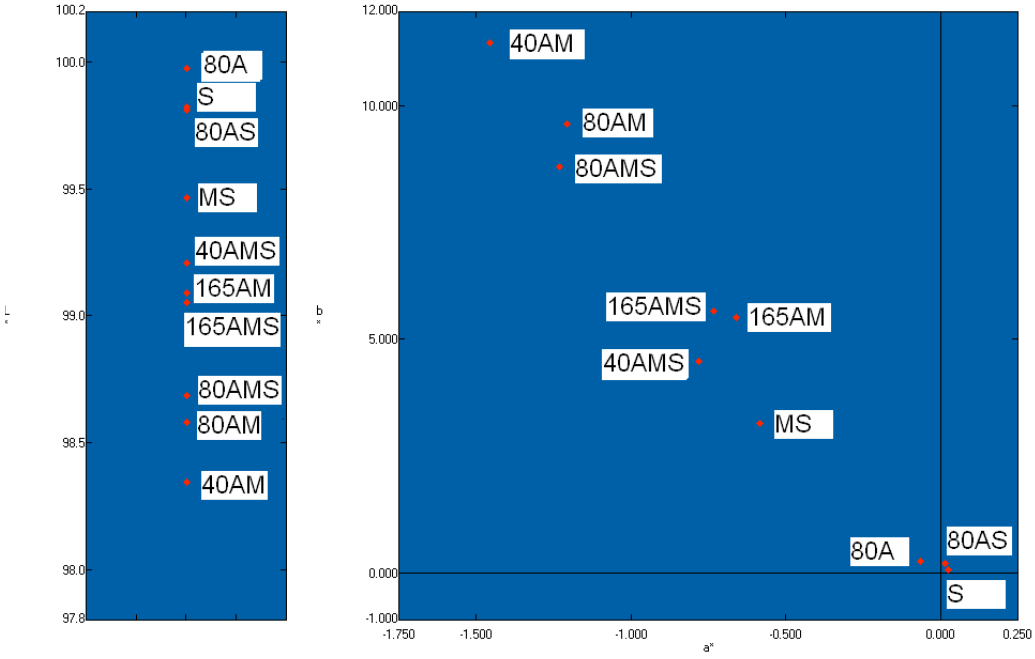


Figure 6.9 demonstrates the importance of metal ions in the formation of the yellow xanthylium cation pigments from the (+)-MHF-8-catechin. The impact of the initial sulfur dioxide in the 80 mg/L ascorbic acid sample with metal ions is only slight and non-significant ($P = 0.05$). The impact of ascorbic acid concentration, in the presence of metal ions, is also evident in Figure 6.9. Most pigment production occurs at 40 mg/L ascorbic acid/metal and then decreases with increasing ascorbic acid concentration, apart from the 40 mg/L ascorbic acid sample with added sulfur dioxide. This latter sample had the least xanthylium cations generated, most likely due to its ability to maintain sulfur dioxide in solution for a longer period than the remaining samples (Figure 6.6). This is also consistent with the reduced loss of (+)-catechin in this sample. It is evident that the 165 mg/L ascorbic acid samples have more (+)-MHF-8-catechin primed for conversion to xanthylium cations compared to the 40 mg/L samples, again suggesting that the conversion of (+)-MHF-8-catechin occurs more rapidly as the time after ascorbic acid depletion increases.

The impact of sulfur dioxide is slight for the samples with high and medium ascorbic acid but it has had a large impact on the samples with 40 mg/L ascorbic acid. The sample with sulfur dioxide was four times lower in xanthylium cations than the equivalent sample without sulfur dioxide. This was most likely a consequence of sulfur dioxide both preventing the decay in (+)-catechin in this sample and also slowing the conversion of (+)-MHF-catechin to xanthylium cations. The mechanism behind this latter conversion is not certain but there is evidence to suggest that it requires an oxidation step (Barril et al., 2009) that sulfur dioxide could impede. A similar (+)-MHF-8-catechin accumulation is observed in the 80 mg/L ascorbic acid/sulfur dioxide sample (i.e. 80AS in Figure 6.8) that also contains residual sulfur dioxide after 28 days.

The results of the xanthylium cation production are consistent with the CIELab analysis (Figure 6.10). As discussed in Section 5, the CIELab analysis allows a 3-dimensional representation of colour as described by intensity (L*) and blue (-b*), yellow (+b*), green (-a*), and red (+a*) colours. These data show that the main colour development is the yellow colouration with a hint of green. The production of red colouration, as induced by the presence of caffeic acid (Section 5), is not evident as caffeic acid is not present in these samples. The rank order of samples in the +b* and -L* direction is consistent with the xanthylium cation production (Figure 6.9).

Figure 6.10. CIELab analysis of samples at day 46.



In summary, it appears that ascorbic acid requires sufficient metal ions in order to scavenge oxygen effectively. Without metal ions added, competing side reactions occur that result in a higher consumption ratio for sulfur dioxide to ascorbic acid. This result places the metal ion content in wine in a new light and may mean that speciation of metal ions in wine may provide insight into the efficiency of ascorbic acid as an oxygen scavenger in a particular wine. If a wine has appreciable levels of metal ions such as copper or iron but they are predominantly bound tightly in complexes, they may not assist ascorbic acid in its scavenging of oxygen. Alternatively, too much metal can result in other problems including spoilage haze formation. Furthermore, if ascorbic acid consumes oxygen at a sufficiently high rate then the hydrogen peroxide produced may not be efficiently scavenged by sulfur dioxide. Such a situation may be more relevant to wine at bottling where dissolved and head-space oxygen can become high. Further studies need to be conducted to assess the levels and speciation of metal ions in wine that would provide the necessary efficiency of ascorbic acid to scavenge molecular oxygen.

Dehydroascorbic acid, and its subsequent degradation products, are not significant binders of ‘free’ sulfur dioxide. This was observed from the consumption ratios of sulfur dioxide to ascorbic acid. Sulfur dioxide did appear to inhibit the reaction product between (+)-catechin and xylosone, the latter product a degradation product of dehydroascorbic acid, but this was only significant in the presence of metal ions when the ratio of sulfur dioxide to ascorbic acid was high. Previously, it had been assumed by some that sulfur dioxide was significantly bound to ascorbic acid degradation products. The ratio of consumed oxygen to ‘free’ sulfur dioxide in the presence of ascorbic acid and metal ions is around 1:1 whist in the absence of ascorbic acid it has been shown to be 1:2. This lower consumption ratio in the presence of ascorbic acid has critical implications for increasing the shelf life of white wine. In the model wine system with excessive oxygen present, the longer that ascorbic acid or free sulfur dioxide remained in the wine, the longer it took for the flavanol/ascorbic acid pigment precursor to convert to the yellow coloured product. However, further work is required to establish whether sulfur dioxide would also prevent conversion of this pigment precursor in bottled wine over a time-frame measured in years.

It must be noted that studies conducted here have been performed in a much larger excess of oxygen than would normally be encountered by a wine bottled under either cork or ROTE screw cap. However, such conditions have allowed determination of the consumption ratios

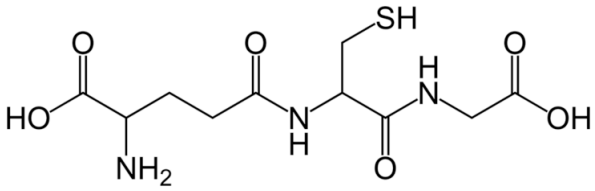
of sulfur dioxide and ascorbic acid, the impact of metal ions and ascorbic acid concentration on these ratios, and also the impact of sulfur dioxide on the degradation chemistry of ascorbic acid and pigment production. That is, the basic mechanistic processes have been evaluated and further work is required to assess how these various mechanisms operate at lower and varying oxygen concentrations.

7. Glutathione chemistry in relation to ascorbic acid and sulfur dioxide

Given the results described in this report, it is apparent that the ascorbic acid/sulfur dioxide system is efficient in its protection of the model wine system from oxidative colouration provided at least one of ascorbic acid or sulfur dioxide remain in the wine medium. Of course, when also considering the microbial stability of wine, it is certainly critical for at least some sulfur dioxide to remain in the wine.

Another protective antioxidant that is naturally present in grapes has recently received much interest. The tri-peptide glutathione (Figure 7.1), which contains a reactive thiol moiety, has been shown to offer increased protection for desirable aroma compounds in white wine at various sulfur dioxide levels (Roussis et al. 2007). Glutathione is not currently a legal additive to wine in Australia but its concentration in the final wine can be manipulated by wine production processes (du Toit et al. 2007).

Figure 7.1. The structure of glutathione



Under physiological conditions (i.e. pH 7.0), glutathione is a well-established reductant and is even capable of reducing dehydroascorbic acid to ascorbic acid. It is also known to react with hydrogen peroxide (Yin et al. 2004) and carbonyl compounds (Gunshore et al. 2006, Kanchuger and Byers 1979). Rate constants for the reaction between hydrogen peroxide and glutathione at pH 4.5 have been reported and are known to increase at higher pH values (Yin et al. 2004).

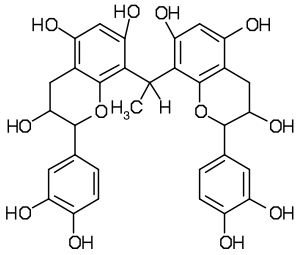
It has been well established for some time that glutathione can also undergo addition reactions with *ortho*-quinone compounds and convert them back to less reactive phenolic compounds (Cheynier et al. 1986, Cheynier and Van Hulst 1988). This is known to occur during the enzymatic oxidation of phenolic compounds, such as caftaric acid, when grapes are first crushed, and also during the non-enzymatic oxidation that is more prevalent after fermentation. Indeed, it is in wines whose must has been protected from oxygen during processing that have the highest levels of glutathione (du Toit et al. 2007).

These reactions for the protective effect of glutathione are essentially the same reactions by which sulfur dioxide acts as an antioxidant in wine (i.e. scavenging hydrogen peroxide, *ortho*-quinone compounds, and carbonyl compounds). Consequently, glutathione could potentially allow lower levels of sulfur dioxide to be used during the bottling of white wine, which would be advantageous for consumers with allergies to sulfur dioxide. Sulfur dioxide itself can impact negatively on wine aroma particularly when at high concentrations (Ribèreau-Gayon et al. 2006a) and can induce the decrease in certain beneficial aroma compounds (Daniel et al. 2004). However, the ability of glutathione to efficiently bind carbonyl compounds in wine conditions has not been fully explored.

This study investigates glutathione as a complement to sulfur dioxide when used with ascorbic acid in the oxidative protection of white wine. Initially studies were conducted to assess the ability of glutathione to bind to carbonyl compounds in wine-like conditions, and

then to assess its ability to prevent colouration in more complex model wine systems containing ascorbic acid.

Figure 7.2. A methylmethine-linked (+)-catechin dimer (8-8 linkage isomer) formed from acetaldehyde and (+)-catechin.



Model wine systems were prepared with the phenolic compound (+)-catechin (0.5 mM) and either acetaldehyde (0.25 mM) or glyoxylic acid (0.25 mM), at concentrations of 0, 0.25 and 2.5 mM glutathione. Both these carbonyl compounds are known to be reactive with wine flavanol compounds. The reaction between acetaldehyde and (+)-catechin is known to afford the methylmethine-linked (+)-catechin dimers such as that in Figure 7.2, and the glyoxylic acid and (+)-catechin reactions provide the products as shown in Figure 2.5. Although these concentrations of carbonyl compounds (i.e. acetaldehyde and glyoxylic acid) and 2.5 mM glutathione were much higher than those found in real wine conditions, it is the ratio of carbonyl compound to glutathione (i.e. 0.25:0, 1:1 and 1:10) that is important for this experiment to determine the ability of glutathione to prevent the reaction between the aldehyde and (+)-catechin. The reactions were followed by UPLC-DAD and products identified via their UV/Visible spectra and LC-MS parent ion and fragmentation data (data not shown).

Figure 7.3. Impact of glutathione on the formation of bridged-(+)-catechin dimers from (+)-catechin and acetaldehyde. Acet = acetaldehyde, LG = 0.25 mM glutathione, HG = 2.5 mM glutathione, Metal = 5mg/L iron(II) and 0.2 mg/L copper(II).

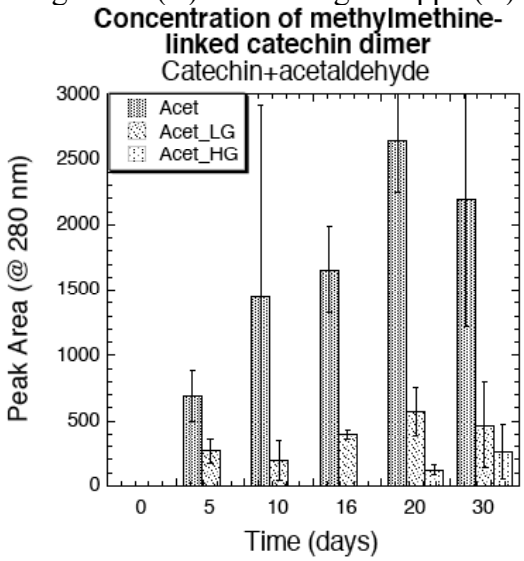


Figure 7.4. Impact of glutathione on the formation of bridged-(+)-catechin dimers from (+)-catechin and glyoxylic acid. Gly = gloxylic acid, LG = 0.25 mM glutathione, HG = 2.5 mM glutathione, Metal = 5mg/L iron(II) and 0.2 mg/L copper(II). Note the different y-scale in the graphs below.

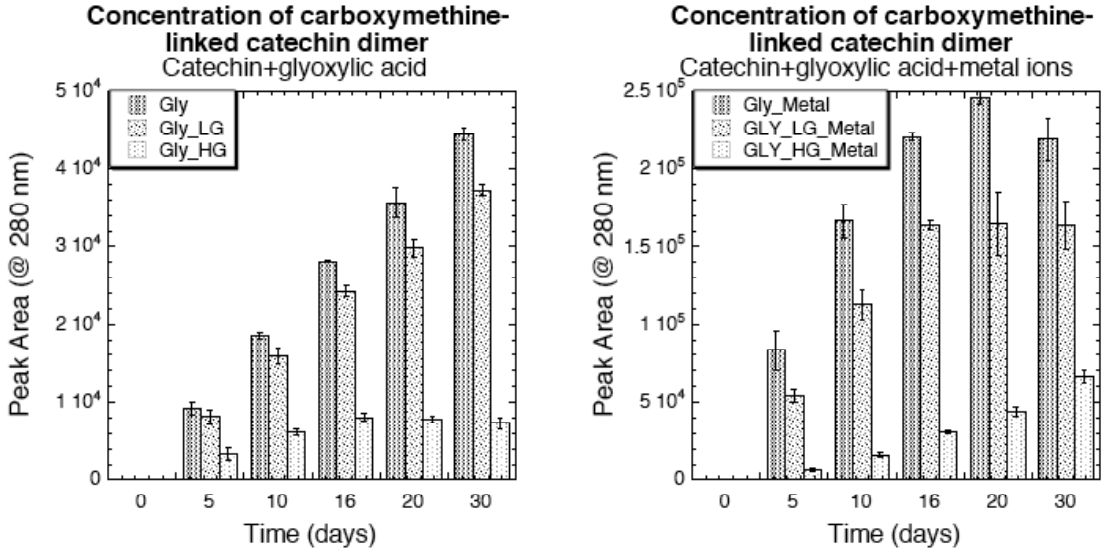


Table 7.1. Peak area of xanthylium cations after 30 days of reaction.
Peak areas have been divided by 10^4.

Sample	0 mM Glutathione	0.25 mM Glutathione	2.5 mM Glutathione
Catechin+glyoxylic acid	0.22 ± 0.03	0.217 ± 0.006	0.08 ± 0.08
Catechin+glyoxylic acid+metal	8 ± 1	5.4 ± 0.1	1.15 ± 0.09

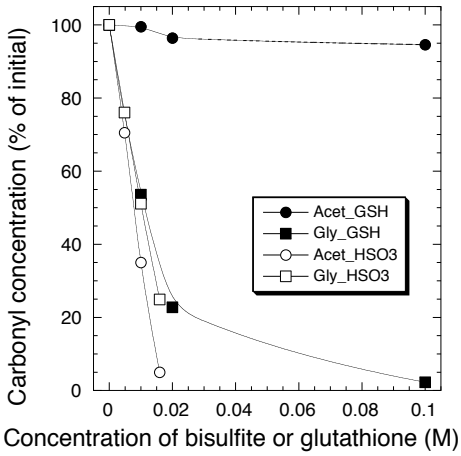
Figures 7.3 and 7.4 show that after 30 days of incubation, the bridged-(+)-catechin dimers were the highest at 0 mM glutathione and the lowest at 2.5 mM glutathione. Furthermore, the terminal product in the glyoxylic acid/catechin system, that is the xanthylium cations, were similarly impacted by glutathione (Table 7.1). This demonstrates that provided a high enough glutathione to carbonyl ratio is established, the addition reaction between (+)-catechin and carbonyl compounds can be significantly inhibited. The results at a ratio of 1:1 carbonyl to glutathione provided mixed results. The addition reaction was greatly inhibited in the acetaldehyde-case but only slightly for the glyoxylic acid reaction, both for the initial product and the terminal product. This suggests a limited effectiveness for glutathione as a competitive binder of glyoxylic acid when the concentration of the thiol becomes low. Glyoxylic acid is known to be more reactive with (+)-catechin than acetaldehyde (Clark et al. 2002) and for this reason may require higher concentrations of glutathione to inhibit the reaction.

The impact of temperature (45 °C) and metal ions (5 mg/L iron ions and 0.2 mg/L copper ions) on the ability of glutathione to inhibit bridged-(+)-catechin dimer production were separately investigated. Although higher concentrations of products were obtained under these conditions (for example see Figure 7.4, compare graphs for metal impact), their sample rank order for 440 nm absorbance intensity by the end of the experiment (i.e. 0 > 0.25 > 2.5 mM glutathione) was similar to the results for (+)-catechin/glyoxylic acid at 20 °C (Figure 7.4, left-hand side).

To confirm the binding of glutathione to carbonyl compounds, an experiment was conducted at 0.02 M carbonyl compound with incremental additions of either glutathione or sulfur dioxide. All the samples were prepared in triplicate, in 2.0 mL capped vials with no head space and held overnight at 20°C before being injected into the HPLC. Sulfur dioxide was included in the study to compare binding ability with that of glutathione. The carbonyl

concentration in the samples was then analysed by HPLC-DAD-RI using an ion exclusion column as described by Clark et al. (2007).

Figure 7.5. Concentration of carbonyl compound with increasing additions of sulfur dioxide or glutathione.



As expected, the results (Figure 7.5) show significant decreases in carbonyl concentrations upon sulfur dioxide addition. However, for glutathione the decrease is evident for glyoxylic acid but not significant for acetaldehyde. Such a result is consistent with the equilibrium constant data reported for the dissociation of glutathione addition products, albeit at non-wine pH values, where dissociation constants are 50-fold smaller for glyoxylic acid (Gunshore et al. (1985), Kanchuger and Byers (1979)). Future work will be conducted with NMR to gain an accurate measure for the dissociation constants of the glutathione-aldehyde addition products.

Despite the low binding of glutathione with acetaldehyde, the results in Figure 7.5 demonstrate that glutathione can still inhibit the product from the reaction between acetaldehyde and (+)-catechin. Intriguingly, this was the case even at the lower 1:1 acetaldehyde to glutathione ratio, at which glutathione could not effectively inhibit the glyoxylic acid/catechin reaction despite glutathione binding more strongly to glyoxylic acid (Figure 7.5). Presumably this apparent contradiction is due to the slower reaction rate between acetaldehyde and (+)-catechin, compared to glyoxylic acid and (+)-catechin, meaning that the production of the methylmethine-linked (+)-catechin dimer is particularly sensitive to the free acetaldehyde concentration.

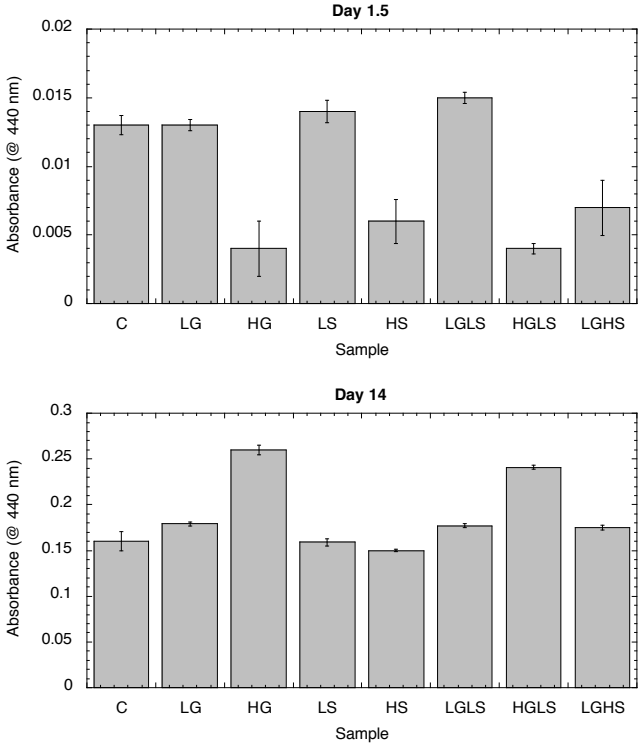
Based on the results above it was decided to utilise glutathione in combination with ascorbic acid and sulfur dioxide in the model wine system to assess its impact on the model wine system during storage. In this case, the model wine system consisted of 12%(v/v) aqueous ethanol buffered to pH 3.2 with tartaric acid/potassium hydrogen tartrate, and 18.4 g/L (0.2 M) glycerol, 100 mg/L ascorbic acid, 300 mg/L caffeic acid, 25 mg/L (+)-catechin, 5 mg/L iron ions, and 0.2 mg/L copper ions. All these concentrations are typical concentrations for these components in real white wines, on the basis that caffeic acid represents the total non-flavonoid phenolic compounds present and (+)-catechin the flavanol content. From the model wine system the following samples were prepared in triplicate and stored in saturated oxygen conditions at 45°C in the darkness:

1. C = Control
2. LG = Low glutathione (30 mg/L, 1.0×10^{-4} M)
3. HG = High glutathione (300 mg/L, 9.8×10^{-4} M)

4. LS = Low sulfur dioxide (20 mg/L, $3.2x10^{-4}$ M)
5. HS = High sulfur dioxide (40 mg/L, $6.3x10^{-4}$ M)
6. LGLS = Low glutathione and low sulfur dioxide
7. HGLS = High glutathione and low sulfur dioxide
8. LGHS = Low glutathione and high sulfur dioxide

Based on the study of du Toit et al. (2007), 30 mg/L glutathione was at the upper range of glutathione concentration measured in wine, and the 300 mg/L glutathione level was used as an extreme level that corresponded to a ratio of 1:1.7 for ascorbic acid to glutathione. The concentrations of sulfur dioxide were chosen as levels that are relatively low and high for the bottling of white wine.

Figure 7.6. The 440 nm absorbance of samples after 1.5 days and 14 days. Error bars represent the standard deviation.

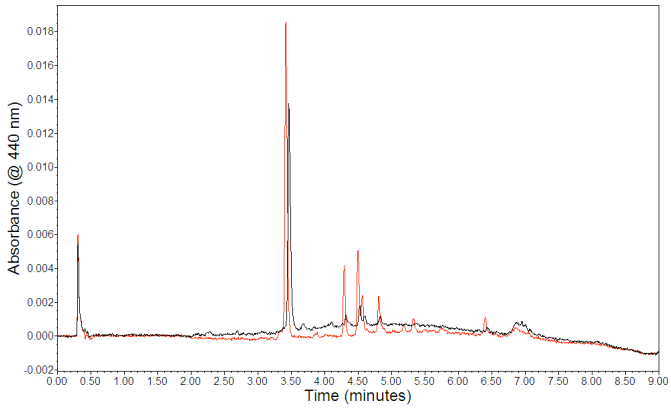


At day 1.5, when ascorbic acid still remained in all the samples, the samples could be separated into two groups based on the 440 nm absorbance values (Figure 7.6, upper graph). The samples with 300 mg/L glutathione or 40 mg/L sulfur dioxide had the least amount of colour whilst the remaining samples had similar levels of colour. Alternatively, after 14 days of the induced browning process (Figure 7.6, lower graph), the samples containing high levels of glutathione reached the highest level of 440 nm absorbance. The remaining samples were similar to the control, with the 40 mg/L sulfur dioxide sample (HS) having the least absorbance.

The behaviour of the high glutathione sample is reminiscent of our observations with ascorbic acid, that there is a period of a protective effect followed by a crossover whereby oxidative colouration is promoted. In the case of the pigments generated, the samples with high glutathione seemed to inhibit the production of xanthylium cations but increased the general area under the UPLC chromatographic baseline (Figure 7.7). This suggested that the higher glutathione concentrations were inducing the formation of a range of polymeric pigments, and the combined absorbance of these pigments was providing the extra

absorbance after 14-days. The inhibition of the xanthylium cation pigments at the high glutathione concentrations was consistent with the ability of glutathione to bind glyoxylic acid provided its concentration was sufficiently high (see Figure7.4), but may have also occurred due to scavenging of *ortho*-quinone compounds and hydrogen peroxide.

Figure 7.7. The 440 nm chromatograms of the model wine system with 0 mg/L glutathione (red trace) and 300 mg/L glutathione (black trace) after 14 days at 45 °C. The composition of the model wine is described in the text.



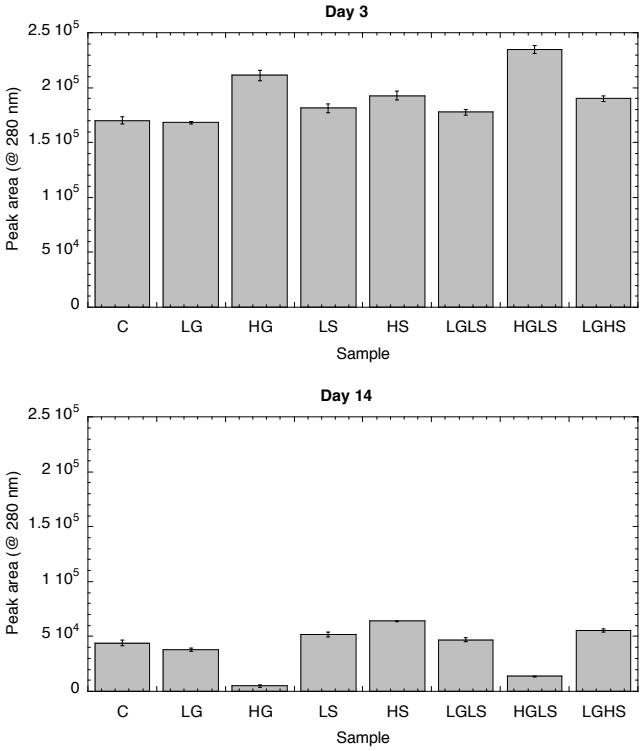
The protective effect of the 300 mg/L glutathione in the early stages of days of the experiment appeared to be linked to its ability to slow the decay in ascorbic acid and sulfur dioxide (Table 7.2). The most likely mechanisms for the slowed decay of the antioxidants again include the ability of glutathione to scavenge hydrogen peroxide, *ortho*-quinone compounds and carbonyl compounds. Also possible, but perhaps less likely, would be the ability of glutathione to reduce the oxidised form of ascorbic acid. The absence of any significant inhibition by the lower glutathione concentration indicates that the concentration of the glutathione was critical under the conditions adopted in this study.

Figure 7.8 shows that the samples with 300 mg/L glutathione (HG and HGLS) had the least depletion of (+)-catechin after 3 days, followed by the samples with 40 mg/L sulfur dioxide (HS). The remaining samples were at similar levels. After 14 days, the samples with 300 mg/L glutathione became the highest consumers of (+)-catechin whilst the samples with 40 mg/L sulfur dioxide the least.

Table 7.2. Time (days) at which the sample was depleted in either ascorbic acid or free sulfur dioxide. Measurements were performed at day 0, 0.5, 1.0, 1.5, 2.0, and 3.0.

	C	LG	HG	LS	HS	LGLS	HGLS	LGHS
Ascorbic acid	0.5	0.5	2.0	0.5	1.0	1.0	3.0	1.0
Free SO_2	n/a	n/a	n/a	0.5	1.0	0.5	2.0	1.0

Figure 7.8. The peak area for (+)-catechin in samples after 3 days and 14 days. Error bars represent the standard deviation.



The preliminary results on ascorbic acid, sulfur dioxide and glutathione provide an insight into the potential for glutathione to inhibit oxidative colouration in white wine. At a high ratio of glutathione to carbonyl compound, glutathione can inhibit the reaction between (+)-catechin and the carbonyl compounds used here (i.e. acetaldehyde and glyoxylic acid). Further, glutathione appears to be a particularly strong binder to the latter.

In a model wine constantly saturated with oxygen, high levels of glutathione initially provided protection against oxidative colouration but eventually induced the production of a diverse range of pigments and consequently the highest levels of oxidative colouration. In the initial protective period, glutathione was able to significantly delay the loss of ascorbic acid and free sulfur dioxide. Given that under normal bottling and storage regimes most wines would consume less oxygen than that consumed in the first three days of this experiment, these results demonstrate the potential for glutathione to offer a protective role. Future experiments at room temperature and reduced oxygen levels would allow an assessment of the optimum ratios/concentrations of ascorbic acid to sulfur dioxide to glutathione to allow protection against the oxidative colouration of white wine. Further studies should also consider the ability of glutathione to prevent oxidative aroma development and if its disulfide oxidised form contributes to oxidative aroma.

PROJECT OUTCOMES AND CONCLUSIONS

This project has examined in detail the chemistry of ascorbic acid in a wine-like model system. For the most part of this study, near O_2-saturation conditions were used and while this condition is extreme with respect to bottled wine, the results of the research have provided a strong basis for clarifying the antioxidant and pro-oxidant effect of ascorbic acid.

The main chemical outcomes of this project are:

- The degradation conditions such as the composition of the model wine system, degradation temperature, below 50 °C, and initial concentration of the ascorbic acid starting reagent only influence the formation and/or degradation kinetics of the degradation products and not the range of compounds formed;

- 3-hydroxy-2-pyrone and 2-furoic acid were identified as the main aerobic degradation products from ascorbic acid. Furfural was identified as a non-oxidative degradation product.
- Ascorbic acid enhances the extent of xanthylium cation pigment production, but does this only after it is completely oxidised;
- The ascorbic acid degradation process produces an aldehyde that is capable of bridging catechin-type phenolic compounds that leads to the glyoxylic acid-derived xanthylium cation pigments;
- The xanthylium cation pigment production occurs in both tartaric acid-based and formic acid-based model systems confirming that it is ascorbic acid that promotes the enhanced colour development;
- The results from the tartaric acid-based and formic acid-based model systems also confirm that hydrogen peroxide production from the oxidation of ascorbic acid is not sufficient to account for the enhanced colour development. Rather, a compound derived from ascorbic acid itself is critical to pigment production;
- L-xylosone, an aldehyde formed as one of the degradation products of ascorbic acid, was shown to react with (+)-catechin to form two furan ring-based addition isomers, known as (+)-1”-methylene-6”-hydroxy-*2H*-furan-5”-one-6-catechin and (+)-1”-methylene-6”-hydroxy-*2H*-furan-5”-one-8-catechin. The two isomers of these new compounds are the first reported products to form between a carbon-based degradation product of ascorbic acid and (+)-catechin;
- The formation of these new compounds confirms that (+)-catechin is not being directly oxidised in the presence of ascorbic acid but rather it is forming an addition product with a degradation compound of ascorbic acid. These results highlight the multifaceted role that ascorbic acid may have in wine or other food systems. While on the one hand, ascorbic acid undoubtedly acts as an antioxidant, on the other, breakdown products of ascorbic acid are capable of further reactions;
- A mechanistic link between the formation of the new compounds and xanthylium pigment production has been established. The reactivity of ascorbic acid is far more complex than the commonly described oxidation involving the simple transfer of two electrons and two protons. This demonstrates that ascorbic acid is a highly unpredictable molecule capable of inducing a variety of reaction processes;
- A comparison of ascorbic acid and erythorbic acid in terms of pigment production from flavanols showed that the isomeric influences are significant: erythorbic acid produced less oxidative colouration, at wavelengths corresponding to yellow and red, than the equivalent solutions containing ascorbic acid;
- Stereochemical effects were also found to impact the oxidative degradation of both antioxidants and flavanols: erythorbic acid was oxidised faster than ascorbic acid and (-)-epicatechin was oxidised faster than (+)-catechin;
- In a winemaking situation, the potential advantages of using erythorbic acid, in place of ascorbic acid, are that erythorbic acid:
    - produces less colour when oxidised than ascorbic acid
    - produces less red colour
    - is less expensive than ascorbic acid
- Caffeic acid degrades to dihydroxybenzaldehyde and this oxidative process is enhanced by ascorbic acid. The dihydroxybenzaldehyde reacts with catechin phenolics to produce orange/red-coloured pigments;
- Glycerol has a small inhibitory effect on the ascorbic acid-enhanced xanthylium pigment production, opening up the possibility of using higher glycerol concentrations as an added form of antioxidant protection;
- Glutathione, at high concentrations (above natural levels in wine), initially provided protection against oxidative colouration but eventually induced the production of a

diverse range of pigments and consequently the highest levels of oxidative colouration;

- In the initial protective period, glutathione was able to significantly delay the loss of ascorbic acid and free sulfur dioxide, demonstrating the possibility that, at low oxygen concentrations, there is the potential for glutathione to offer additional protection against oxidative processes;
- The degradation of ascorbic acid in the presence of sulfur dioxide is highly complex and depends on several factors including the ratio of sulfur dioxide to ascorbic acid, the oxygen concentration in the medium and the activity of metal ions that are present in the reaction matrix;
- Based on the consumption ratios of sulfur dioxide to ascorbic acid, it was found that dehydroascorbic acid, and its subsequent degradation products, are not significant binders of free sulfur dioxide. This result implies that the amount of free sulfur dioxide consumed per oxygen consumed is lower in the presence of ascorbic acid, confirming the anecdotal evidence for the synergistic relationship in the sulfur dioxide/ascorbic acid antioxidant system.
- Sulfur dioxide significantly inhibited the production of pigmented phenolic compounds and their precursors provided that the sulfur dioxide to ascorbic acid ratio was high (i.e. 2:1). This result suggests a way forward to ensure that pigmentation development in white wine can be minimised;
- Ascorbic acid was found to be significantly less efficient at scavenging oxygen at low metal concentrations. This result places the metal ion content in wine in a new light and suggests that speciation of metal ions may provide insight into the efficiency of ascorbic acid as an oxygen scavenger in a particular wine.

The outputs of the project have been widely disseminated in the winemaking and scientific communities. Dissemination has included:

- 1 book chapter;
- 6 scientific publications with 3 publications in preparation;
- 3 wine industry technical publications;
- 8 conference presentations (international and AWITCs);
- 2 presentations at regional winemaker meetings;
- on-going presentation in wine science/wine chemistry subjects at CSU.

In summary, this project has identified conditions for which ascorbic acid can act as an antioxidant and the basis for the crossover from anti-oxidant to prooxidant. The contribution of a degradation product of ascorbic acid to the enhancement of oxidative pigmentation, rather than hydrogen peroxide, has been confirmed. This result is significant as it requires a review of the basic textbook chemistry of some of the oxidative processes in white wine. The synergistic relationship between sulfur dioxide and ascorbic acid has also been confirmed, provided the amount of sulfur dioxide is at least double that of the ascorbic acid. The relationship between the oxygen scavenging capacity of ascorbic acid and concentration of metal ions, such as iron and copper, has been demonstrated for the first time. The scientific and winemaking outputs of the project have been widely disseminated and will continue to be so through further scientific and industry publications as well as through teaching programs in oenology.

APPENDIX 1

COMMUNICATION

Communication of project outcomes

The strategies used to communicate the project results were

- Publications in the scientific literature (refereed)
- Wine industry technical publications
- International scientific conferences
- Australian wine industry technical conferences
- Wine industry regional meetings
- NWGIC seminar series
- CSU teaching program

Publications in the scientific literature (refereed)

1. Célia Barril, Andrew C Clark and Geoffrey R Scollary (2008) Understanding the contribution of ascorbic acid to the pigment development in model white wine systems using liquid chromatography with diode array and mass spectrometry detection techniques. *Analytica Chimica Acta*, 621, 44-51.

2. Andrew C Clark, Fabien Pedretti, Paul D Prenzler and Geoffrey R Scollary (2008). The impact of ascorbic acid on the oxidative colouration and associated reactions of a model wine solution containing (+)-catechin, caffeic acid and iron. *Australian Journal of Grape and Wine Research*, 14(3), 238-249.

3. Andrew C Clark (2008) The production of yellow pigments from (+)-catechin and dihydroxyfumaric acid in a model wine system. *European Food Research and Technology*, 226, 925-931.

4. Célia Barril, Andrew C. Clark, Paul D. Prenzler, Peter Karuso and Geoffrey R. Scollary (2009). Formation of pigment precursor (+)-1”-methylene-6”-hydroxy-2H-furan-5”-one-catechin isomers from (+)-catechin and a degradation product of ascorbic acid in a model wine system. *Journal of Agricultural and Food Chemistry*, 57(20), 9539-9546.

5. Andrew C Clark, Jochen Vestner, Celia Barril, Chantal Maury, Paul D Prenzler, Geoffrey R Scollary (2010). The influence of stereochemistry of antioxidants and flavanols on oxidation processes in a model wine system: ascorbic acid, erythorbic acid, (+)-catechin and (-)-epicatechin. *Journal of Agricultural and Food Chemistry*, 58(2), 1004-1011.

6. Marc P. Bradshaw, Celia Barril, Andrew C Clark, Paul D. Prenzler and Geoffrey R. Scollary (2010). Ascorbic acid: A review of its chemistry and reactivity in relation to a wine environment. *Critical Reviews in Food Science and Nutrition*. In press.

Publications in preparation

7. Francesca Sonni, Andrew C Clark, Evan Moore, Fabio Chinnici, Claudio Riponi, Geoffrey R Scollary (2010) The impact of glutathione on the formation of xanthylium cations from (+)-catechin and glyoxylic acid in a model wine system. *Food Chemistry*, In preparation

8. Célia Barril, Andrew C Clark, Daniel Dias, Julie Niere and Geoffrey Scollary (2010) The degradation of ascorbic acid in a model wine: identifying the degradation productions. *Food Chemistry*, In preparation.

9. Célia Barril, Andrew C Clark, Paul D Prenzler and Geoffrey Scollary (2010) The impact of metal ions and sulfur dioxide on the decay and degradation products of ascorbic acid in a model wine system. *Journal of Agricultural and Food Chemistry*. In preparation.

Book Chapter

Felipe V Laurie and Andrew C Clark (2010) Chapter 30: Wine oxidation, In: *Oxidation in foods and beverages and antioxidant applications*, Editors: Decker, E. and Elias, R., Woodhead Publishing, Cambridge (UK).

Wine industry technical publications

Fabien Pedretti, Célia Barril and Andrew C Clark (2007) The colourful world of ascorbic acid. *Australian and New Zealand Grapegrower and Winemaker. 519, 51-54.*

Célia Barril, Chantal Maury and Andrew C Clark (2006) White wine antioxidants: ascorbic acid or erythorbic acid. What's the difference? *Australian and New Zealand Grapegrower and Winemaker.* 512, 56-60.

Andrew Clark (2006) Chemistry used to explain discolouring. *GWRDC R&D at Work*, January, 14.

International scientific conferences

Oral presentations:

Célia Barril, Andrew C Clark and Geoffrey R Scollary. Identification of the coloured pigments formed from the ascorbic acid induced oxidation of (+)-catechin in model wine sytesms. *In Vino Analytical Scientia* July 22-25, **2007**, Melbourne (Australia).

Andrew Clark, Célia Barril, Paul D Prenzler and Geoffrey R Scollary. Analytical approaches to modelling the oxidative colouration of white wine. *In Vino Analytical Scientia* July 22-25, **2007**, Melbourne (Australia).

Geoffrey R Scollary. Analytical methods and wine analysis: present status and future challenges (*Opening Plenary Lecture*). *In Vino Analytical Scientia* July 2-4, **2009**, Angers (France).

Célia Barril, Andrew C Clark, Paul D Prenzler, Peter Karuso and Geoffrey R Scollary. Formation of (+)-3'-hydroxy-2'-methylfuran-4'(2H)-one-catechin isomers from (+)-catechin and a degradation product of ascorbic acid in a model wine system. *In Vino Analytical Scientia* July 2-4, **2009**, Angers (France).

Poster presentations:

Andrew C Clark, Jochen Vestner, Célia Barril, Chantal Maury, Paul D Prenzler and Geoffrey R Scollary. The isomeric influence of antioxidant and phenolic compounds on the oxidation of a model wine system: ascorbic acid, erythorbic acid, (+)-catechin and (-)-epicatehin. *In Vino Analytical Scientia* July 2-4, **2009**, Angers (France).

Australian wine industry technical conferences

Poster presentations:

Andrew C Clark and Geoffrey R Scollary. Modelling the oxidation colouration of white wine. 13th *Australian Wine Industry and Technical Conference* July 28 – 2 August, **2007**, Adelaide.

Célia Barril, Andrew C Clark and Geoffrey R Scollary. Ascorbic acid and white wine browning. 13th *Australian Wine Industry and Technical Conference* July 28 – 2 August, **2007**, Adelaide.

Workshop presentations:
Within 'Workshop 13: White wine colour: how to measure it and what the data mean.' at the 13th *Australian Wine Industry and Technical Conference* July 28 – 2 August, **2007**, Adelaide.
- Geoffrey R Scollary. An overview of the chemistry of white wine oxidation.
- Célia Barril. Modelling the white wine oxidation process.

Wine industry regional meetings
Workshop for winemakers and viticulturists in the Hunter Valley, *13-14 August 2009*:
Andrew C Clark. White wine oxidation: ascorbic acid, sulfur dioxide and colour formation.

Workshop at the Canberra International Riesling Challenge (*16 October 2009*):
Andrew C Clark. Creating the signature Riesling: Riesling making – oxidative impacts during winemaking, and bottling and aging.

NWGIC/CSU seminars
Célia Barril. The chemistry of ascorbic acid and sulfur dioxide as an anti-oxidant system in white wine. 22 February 2006.

Célia Barril. Chemistry of ascorbic acid in a model white wine system. 19 July 2006.

Andrew C Clark. The chemistry behind wine and molecular oxygen, and 11 years of acid baths. 4 March 2009.

CSU teaching program
Results from this project (CSU 03/02) have been integrated into the following courses at Charles Sturt University; i) the Bachelor of Wine Science, ii) the Bachelor of Viticulture, and iii) the Bachelor of Viticulture and Wine Science. The specific subjects are WSC101 Wine Science 1 (first-year subject) and WSC312 Wine Science 2 (third-year subject). Both these subjects deal with white wine oxidation and approaches to limit the occurrence of oxidative spoilage, including the use of ascorbic acid to complement sulfur dioxide. Material relevant to this project (CSU 03/02) has been progressively inserted into these subjects since 2006 and the number of students enrolled in these subjects since this time is shown in the table below:

Year/subject	WSC101 Wine Science 1 (student enrolments)	WSC312 Wine Science 2 (student enrolments)
2010	n/a	27
2009	47	53
2008	35	45
2007	44	77
2006	52	71

APPENDIX 2

INTELLECTUAL PROPERTY

Intellectual property

The project outputs are in the form of new scientific knowledge that is and will be published in scientific and wine industry technical journals. The project outputs have generated scientific 'know-how' for industry's benefit.

APPENDIX 3

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APPENDIX 4

STAFF

Project staff

The following table lists staff involved in the project. The role of each person and the funding source is also identified.

Name	Agency	Years in project	Role in project	Funding
Dr Andrew Clark	NWGIC, CSU	Full project	Project Supervisor 2008 - 2010 Chief Investigator Full-project	CSU in-kind
Célia Barril	NWGIC, CSU	Full project Fulltime 2005 - 2008 Part-time 2008 - 2010	PhD student	GWRDC from 2005 - 2008
Professor Geoffrey Scollary	NWGIC, CSU The University of Melbourne	Full project	Project Supervisor 2005 - 2008 Chief Investigator 2008 - 2010	CSU in-kind 2005 - 2007
Professor Douglas Rutledge	AgroParisTech	Full project	Chief Investigator	AgroParis Tech in-kind
Dr Paul Prenzler	NWGIC, CSU	2007-2010	Collaborator	CSU in-kind
Fabien Pedretti	Université de Poitiers (France)	2007 (4-months)	Visiting student	CSU (NWGIC) funds
Jochen Vestner	Forschungsanstalt Geisenheim (Germany)	2008 (3-months)	Visiting student	CSU (NWGIC) funds
Francesca Sonni	University of Bologna (Italy)	2009 (3-months)	Visiting student	CSU competitive grant

The project team developed the following collaborations:

Assoc. Prof. Peter Karuso, from Macquarie University, whom has expertise in NMR techniques including structure and dynamics of bio-molecules using high-field NMR spectroscopy.

Dr Julie Niere and Dr Daniel Dias, from the Royal Melbourne Institute of Technology (RMIT), whom have expertise in LC-NMR techniques.

Mr John Allen, from the Australian National University (Research School of Chemistry), whom has expertise in High Resolution Mass Spectrometry.

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Understanding the contribution of ascorbic acid to the pigment development in model white wine systems using liquid chromatography with diode array and mass spectrometry detection techniques

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ABSTRACT

The present study investigated the contribution of ascorbic acid to the formation of coloured species in model white wine systems containing (+)-catechin as the oxidisable phenolic substrate. Reactions were carried out in the presence or absence of ascorbic acid in model wine systems buffered with either tartaric acid or formic acid. High performance liquid chromatography with diode array detector (HPLC–DAD) or mass spectrometry (HPLC–MS) analyses demonstrated that glyoxylic acid-derived xanthylium pigments were the main coloured species produced in all samples except those containing just (+)-catechin and formic acid. Higher concentrations of these pigments were detected in the tartaric acid based model system containing both (+)-catechin and ascorbic acid than in the corresponding formic acid model system. The inability of formic acid to form an aldehyde, unlike the known oxidative formation of aldehydes from tartaric acid, contributes to the lower colour development in the formic acid model system. Significantly, these observations imply that ascorbic acid must break down to provide an aldehyde, or ketone, capable of reacting with (+)-catechin to generate the glyoxylic acid-derived xanthylium cations.

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1. Introduction

During storage, wine can be subject to oxidation resulting in the development of off flavour as well as colouration. The latter is particularly detrimental to white wine as it appears to the consumer before purchasing and tasting the wine.

Flavanols, such as (+)-catechin (Fig. 1), are the main polyphenolic compounds correlated with white wine colouration [1–3]. In model white wine, these flavonoid compounds have been shown to react with aldehydes [4–7] and in particular, glyoxylic acid, an oxidation product of tartaric acid. The latter acid is one of the major organic acids found in grapes and wine. (+)-Catechin and glyoxylic acid can initiate a reaction sequence leading to the formation of coloured compounds known as xanthylium cations [7–9] (Fig. 1). The UV–vis spectrum of these pigments shows an absorbance maximum at 440 nm, which corresponds to a yellow colour. Recent studies in model wine systems have also shown that a hydroxycinnamic non-flavonoid, caffeic acid, had an impact on the oxidative colouration and the stability of the xanthylium

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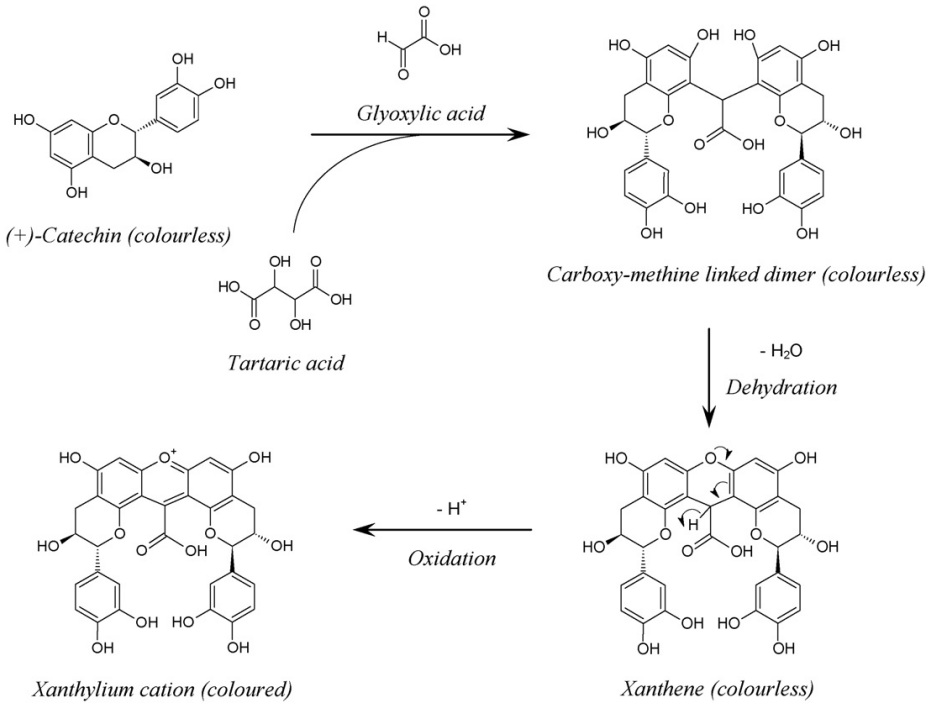


Fig. 1 – Formation of xanthylium cations from (+)-catechin and glyoxylic acid.

cation pigments [10,11]. These pigments have been detected in red wine [9] but not in white wine, presumably as a result of the lower flavonoid concentration in white wine and instability of the pigments in the presence of non-flavonoids [11].

To prevent oxidative white wine spoilage, anti-oxidants such as ascorbic acid (Fig. 2) may be added to the wine [12–15]. However, it is now recognised that the addition of ascorbic acid to a model wine system can eventually lead to a crossover effect from anti-oxidant activity (little colouration) to pro-oxidant activity (accelerated colouration) [16–18]. However, the onset of colouration is known to take place only after the solution was nearly depleted in ascorbic acid [19]. Xanthylium cations were proposed to be responsible for the enhanced colour development [19], but no confirmation or insight into the formation mechanism has been provided. Hydrogen peroxide, an initial degradation product of ascorbic acid (Fig. 2), was initially proposed to be responsible for the pro-oxidant activity [20] due to its strong oxidising power and its possible interaction with tartaric acid. Indeed, in the presence of trace amounts of metal ions (Fenton chemistry [21]), hydrogen peroxide generates hydroxyl radicals which can then induce degradation of tartaric acid to glyoxylic acid [22]. However, further study [18] demonstrated that hydrogen peroxide alone could not account for the enhanced colouration observed in model wine systems with added ascorbic acid. Consequently, additional degradation products of ascorbic acid were thought to be involved in the induced pro-oxidant activity.

The identification of ascorbic acid degradation products has been the focus of many studies [23–27] but only few studies were performed with conditions relevant to those of wine. Organic acid, ketone and aldehyde compounds [12,28–31] (Fig. 3) have been reported as being produced through ascorbic acid or dehydroascorbic acid degradation.

Aldehydes, and also potentially ketones, are reactive towards the nucleophilic flavonoids and thus (+)-catechin could potentially react with many of the ascorbic acid degradation compounds shown in Fig. 3. In fact, furfural and 5-hydroxymethylfurfural [32], similar to a reported degradation product of ascorbic acid (Fig. 3) are already known to

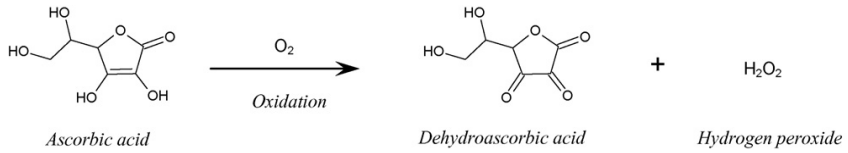


Fig. 2 – Anti-oxidant action of ascorbic acid.

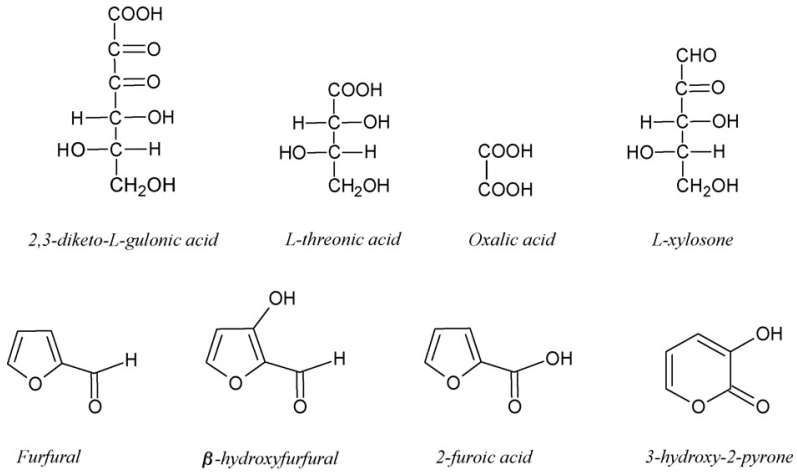


Fig. 3 – Reported ascorbic acid additional degradation products at wine pH (non-exhaustive list).

react with (+)-catechin and generate coloured xanthylium cations. Therefore, some ascorbic acid degradation products besides hydrogen peroxide could be involved in (+)-catechin addition and subsequent pigment production. However, the specific amounts and reactivity of ascorbic acid degradation products towards (+)-catechin under wine conditions are not known.

The aim of this work was to investigate how ascorbic acid can promote the colouration of (+)-catechin model systems. Different model wine systems were employed to determine the contribution of hydrogen peroxide and/or other degradation products of ascorbic acid. Reactions were compared in tartaric acid and formic acid buffers as any pigment development in the formic acid based system would not include any contribution from glyoxylic acid derived from the degradation of tartaric acid. The combination of absorbance measurements and sensitive chromatographic techniques (high performance liquid chromatography with diode array detector (HPLC–DAD) or mass spectrometry (HPLC–MS)) allowed the detection and identification of the coloured pigments ultimately induced by ascorbic acid, as well as indicating their mode of formation.

2. Experimental

2.1. Reagents and chemicals

Water purified through a Milli-Q (Millipore) water system (ISO 9001) was used for all solution preparations and dilutions. All glassware was soaked overnight in a 10% nitric acid (BDH, AnalR) and then rinsed with copious amounts of water. L-Ascorbic acid (99%), (+)-catechin hydrate (98%) and potassium bitartrate (99%) were purchased from Sigma–Aldrich. Potassium hydroxide (AR grade, >85%) was obtained from BDH and sulfuric acid (AR grade, >95%) from Ajax Fine Chemicals. Ethanol (AR grade, >99.5%, Ajax Fine Chemicals), glacial acetic acid (AR grade, >99.7%, APS Ajax Fine Chemicals), formic acid (98%, Fluka) and acetonitrile (HPLC grade, >99.9%, Ajax Fine Chemicals) were used without further purification.

2.2. Preparation of analytical solutions

2.2.1. Model white wine

Tartaric acid-buffered solution was prepared by dissolving 2.09 g of potassium bitartrate and 0.990 g of potassium hydroxide in 1 L of 12% (v/v) aqueous ethanol, resulting in a tartaric acid concentration of 0.011 M and a potassium concentration of 0.026 M (1 g L^{-1}). Formic acid-buffered solution was prepared by dissolving 1.72 g of potassium hydroxide (0.026 M) in 1 L of 12% (v/v) aqueous ethanol acidified with 423 μL of 26 M formic acid (0.011 M). The pH of the buffered solutions was adjusted to 3.2 with 10% (v/v) sulfuric acid using a Cyberscan 510 ion pH meter and a EUTECH Instruments pH electrode.

Tartaric acid- and formic acid-buffered solutions without ethanol were prepared as above, but without ethanol addition.

2.2.2. Ascorbic acid and (+)-catechin model white wine solutions

Either ascorbic acid (500 mg L^{-1}) or (+)-catechin (250 mg L^{-1}) or a combination of both (ascorbic acid 500 mg L^{-1}, (+)-catechin 250 mg L^{-1}) was dissolved in 100 mL of the appropriate model white wine. Samples were prepared in triplicate. The concentrations of the reactants are higher than those generally found in wine but were required to generate sufficient amounts of oxidation products. It has been shown that the high concentration did not affect the species formed but only their rate of formation [19].

All degradation solutions were prepared in sterile conditions to avoid any microbial growth in ethanol-free solutions. Solutions were sterile filtered (0.22 μm) into previously sterilised 250 mL Schott bottles (soaked in a 70% ethanol solution, lids were loosely screwed and bottles placed into an oven at

90 °C for a few hours). During the 24 days of the experiment, samples were maintained in darkness at 45 °C. Flasks were exposed to the air (in a sterile environment) with 2 min stirring on a daily basis to replenish the molecular oxygen content in the samples.

2.2.3. *Glyoxylic acid-derived xanthylium cation and corresponding ethyl ester reference solution*

The preparation of xanthylium cation pigments was conducted as described by Clark et al. [33]. A solution of (+)-catechin (150 mg L^{-1}, 5 mM), glyoxylic acid (2.5 mM) and copper(II) (0.6 mg L^{-1}) was prepared in a tartaric acid-buffered model white wine. The solution was left to incubate at 45 °C over 3 days.

2.3. *Experimental procedure*

2.3.1. *Absorbance measurements*

Plate reader analyses were performed using a μQuant Universal microplate spectrophotometer (Biotek Instruments) run by the software KC4 v 3.0 (Biotek Instruments). Absorbance measurements were recorded over the range 200–750 nm. The 96 well plates were prepared by filling the first three wells with water (blank) and for each of the samples, filling three wells with 300 μL each.

2.3.2. *Monitoring of the products using HPLC–DAD*

HPLC–DAD analyses were conducted every 4 days on a Waters 2690 separation module connected to a Waters 2996 Photodiode Array Detector both run by Millenium^{32} chromatography manager software. The column was a LiChrosphere RP C18 (250 mm × 4 mm) of 5 μm particle diameter with integrated guard column of the same type. The temperature of the autosampler was set up at 8 °C. Injection volume was 100 μL and the elution gradient consisted of solvent A: 2% formic acid in water and B: 2% formic acid in 80% acetonitrile, as follows (expressed in solvent A): from 95 to 50% over 45 min, down to 20% in 5 min and to 0% in 10 min; after 10 min at 0%, up to 95% in 5 min followed by 15 min at 95%. The flow rate was 0.8 mL min^{-1}. Chromatograms and UV–vis spectra were recorded over the range 200–700 nm; ascorbic acid and (+)-catechin were quantified at 280 nm and final products were detected at 440 nm.

2.3.3. *HPLC–MS identification of the pigments*

HPLC–MS analyses were performed at the end of the experiment (24 days) using a SpectraSYSTEM LC run by Xcalibur software with a P4000 sample pump and a AS3000 autosampler. Column was as previously described but the elution gradient had to be modified to satisfy mass spectrometry detection requirements; solvent A: 0.3% formic acid in water and B: 0.3% formic acid in 80% acetonitrile, as follows (expressed in solvent A): from 95 to 65% over 5 min, down to 50% in 15 min, then to 20% in 5 min and to 0% in 15 min; after 30 min at 0%, up to 95% in 10 min followed by 10 min at 95%. Flow from the column was split so that it decreased from 0.8 to 0.1 mL min^{-1} with an injection volume of 20 μL. The Finnigan AQA quadruple MS was equipped with an electrospray source to achieve ionisation (ESI). Pigments were detected in the positive ionisation mode with probe voltage set up at +3 kV, cone voltage at +10 V and probe temperature set up according to the flow rate at 190 °C.

3. Results and discussion

3.1. *Colour development by spectrophotometric measurements*

At the beginning of the experiment, all the samples were transparent and essentially colourless to the eye. During the experiment, the samples containing both ascorbic acid and (+)-catechin, regardless of the supporting buffer, intensified in yellow colouration while the other solutions remained either colourless or faintly yellow. The colouration of the samples containing both (+)-catechin and ascorbic acid was slightly more intense in the tartaric acid-buffered sample.

The UV–vis spectrum of each sample was recorded every second day for the first 12 days of the experiment and every 4 days for the 12 remaining days. UV–vis spectra of the samples containing both (+)-catechin and ascorbic acid showed the development of an absorbance maximum at 440 nm, which increased over 24 days. A similar absorbance maximum was observed in the sample with just (+)-catechin in the tartaric acid-buffer system. However, samples containing just ascorbic acid had no absorbance maximum at 440 nm but tailing of absorbance bands at lower wavelength, accounting for the slight absorbance observable at 440 nm. This is in agreement with previous work [19] that showed the development of a yellow colour in oxidised ascorbic acid solutions is a consequence of peak tailing into the visible region.

The 440 nm absorbance of the samples, plotted for the duration of the experiment (Fig. 4), shows the highest values for samples containing both ascorbic acid and (+)-catechin. The absorbance values are significantly different ($P = 0.05$), from day 8, between these samples and the others, consistent with the findings that ascorbic acid addition does lead to enhanced colouration of (+)-catechin solutions [18]. However, further insights into the pigment production can be gained

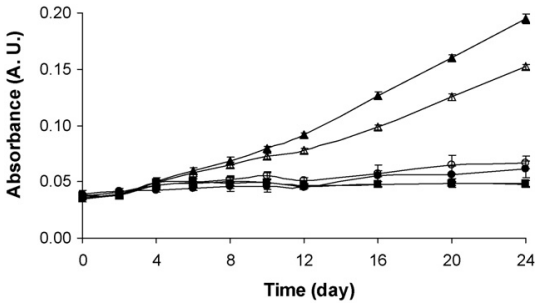


Fig. 4 – Evolution of the absorbance at 440 nm. In formic acid-buffered solutions: (□) AA, (△) AA + Cat, (○) Cat; in tartaric acid-buffered solutions: (■) AA, (▲) AA + Cat, (●) Cat; AA: ascorbic acid; Cat: (+)-catechin. Each point corresponds to the average of nine measurements (three measurements for each of the three replicated samples). Error bars represent the 95% confidence limit ($P = 0.05$) calculated from the nine values measured for each model system.

from the different buffers adopted in the model wine systems. Among the samples containing both ascorbic acid and (+)-catechin, the tartaric acid-buffered solution presented higher absorbances than the equivalent formic acid-buffered sample from day 10 (Fig. 4). This can be explained by the contribution of the hydrogen peroxide, as produced by ascorbic acid, and adventitious trace metals in the model wine system. This situation allows the conversion of hydrogen peroxide into the hydroxyl radical and the subsequent interaction of this radical with the supporting buffer, which in the case of tartaric acid, produces glyoxylic acid, a precursor of the coloured xanthylium cations. However, with formic acid, hydroxyl radicals only lead to the production of carbon dioxide. Therefore, in the formic acid-buffered sample, with both (+)-catechin and ascorbic acid added, the absorbance at 440 nm must be a consequence of (+)-catechin reacting with an ascorbic acid degradation product. Alternatively, the absorbance exhibited in the equivalent tartaric acid-buffered model system must be the result of (+)-catechin reacting with both tartaric acid degradation products and ascorbic acid degradation products.

3.2. *Identification of the reaction products by HPLC–DAD and HPLC–MS*

The formation of the reaction products was monitored by HPLC–DAD, injecting all the replicates every 4 days.

Examination of the DAD-chromatograms over the 24-day experiment revealed the appearance of peaks at identical retention times, observable in the visible region (440 nm), for the samples containing both ascorbic acid and (+)-catechin regardless of the supporting buffer (Fig. 5A and B). The same peaks were observable in the (+)-catechin sample buffered with tartaric acid but in a much smaller amount (data not shown) while no products were detected in the (+)-catechin model system buffered with formic acid. These results suggested that the main pigments generated from the reaction between (+)-catechin and ascorbic acid degradation products were the same as the main pigments generated from the reaction of (+)-catechin and tartaric acid degradation products. Consistent with the absorbance measurements, the intensity of the main pigment peaks was larger in the tartaric acid-

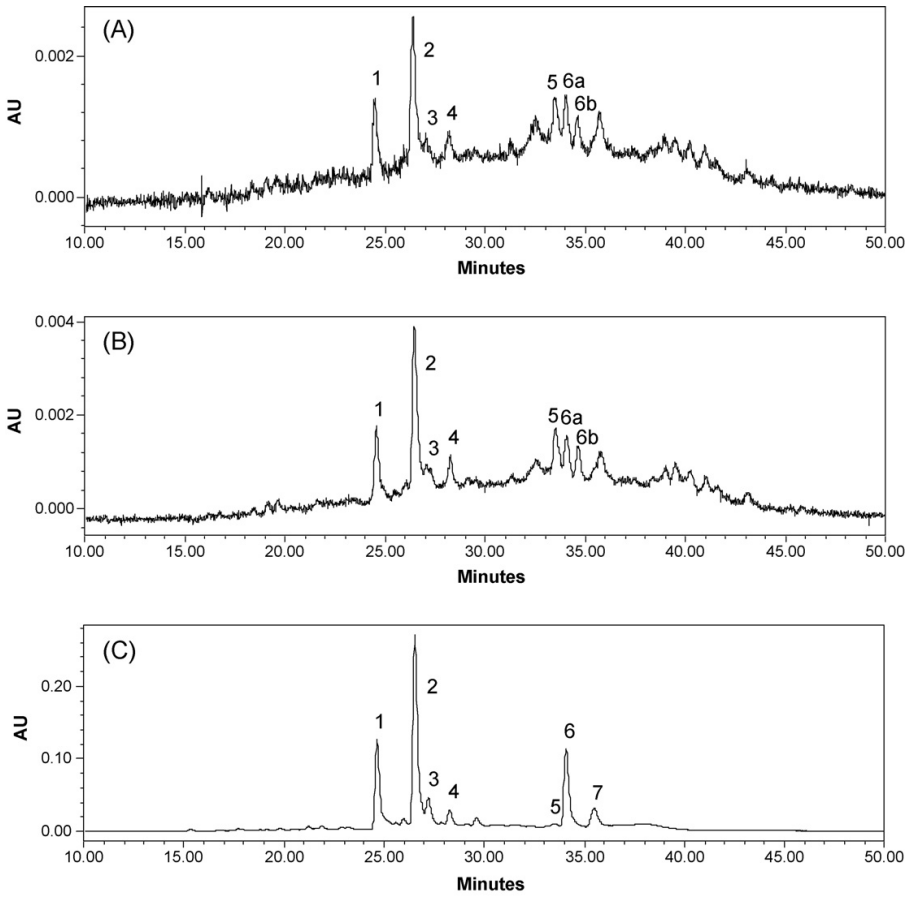


Fig. 5 – HPLC profiles at 440 nm after 24 days of the ascorbic acid and (+)-catechin samples in formic acid- (A) and tartaric acid- (B) buffered solutions and the glyoxylic acid-derived xanthylium cation (peaks 1–4) and corresponding ethyl ester (peaks 5–7) reference solution after 3 days (C).

buffered sample (Fig. 5B) than the equivalent sample with formic acid buffering (Fig. 5A).

Spectral characteristics of the products obtained from HPLC–DAD monitoring, as well as HPLC–MS analyses allowed the identification of the main pigments responsible for the colouration.

All the samples exhibiting significant pigment production at 440 nm, *i.e.* ascorbic acid and (+)-catechin samples in both tartaric and formic acid buffers (Fig. 5A and B) and (+)-catechin in the tartaric acid-buffered system only (data not shown), showed the same pattern of chromatographic peaks. Peaks 1–4 had identical UV–vis spectra with a shoulder at 310 nm and absorbance maxima around 280 and 440 nm, while peaks 5, 6a and 6b all exhibited a shoulder at 310 nm and absorbance maxima around 280 and 460 nm. For comparison, a solution containing (+)-catechin, glyoxylic acid and copper(II) (mediator of the reaction) was injected into the HPLC–DAD system after incubating 3 days at 45 °C. These conditions are known to form xanthylium cations as well as xanthylium ethyl esters [33]. Chromatographic profiles and UV–vis spectra indicated strong similarities between peaks 1 and 4 (Fig. 5A and B) and xanthylium cations (Fig. 5C, peaks 1–4) generated from the polycondensation of (+)-catechin with glyoxylic acid [7–9], as well as peaks 5, 6a and 6b (Fig. 5A and B) with the corresponding xanthylium ethyl esters (Fig. 5C, peaks 5 and 6), formed from the esterification of glyoxylic acid-derived xanthylium cations with ethanol [8]. Peak 6 in Fig. 5C is thought to actually correspond to a non-resolved combination of the two peaks, 6a and 6b (Fig. 5A and B) because of the high intensity of peak 6 in the reference solution (*i.e.* 100 times more intense than peaks 6a and 6b).

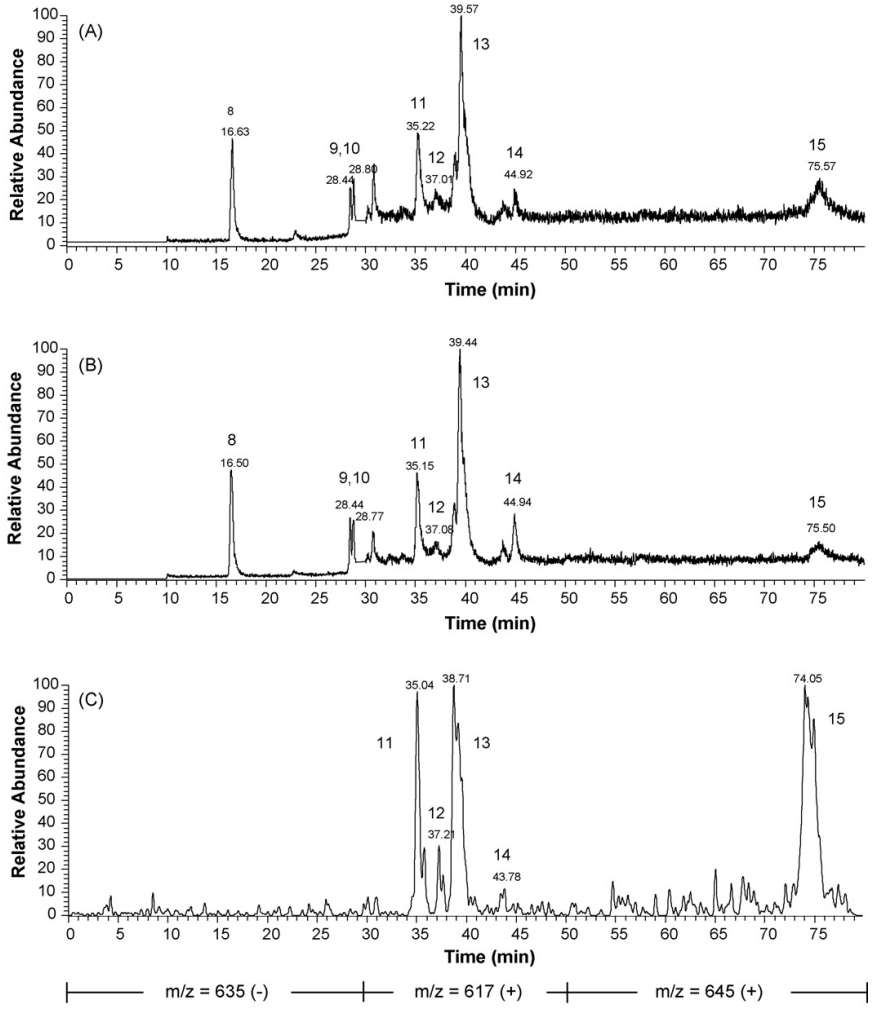


Fig. 6 – Mass chromatograms of the ascorbic acid and (+)-catechin samples in formic acid- (A) and tartaric acid- (B) buffered solutions and the glyoxylic acid-derived xanthylium cation (peaks 11–14) and corresponding ethyl ester (peak 15) reference solution (C). Single ion monitoring as indicated below the chromatograms.

Further analyses were performed by HPLC–MS to confirm the identity of the produced pigments, as detected by HPLC–DAD at 440 nm. HPLC–MS analysis of the reference solution used for DAD-chromatogram comparison is shown in Fig. 6C. The mass chromatogram displays the peaks accounting for the glyoxylic acid-derived xanthylium cations (peaks 11–14) and the corresponding ethyl esters (peak 15), extracted at $m/z = 617$ and $m/z = 645$, respectively, in the positive ionisation mode.

The ascorbic acid and (+)-catechin model systems were analysed under the same HPLC–MS detection conditions and revealed the presence of glyoxylic acid-derived xanthylium cations and corresponding ethyl esters consistent with the peaks observed for the HPLC–DAD analysis. Fig. 6A and B show the single ion monitoring mass chromatograms of the ascorbic acid and (+)-catechin solutions in the different buffer systems. The mass chromatograms A and B exhibit the glyoxylic acid-derived xanthylium cation ($m/z = 617$; peaks 11–14) and corresponding ethyl ester peaks ($m/z = 645$; peak 15), around 35–45 and 75.50 min, respectively. Peaks 8–10 (Fig. 6A and B) were also detected in the negative ionisation mode at 635 m/z and are consistent with carboxymethine-linked (+)-catechin dimers that are known intermediates in the formation of the glyoxylic acid-derived xanthylium cations [5]. These dimers were not detected in the reference solution (Fig. 6C) as the preparatory conditions, including the presence of copper(II) and heating at 45 °C, did not allow sufficient accumulation of the dimers.

HPLC–DAD and HPLC–MS analyses demonstrated that the main pigments produced in the (+)-catechin/ascorbic acid samples, regardless of the supporting buffer, were identical to the glyoxylic acid-derived xanthylium cations and the corresponding ethyl esters. These results imply that ascorbic acid degrades into glyoxylic acid or alternatively, some other compounds capable of reacting with (+)-catechin to form the glyoxylic acid-derived xanthylium cations. For example, other compounds, such as glyoxal and dihydroxyfumaric acid are known to produce the same glyoxylic acid-derived xanthylium cations when incubating in model wine solutions with (+)-catechin [34,35].

3.3. *Influence of ethanol on the pigment formation*

It is known that hydrogen peroxide and metal ions, or hydroxyl radicals directly, can induce the formation of glyoxylic acid from tartaric acid [22]. Furthermore, certain studies have suggested that ascorbic acid may also degrade via an oxidative radical mechanism [36–38]. Alternatively, ethanol can act as an inhibitor of such oxidising conditions when at high concentrations and in oxygenated solutions [39]. Therefore, the experiment was also conducted in the absence of ethanol to assess the impact of ethanol on the type and concentration of pigments generated from both tartaric acid and ascorbic acid degradation products.

The HPLC–DAD and HPLC–MS analyses of the ethanol-free samples showed that the main pigments generated were the same xanthylium cations as identified in the equivalent samples containing ethanol. This was confirmed by their identical retention times, UV–vis spectra and MS data to the glyoxylic acid-derived xanthylium cations (data not shown). The final

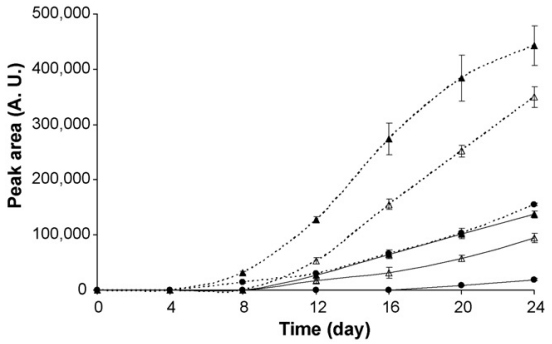


Fig. 7 – Influence of ethanol on the xanthylium cation production. In ethanol model systems (—): formic acid-buffered solutions: (–△–) AA + Cat; tartaric acid-buffered solutions: (–▲–) AA + Cat, (–●–) Cat. In ethanol-free model systems (….): formic acid-buffered solutions: (–△–) AA + Cat; tartaric acid-buffered solutions: (–▲–) AA + Cat, (–●–) Cat; AA: ascorbic acid; Cat: (+)-catechin. Each point corresponds to the average value of the three replicates. Error bars represent the 95% confidence limit ($P = 0.05$) calculated from the three values measured for each model system.

xanthylium cation concentrations were in the same rank order (*i.e.* AA + Cat (tartaric acid) > AA + Cat (formic acid) > Cat (tartaric acid)) regardless of the presence or absence of ethanol. However, apart from the (+)-catechin sample in the formic acid buffer, all the samples had accelerated formation of the glyoxylic acid-derived xanthylium cations in the absence of ethanol compared to the presence of ethanol (Fig. 7). This was also consistent with the visual assessment and 440 nm absorbance measurements conducted on the ethanol-free samples (data not shown). Therefore, although ethanol did not impact on the type of pigment generated it did inhibit the rate of pigment formation.

Intriguingly, the ascorbic acid and (+)-catechin sample buffered with formic acid, had a 4-fold increase in xanthylium cation production when ethanol was omitted from the system. In this case, the xanthylium cation production was not from the degradation of the supporting formic acid buffer, but from the degradation products of ascorbic acid. Therefore, the results suggest that ethanol can have a critical influence on the xanthylium cations derived from the ascorbic acid degradation products. The exact mode by which ethanol can exert this effect is not certain, but may include an inhibitory influence on the formation of the ascorbic acid degradation products and/or an impact on the reactivity of the ascorbic acid degradation products with (+)-catechin. This is the subject of on-going study.

4. Conclusion

The results of this study confirm that xanthylium cations, identical to those derived from glyoxylic acid and (+)-catechin, are the main pigments formed during the accelerated colouration of ascorbic acid and (+)-catechin model wine solutions.

Importantly, this was shown to be the case, regardless of the supporting acid buffer in the model wine system, indicating that a degradation product of ascorbic acid was able to react with (+)-catechin and form the same xanthylium cation as that derived from glyoxylic acid and (+)-catechin. Therefore, the results demonstrate an alternative mechanism for the formation of these particular xanthylium cations in model wine systems. Previously, their mode of formation was only designated to the oxidative degradation of tartaric acid and subsequent production of precursors such as glyoxylic acid, glyoxal and dihydroxyfumaric acid. Ethanol was observed to slow the production of the xanthylium cations regardless of their production from ascorbic acid- or tartaric acid-derived mechanisms. The identification of the critical ascorbic acid degradation products and the influence of ethanol on the production of ascorbic acid derived pigments is the subject of further study. The results of this study will also allow the complexity of the model wine system to be increased, such as the introduction of non-flavonoid compounds to the model system, to allow further insights into the fundamental chemistry behind the oxidative spoilage of white wine.

Acknowledgements

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PUBLICATION 2

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The impact of ascorbic acid on the oxidative colouration and associated reactions of a model wine solution containing (+)-catechin, caffeic acid and iron.

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Impact of ascorbic acid on the oxidative colouration and associated reactions of a model wine solution containing (+)–catechin, caffeic acid and iron

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Abstract

Background and Aims: The aim of this study was to examine the oxidative reactions and associated colouration changes relevant to white wine in a synthetic wine system consisting of different combinations of 200 mg/L caffeic acid, 150 mg/L (+)–catechin and 1000 mg/L ascorbic acid, in the presence of 1.5 mg/L iron(II).

Method and Results: Reactions were monitored by UV/VIS, CIELab, LC-DAD and LC-MS techniques. When ascorbic acid was less than 90% depleted in samples, it induced yellow/green colouration but prevented brown colouration that would otherwise result from catechin- and caffeic acid-derived yellow and red pigments. However, during the loss of ascorbic acid, in the presence of either catechin and/or caffeic acid, reactions were still occurring to 'prime' the system for rapid colour formation. When the ascorbic acid was more than 90% depleted, the samples with catechin and/or caffeic acid had an increased rate of brown colouration compared with those to which ascorbic acid was not added.

Conclusions: This study demonstrates that ascorbic acid is an effective anti-browning agent provided it persists in the wine after bottling, but if depleted, serious brown colouration ensues.

Significance of the Study: Findings detailed in this report will provide useful guidelines for the more effective use of ascorbic acid as an anti-browning agent in bottled wine.

Abbreviations

LC-DAD high performance liquid chromatography with photodiode array detector; **LC-MS** high performance liquid chromatography with mass spectrometry detection; **UV** ultraviolet; **VIS** visible; **CIE** International Commission on Illumination

Keywords: *browning, caffeic acid, catechin, iron, oxidation, pinking, white wine, xanthylium cation*

Introduction

The oxidation of white wine is accompanied by various colour changes, particularly the appearance of a 'brown' colour, that give a visual clue to potential deterioration of the flavour and aroma of the wine. As such, wine colour is an important sensory property and there is considerable research devoted to understanding the formation of new pigments during wine spoilage and aging. A simple measure for the oxidative discolouration of white wine is the absorbance of light at 420 nm (Simpson 1982), a wavelength that is actually a measure of the yellow colour of the wine. More recently, Skouroumounis et al. (2005) showed that for several white wine varieties, the perceived browning after their oxidation was more consistent with the increase in absorbance at 500 nm, a wavelength corresponding to red colouration, rather than 420 nm. Therefore, this recent research highlighted the importance of the specific wavelength used to measure the oxidative colouration of white wine, a factor that has not been considered in many wine and model wine oxidation studies.

Model wine systems have been routinely used in studying the chemistry involved during the oxidation of wine-related components. Previous studies have found that in model systems that contained (+)–catechin (Figure 1; a flavanol) as the only phenolic compound present, the solution turned yellow, with a distinct absorbance maximum at 440 nm, after an accelerated oxidation procedure (Oszmianski et al. 1996, Clark and Scollary 2002). The pigments responsible for the yellow colour were xanthylium cations (Figure 1) formed from catechin and oxidation products of tartaric acid, used to pH buffer the model wine system. However, a model wine containing both catechin and caffeic acid (Figure 1; a hydroxycinammic acid) turned brown, with a broader absorbance over 400–500 nm, after the same accelerated oxidation procedure (George et al. 2006). The yellow xanthylium cations were again identified, but additional catechin/

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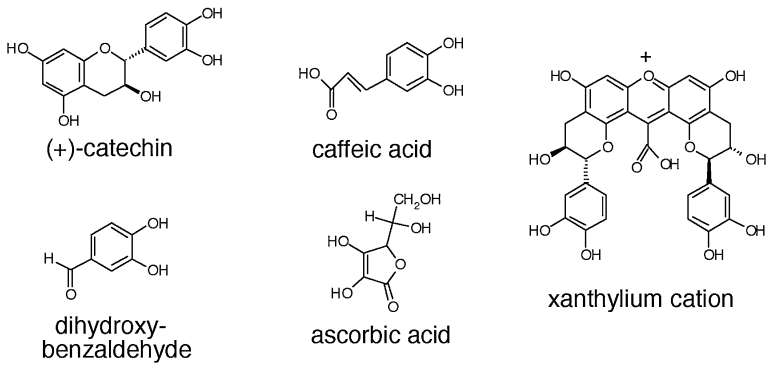


Figure 1. Chemical structures for several compounds present in the model wine solutions.

caffeic acid-derived red pigments were also identified. Consequently, the mixture of red and yellow pigments resulted in the perceived brown colouration of the oxidised model wine, emphasising the need to consider several wavelengths in the oxidation study.

There are various ways to prevent oxidative spoilage of wine and the associated colour changes. Preventing or minimising oxygen ingress at bottling is one means, but antioxidants, such as sulphur dioxide or ascorbic acid (Figure 1), are often added to a wine to react with residual oxygen and/or inhibit further oxidative reactions. However, recent work in catechin and ascorbic acid model systems has shown that ascorbic acid may have a dual role; at times preventing formation of pigments, while under other conditions, greatly enhancing the rate of their formation (Bradshaw et al. 2001). The 'pro-oxidant' effect was ascribed to reactions between the breakdown products of ascorbic acid and catechin to generate pigments, which only formed once the ascorbic acid had been depleted (Bradshaw et al. 2003). However, this study, and related studies with ascorbic acid in model wine systems, have only focused on the pigments detected at 420 nm rather than considering those with significant absorbance at higher wavelengths, and thus potentially contributing to brown colour.

Given that in model white wine systems of greater complexity (i.e. containing both catechin and caffeic acid) brown colours were generated, it was of interest to explore the role ascorbic acid may play in this system. Specifically, we have investigated whether ascorbic acid is able to prevent the formation of the pigments contributing to the brown colour and whether new pigments are formed due to reactions between breakdown products of ascorbic acid and caffeic acid (and whether these are coloured). Furthermore, we are now able to report colour changes in our model systems using CIELab, which gives a more holistic view of perceived colour than a simple absorbance measurement at a particular wavelength. Thus, we present CIELab data for model wines containing single components (ascorbic acid, catechin, and caffeic acid), as well as binary and ternary mixtures, kinetic and chromatographic data. Together, the data give new insights into colour changes and pigment formation in model white wines.

Materials and methods

Reagents and apparatus

All glassware and plastic-ware were soaked for at least 16 h in 10% nitric acid (BDH, Poole, England; AnalaR) and then rinsed with copious amounts of Grade 1 water (ISO 3696). Solutions and dilutions were prepared using Grade 1 water. Chemicals were obtained from Sigma (St Louis, MO, USA) (potassium hydrogen tartrate (>99%), L(+)-tartaric acid (>99.5%), caffeic acid (99%), (+)–catechin monohydrate (98%)), Aldrich (Milwaukee, WI, USA) (L-ascorbic acid (>99%)) and AJAX (Auburn, NSW, Australia) (iron(II) sulfate heptahydrate (AR)).

Absorbance measurements and spectra were recorded on a μQuant Universal Microplate Spectrophotometer (Biotek Instruments) with the software KC4 v3.0 (BioTek Instruments, Winooski, VT, USA) using 96 well plates (UV-Star, Greiner Bio-one, Frickenhausen, Germany) and a sample volume of 300 μL. The wine-like solution, described in the *Reactions* section, was used to zero the microplate spectrophotometer for all measurements.

CIELab measurements were conducted on a Shimadzu UV-1700 UV-Visible spectrophotomer with UVPC Colour Analysis software (version 3.00; Shimadzu Scientific Instruments, Kyoto, Japan). The L*, a* and b* CIELab values were calculated at the daylight illuminant D65 and with a 10-degree observer angle. The transmission was scanned over the range 380 to 780 nm with samples in 10 mm quartz cuvettes. The wine-like solution was used as the blank solution.

LC-DAD experiments were with instrumentation as described previously (Clark and Scollary 2002). The column was a LiChrosphere RP C18 (250 × 4 mm) of 5 μm particle diameter with integrated guard column of the same type. The temperature of the autosampler was set at 8°C. Injection volume was 100 μL and the elution gradient consisted of solvent A: 2% formic acid in water and B: 2% formic acid in 80% acetonitrile, as follows (expressed in solvent A): from 95 to 50% over 45 min, down to 20% in 5 min and to 0% in 10 min; after 10 min at 0%, up to 95% in 5 min followed by 15 min at 95%. The flow rate was 0.8 mL/min. Chromatograms and UV-visible spectra were recorded over the range 200 to 700 nm, ascorbic acid and catechin were quantified at 280 nm and caffeic acid was quantified at 380 nm. The

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oxidation products of tartaric acid, including glyoxylic acid, were not monitored in the reaction system as the presence of formic acid in the liquid chromatography mobile phase prevented their detection. LC-MS was conducted as described by Lutter et al. (2007). Ascorbic acid was also quantified by titration with 2,6-dichlorophenolindophenol dye (Iland et al. 2000).

Reactions

Samples were prepared in a model wine system containing 1.5 mg/L iron(II) with various combinations of 150 mg/L catechin, 200 mg/L caffeic acid and 1000 mg/L ascorbic acid. The concentrations for the phenolic compounds were adopted to be consistent with a past model wine oxidation study (George et al. 2006), while the ascorbic acid concentration was known to influence the oxidative colouration in simpler model wine systems containing only catechin (Bradshaw et al. 2003). In Bradshaw et al.'s (2003) work, a high concentration of ascorbic acid was required in order to understand the chemistry occurring during ascorbic acid breakdown. It was shown that the concentration of ascorbic acid did not affect the species formed, only their rate of formation (Bradshaw et al. 2003). Iron is added at the rate commonly found in wines (Cacho et al. 1995, Riganakos and Veltsistas 2003), and its inclusion will mediate reactions between oxygen and other components (Powell and Taylor 1982).

The wine-like solution was prepared by adding 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid to aqueous ethanol (12% v/v) and stirring overnight at room temperature. The pH of the wine-like solution was 3.2 ± 0.1. (+)–Catechin (150 mg/L), caffeic acid (200 mg/L) and/or ascorbic acid (1000 mg/L) were added to this solution to form the model white wines. The different combinations of these components were prepared in 500-mL volumes and using a 2.8-mg/L iron(II) sulfate heptahydrate stock solution, an addition of iron(II) (1.5 mg/L) was made directly to each 500-mL model wine solution. The iron(II) stock solution was prepared fresh in the wine-like solution and used immediately.

After the addition of the iron(II) to the model white wine, three 150 mL portions were taken and placed in separate 250 mL Schott bottles with screw top lids. The samples were held in darkness at 45°C, unless stated otherwise, and the sample bottles were aerated, with vigorous stirring to achieve a vortex, for 5 min each day. Data were collected every 2 days, including concentration of components, absorbance spectra of solutions and CIELab, and LC-DAD chromatograms. All samples were prepared in triplicate and the plotted data are the means of the replicates, and significant differences ($P = 0.05$) (Miller and Millier 1993) are indicated within the text.

Results and discussion

Colour changes during oxidation

At day 0, all samples were essentially colourless except those containing caffeic acid, which had a faint yellow colour due to tailing into the visible region of the absorbance spectrum by caffeic acid (George et al. 2006). During the storage of these samples in darkness at 45°C, with daily exposure to atmospheric oxygen, all samples, other than the control, gradually became coloured, as assessed visually over the 17-day experiment (Figure 2).

Up to day 8 of the experiment, the samples with catechin and catechin/caffeic acid exhibited distinct absorbance bands in the visible spectrum (Figure 3a) as previously described (George et al. 2006). The absorbance of the caffeic acid/catechin sample at both 440 nm and higher wavelengths (i.e. 480–520 nm) explained the 'brown' appearance of this sample compared with the yellow appearance of the catechin sample (i.e. maximum at 440 nm). The presence of ascorbic acid in binary and ternary mixtures completely suppressed the appearance of these bands (Figure 3a) and in fact, samples containing ascorbic acid exhibited very similar visible spectra with minimal absorbances (<0.03 absorbance units) above wavelengths of 480 nm. Consequently, these latter samples with ascorbic acid were yellow in colour. This clearly demonstrated the ability of ascorbic acid to suppress the pigments responsible for the absorbance above 480 nm in the catechin/caffeic acid sample, and hence suppress the brown colour in favour of a yellow colour. Of significance, Figure 3a demonstrates that the absorbance intensity at 420 nm is not an indicator for the brown colour in these model wine samples.

The visible spectra for the samples began to show substantial differences after day 8. Figure 3b shows the visible spectra of the samples on day 17, and the sample containing all components, ascorbic acid/catechin/caffeic acid, had the highest absorbance at all visible wavelengths. This was consistent with the visual observation that this sample was the most brown by day 17 (Figure 2). The visible spectrum for ascorbic acid shows minor absorbance above 440 nm, in contrast to binary and ternary mixtures containing ascorbic acid. This points to the likely involvement of ascorbic acid breakdown products in pigment formation during days 8–17. Also of significance is that spectra of catechin and catechin/caffeic acid maintain their distinct absorbance maxima in the visible region, whereas other samples with these compo-

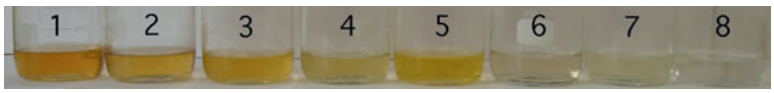


Figure 2. The model wine samples after 17 days of the induced oxidation. The samples all contained 1.5 mg/L of added iron(II), as well as: 1, catechin/caffeic acid/ascorbic acid; 2, catechin/caffeic acid; 3, catechin/ascorbic acid; 4, caffeic acid/ascorbic acid; 5, catechin; 6, caffeic acid; 7, ascorbic acid; and 8, no addition.

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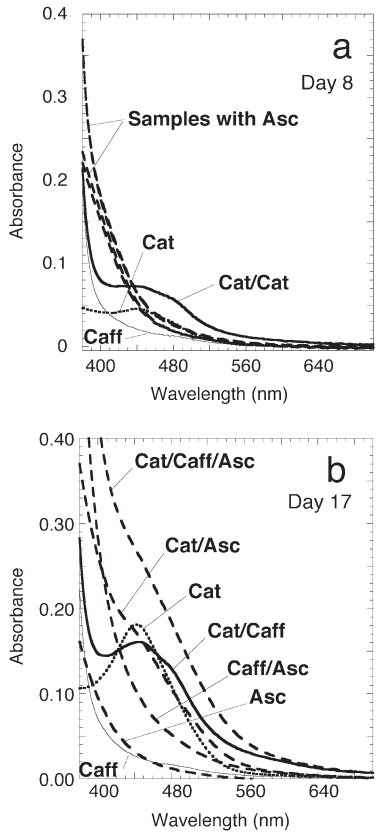


Figure 3. The UV/VIS spectra of the model wine samples after 8 (a) and 17 (b) days of induced oxidation. The spectra with dashed lines correspond to those containing ascorbic acid, the dotted line to catechin, the thick solid line to catechin/caffeic acid, and the thin solid line corresponds to caffeic acid.

nents and ascorbic acid have spectra with shoulders tailing into the visible region. Figure 3b also illustrates that only two solutions, catechin/caffeic acid and catechin/caffeic acid/ascorbic acid, have spectra that tail out to 700 nm.

Figure 4a shows the A_{440} and A_{500} time course data for the ascorbic acid sample during the experiment and demonstrates the typical A_{440} increase, in this case to day 8, and decrease that has been previously reported for ascorbic acid in a similar model wine system (Bradshaw et al. 2003). The A_{500} time course data, not previously reported, are significantly ($P = 0.05$) less intense than the A_{440} data (<0.015 absorbance units) and show less pronounced changes throughout the experiment, consistent with the lack of brown colouration observed in the ascorbic acid sample.

Figure 4b and 4c show absorbance time course data at 440 nm and 500 nm, respectively, for the remaining samples in the experiment. In this time course data, the absorbances for all the samples containing ascorbic acid (Figure 4b,c) have been corrected for the absorbance provided by ascorbic acid alone (Figure 4a). The notation '*A'

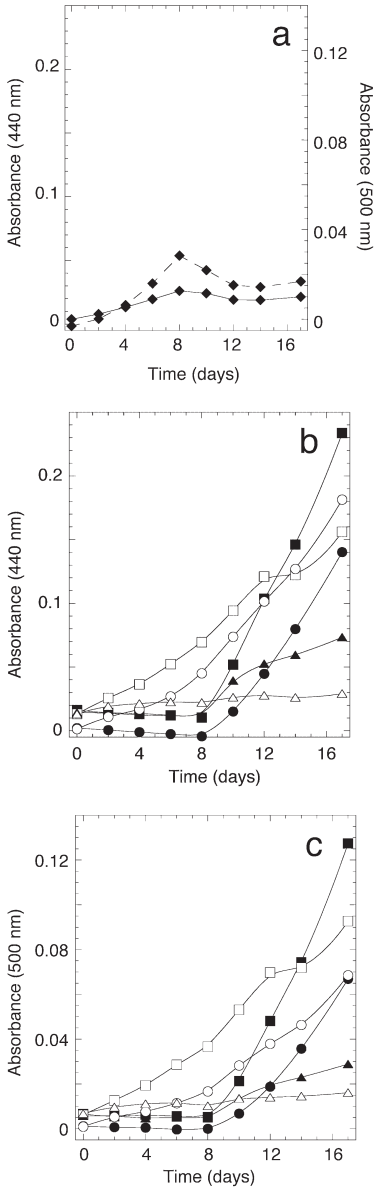


Figure 4. The A_{440} (dashed line) and A_{500} (solid line) time course data for ascorbic acid (a), and the $*A_{440}$ (b) and $*A_{500}$ (c) time course data for all other samples. In the *A data, the samples with ascorbic acid are corrected for the absorbance due to ascorbic acid alone. The samples are: (■) caffeic acid/catechin/ascorbic acid, (□) caffeic acid/catechin, (●) catechin/ascorbic acid, (○) catechin, (▲) caffeic acid/ascorbic acid, (△) caffeic acid and (◆) ascorbic acid.

will be used to denote this corrected absorbance. The data in Figure 4b and 4c clearly demonstrate that from days 0 to 8, the samples with ascorbic acid have little $*A_{440}$ or $*A_{500}$ intensity. Hence, the main absorbance from days 0 to 8 in the samples with ascorbic acid would be that explained by ascorbic acid alone (Figure 4a). Alternatively, during the same period, the samples without ascorbic acid are all steadily increasing in $*A_{440}$ or $*A_{500}$

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Table 1. CIELab values for the model wine samples.

Sample	Day 0			Day 8			Day 17			Change in values Day 8–Day 17		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
Catechin/caffeic acid/ascorbic acid	99.8	−0.4	1.8	98.9^{g}	−2.1^{i}	8.6^{k,l}	92.6	1.5^{n}	29.1	−6.3	3.6	20.5
Catechin/caffeic acid	99.6^{a}	−0.3^{d}	1.5^{e}	98.1	−0.2^{j}	7.5^{l}	94.9	1.0^{n}	17.0^{q}	−3.2	1.2	9.5
Catechin/ascorbic acid	100.0^{b}	0	−0.2^{f}	99.3^{g,h}	−1.8^{i}	6.9^{k,l}	96.1^{m}	−0.9^{p}	20.5^{q,r}	−3.2	0.9	13.6
Caffeic acid/ascorbic acid	99.8	−0.4^{d}	1.5^{e}	98.9^{g}	−2.2^{i}	8.9^{k}	97.5	−2.1^{o}	12.8	−1.4	0.1	3.9
Catechin	100.0^{b}	−0.1	0.1	99.0^{g}	−0.8	6.1^{k}	96.7^{m}	−2.0^{o}	21.5^{r}	−2.3	−1.2	15.4
Caffeic acid	99.7^{a}	−0.3^{d}	1.3	99.4^{h}	−0.4	2.8	98.9	−0.2	2.9	−0.5	0.2	0.1
Ascorbic acid	100.1^{c}	0	−0.4	99.1^{g}	−1.9^{j}	7.3^{k,l}	99.1	−0.9^{p}	3.8	0	1	−3.5
No addition	100.0^{b,c}	−0.1	−0.2^{f}	99.8	−0.1^{j}	0.4	99.6	0	−0.2	−0.2	0.1	−0.6

The values quoted are the average CIELab values ($n = 3$) for samples at days 0, 8 and 17 of the experiment. Values are significantly different ($P = 0.05$) from other values within a column unless they have at least one similar superscript letter. The change in the mean CIELab values ($n = 3$) for samples from day 8 to day 17 is also shown.

absorbance. After day 8, the increase in absorbance with time in the ascorbic acid-binary and ternary mixtures is more rapid than when ascorbic acid is absent. In support of the spectra already presented (Figure 3), this suggests that ascorbic acid can inhibit the production of caffeic-derived and/or catechin-derived pigments for a period of time, but eventually accelerated production of pigments occurs. Importantly, in the first 8 days of the experiment, the $*A_{500}$ of the ternary mixture is prevented by the presence of ascorbic acid, and this leads to the sample appearing yellow but not brown.

The CIELab analysis of samples (Table 1) was also performed as this allows both quantitative and descriptive analysis of the samples that is comparable with their visual assessment. The L* parameter gives an indication of the intensity of colour from essentially none (100) to maximum (0), a* gives an indication of green (−a*) to red (+a*) colouration and b* gives an indication of yellow (+b*) to blue (−b*).

The CIELab data at day 8 showed that the caffeic acid/catechin sample had the highest colour intensity (L*). The a* parameter was negative for all samples indicating a slight green colour, but for ascorbic acid and mixtures with ascorbic acid, this was more pronounced. Thus, the presence of ascorbic acid is probably the main cause for negative a* values. Other than the control (Table 1, No addition), the caffeic acid/catechin sample had an a* value that was closest to red portion of the axis which was consistent with the spectra data showing increased absorbance at 480–520 nm wavelengths. All samples tended to the yellow side of the CIELab axes as indicated by positive b* values. In terms of single component systems, caffeic acid is the least yellow, while ascorbic acid is the most yellow and catechin is similar. There are no clear trends for the yellowness of mixtures, but as observed from the spectra data, the colouration is not additive (i.e. catechin + ascorbic acid might be expected to be 6.1 + 7.3 = 13.4, whereas b* is only 6.9 for this mixture).

The CIELab data at day 17 show that all mixtures are more intensely coloured (L*) than single component samples, the exception being catechin. The a* parameter is now positive for catechin/caffeic acid and catechin/caffeic acid/ascorbic acid indicating an increase in red colour. With the concurrent presence of yellow colouration (see below), the mixture of colours provides the perceived brown colouration in the samples evident in Figure 2. It is informative to look also at the change in a*, which shows that all solutions have either had no shift or a shift towards more red colouration. The exception again is catechin, which is the only sample to show a shift to increasing green (while also becoming intensely yellow, see below).

The b* parameter shows the biggest divergence among the samples from days 8 to 17. Consistent with the data in Figure 4, b* decreases for ascorbic acid, whereas in all mixtures with ascorbic acid, b* increases. Catechin is again unique among the single component samples in that it continued to show a large increase in yellow, which was accelerated during days 8–17 compared to days 0–8. Previous work (Es-Safi et al. 2000b, Clark and Scollary 2002) has shown that the yellow colour can be attributed to xanthylium cations, which can be formed from the reaction between glyoxylic acid (a degradation product of tartaric acid) and two molecules of catechin. The CIELab data indicate that when catechin is in binary mixtures with either caffeic acid or ascorbic acid, the increase in yellow colour from days 8 to 17 is inhibited, with a corresponding shift to red colouration. Overall, the largest change in b* was found in the ternary mixture, which also showed the largest change in a*. However, by examining the various binary combinations, it can be concluded that catechin/caffeic acid is mostly responsible for the red colour, while catechin/ascorbic acid is driving increases in the yellow colour.

These data demonstrate that before day 8 of the experiment, the samples with added ascorbic acid maintained a similar yellow/green colouration in the model

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wine samples, regardless of the presence of caffeic acid and/or catechin. Importantly, the results demonstrate that ascorbic acid can suppress the absorbance at higher wavelengths (480–520 nm), which would otherwise be present in the catechin/caffeic acid samples, and therefore maintain a yellow/green colour rather than a brown colour. However, any non-coloured species cannot be determined by the techniques discussed above. Further insights into how the various components, especially ascorbic acid, impact on reactions to form colourless (and/or coloured) compounds may be inferred from the kinetic data shown in Figure 5.

Kinetic study on the model wine solution components during oxidation

At 2-day intervals, concentrations of phenolic compounds and ascorbic acid were measured by LC-DAD, and the ascorbic acid concentration was also determined via titration with the dichlorophenolindophenol reagent (Iland et al. 2000). The concentrations of ascorbic acid measured by these two techniques were closely correlated (gradient = 1.05, $R^2 = 0.9805$, $n = 20$).

No significant difference ($P = 0.05$) was observed for the rate of ascorbic acid decay in any of the samples (Figure 5a). This suggested that the decay of ascorbic acid in the model wine system was independent of the presence of catechin and caffeic acid, i.e. there is no direct reaction between ascorbic acid and catechin or caffeic acid to form new species, coloured or otherwise. All the significant reactions involving ascorbic acid and colour formation occur after an initial reaction involving ascorbic acid alone. The independence of ascorbic acid decay is consistent with its lower redox potential compared with phenolic compounds such as catechin and caffeic acid (Danilewicz 2003). At day 10, around 90% of the ascorbic acid is depleted in the samples, and it is at this time that binary and ternary mixtures containing ascorbic acid had a rapid increase in colour (Figure 4b,c). This observation has been reported previously for a simpler model system containing just catechin and ascorbic acid (Bradshaw et al. 2003), without added iron(II). Kinetic analysis of the data in Figure 5a shows that ascorbic acid decays by a two-stage process, and both stages are pseudo-first order (k(0–6 days) = 0.11/day; k(8–17 days) = 1.22/day). The faster decay rate in the second stage is independent of catechin, caffeic acid and oxygen (the latter was maintained at a constant dose rate). Therefore, it is possible that breakdown products of ascorbic acid accumulate to the point where they start to react with ascorbic acid and enhance its decay.

The decay of catechin appears to be slightly enhanced ($P = 0.05$) in the presence of caffeic acid (Figure 5b) compared with catechin alone, and the products of these reactions are different (Figure 3). In both cases, the loss of catechin follows pseudo-zero order kinetics ($k = 5.2 \times 10^{-6}$ mol/L/day for catechin, $k = 6.9 \times 10^{-6}$ mol/L/day for catechin/caffeic acid), indicating that some slower reaction (not involving catechin) is involved prior to reactions with catechin. In terms of pigment formation, coloured compounds are formed in a reaction where the rate-determining step does not involve catechin.

Caffeic acid shows similar decay kinetics to catechin in that the reactions are pseudo-zero order (after a very rapid drop from day 0 to day 2) regardless of whether caffeic acid is alone, or in mixture with catechin. While

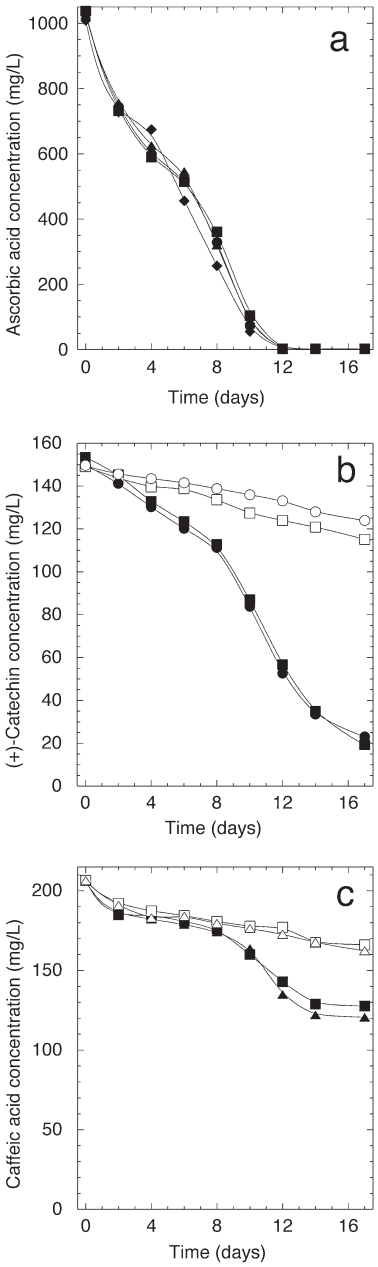


Figure 5. The change in concentration of (a) ascorbic acid, (b) catechin and (c) caffeic acid in the model wine systems with 1.5 mg/L iron(II). The samples are: (■) caffeic acid/catechin/ascorbic acid, (□) caffeic acid/catechin, (●) catechin/ascorbic acid, (○) catechin, (▲) caffeic acid/ascorbic acid, (△) caffeic acid and (◆) ascorbic acid.

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the decay of caffeic acid does not give rise to pigmented species, coloured compounds are formed in the presence of catechin, but this does not occur via a direct reaction between catechin and caffeic acid as the rate-determining step does not involve caffeic acid. The pseudo-zero order rate constant is 1.4×10^{-5} mol/L/day, which is double the rate of decay of catechin.

The addition of ascorbic acid has a significant effect on the decay of both catechin and caffeic acid (Figure 5b,c), giving rise to a two-stage process for both phenolic compounds: a 'slow' step, days 0–8; and a 'fast' step, days 8–17. For catechin, both stages are affected by ascorbic acid, whereas for caffeic acid, it is only the fast step where the influence of the added ascorbic acid becomes apparent.

The decay of catechin in the catechin/ascorbic acid solution is still pseudo-zero order in the slow stage, with $k = 1.7 \times 10^{-5}$ mol/L/day, some three times faster than in the absence of ascorbic acid. It can be inferred that the breakdown products of ascorbic acid, formed in a rate-determining step, are reacting with catechin in a reaction that is more rapid than that of the auto-decay of catechin. The products of this reaction are not coloured, as all colours can be accounted for from ascorbic acid by-products. The nature of these colourless compounds will be discussed below. In the fast step, the rate of pigment formation is enhanced and the products formed are not simply xanthylium cations as observed with catechin alone. This is evidenced by the absorption spectrum (Figure 3b), showing increasing absorbance at 380 nm for catechin/ascorbic acid, rather than the characteristic maximum of xanthylium cations (i.e. 440–460 nm).

In the caffeic acid/ascorbic acid solution, the effect of added ascorbic acid only becomes evident after day 8. The accelerated loss of caffeic acid is accompanied by formation of yellow pigments (Table 1), but this is due to tailing of strongly UV-absorbing species into the visible region, rather than a distinct absorption maximum as observed for the red/brown pigments from catechin/caffeic acid solutions.

In the ternary mixture, catechin/caffeic acid/ascorbic acid, the kinetics of decay of any one species is independent of the presence of the other two species. It would appear that there are no 'new' reactions occurring that have an impact on the kinetics of the system. On the other hand, new reactions that occur after the rate-determining step may be happening to give a solution with the absorbance spectrum shown in Figure 3b and the most 'brown' of any of the solutions (Figure 2). In order to explore the new compounds forming – both colourless from the catechin/ascorbic acid solution, and coloured from caffeic acid/ascorbic acid and catechin/caffeic acid/ascorbic acid solutions – LC-DAD was used to monitor reaction mixtures.

Analysis of products at Day 8 by LC-DAD

Colourless compounds. The formation of new compounds was followed by LC-DAD, as colourless species containing the phenolic moiety will give peaks in the 280-nm chromatogram. To be classified as colourless, a species must give rise to a peak in the 280-nm chromatogram but not in the 440-nm chromatogram, where coloured species were monitored (and these are reported later). Table 2 provides a summary of the critical LC-DAD peaks detected. Results for single component samples have been reported elsewhere, but results are given here to aid discussion of binary and ternary mixtures. Colourless products of ascorbic acid breakdown in wine-like medium have been reported on several occasions (Bradshaw et al. 2001, 2003) but so far have eluded identification due to their extreme instability out of the sample matrix. For catechin degradation, colourless precursors to the yellow xanthylium cations have been reported (Fulcrand et al. 1997, Es-Safi et al. 2000a). The breakdown of caffeic acid results in numerous components in very small concentrations. Of these, dihydroxybenzaldehyde (Figure 1) was identified in recent work in model wine (Lutter et al. 2007). Results from the current study are consistent with previous reports.

Kinetic analysis of binary mixtures showed that the presence of ascorbic acid had a pronounced effect on catechin degradation, but little effect on caffeic acid degradation (days 0–8). In the catechin/caffeic acid sample, caffeic acid increased the catechin decay slightly, whereas caffeic acid decay was unaffected. The kinetic data presented so far (Figure 5b) demonstrate that a degradation product of ascorbic acid is involved in the accelerated loss of (+)–catechin. The 280-nm chromatogram of the catechin/ascorbic acid sample showed two new peaks (5 and 6) not present in the single component samples (Figure 6a). The extracted LC-DAD UV-visible spectra corresponding to these peaks showed a maximum at 285 nm, with a shoulder at 300 nm and no absorbance in the visible region of the spectrum. Peaks 5 and 6 (Figure 6a) corresponded to *m/z* values of 401 and 403 in the negative and positive ion modes of LC-MS, respectively. Fragmentation signals were also gained at *m/z* 249 and 251 in the negative and positive ion modes of LC-MS, respectively, which supported a retro-Diels-Alder degradation of the middle ring of (+)–catechin (Lutter et al. 2007) and hence substitution of the (+)–catechin A ring by a 113 *m/z* unit. This was further supported by the detection of the remnant retro-Diels-Alder degradation fragment (151 *m/z*, negative ion mode), and also the (+)–catechin fragment (289 *m/z*, negative ion mode). The presence of the two unknown peaks suggested that the (+)–catechin was substituted at either the position 8-carbon or the position 6-carbon to generate the colourless compound. Previous studies have generally identified that the 8-carbon of (+)–catechin is most reactive to electrophilic reagents. Attempts were made to purify and characterise these compounds; however, they were found to be highly unstable and further work is required to establish a method for their purification.

The concentration of the compounds responsible for peaks 5 and 6 were found to accumulate to a maximum at day 8, followed by a loss on the next measurement day (day 10), and reach depletion by day 12 of the experiment (data not shown). This link with the 90% depletion of ascorbic acid at day 10 suggests that, perhaps, an oxi-

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Table 2. Assignment of the peaks present in the LC-DAD chromatograms.

Peak	Retention time (min)	Assignment	Evidence
1–3	0–17	Colourless ascorbic acid degradation products. *Not yet identified*	Present in all samples containing ascorbic acid
4	19	Catechin	Starting reagent, UV-VIS, retention time (standard)
5, 6	22, 23	Catechin with ascorbic acid-derived unit substituted on the A-ring	UV-VIS spectra, LC-MS
7	18.5	Coloured ascorbic acid degradation product. *Not yet identified*	Only present in samples containing ascorbic acid
8	42–50	Glyoxylic acid-derived xanthylium cations	UV-VIS spectra, retention time (standard), LC-MS (George et al. 2006)
9	57–60	Ethyl ester of the glyoxylic acid-derived xathylium cations	UV-VIS spectra, retention time (standard), LC-MS (George et al. 2006)
10	18.5	Dihydroxybenzaldehyde	UV-VIS, retention time (standard), LC-MS (Lutter et al. 2007)
11	28	Caffeic acid	Starting reagent, UV-VIS, retention time (standard)
12, 13, 14	34 (Broad), 38.5, 46.5	Colourless compounds derived from caffeic acid/ ascorbic acid. *Not yet identified*	Only present in samples containing both caffeic acid and ascorbic acid
15	4 (Broad)	Coloured compound/s derived from binery or ternary mixtures. *Not yet identified*	Not present in single component samples

dation step is involved in the loss of these intermediate (+)–catechin compounds, a loss that is otherwise inhibited by the presence of sufficient amounts of ascorbic acid.

In the caffeic acid/ascorbic acid sample, ascorbic acid does not influence the kinetics of caffeic acid decay between days 0 and 8. The 280-nm chromatogram (peak 10 Figure 7a), however, reveals that dihydroxybenzaldehyde production is enhanced. Unlike the catechin-derived peaks (5 and 6, Figure 6a) above, dihydroxybenzaldehyde (peak 10 Figure 7a) increased in concentration throughout the experiment in a near linear manner ($r^2 = 0.979$, data not shown), and was found 14 times more concentrated than in the equivalent sample without ascorbic acid at day 17 of the experiment (i.e. 34.8 μM vs 2.5 μM). The linear increase in the concentration of dihydroxybenzaldehyde suggests that the presence of ascorbic acid does not inhibit its formation, as the rate of production does not accelerate after day 12 when all the ascorbic acid has decomposed. The concentration of dihydroxybenzaldehyde, generated over the first 8 days of the experiment, would only have accounted to a loss of 1.7% (or 3.4 mg/L) of the total caffeic acid. This small loss of caffeic acid would not be detected as a significant difference between samples with and without ascorbic acid (Figure 5c) over the first 8 days of the experiment.

The binary mixture catechin/caffeic acid has been studied previously (George et al. 2006). In terms of colourless compounds, the degradation product of caffeic acid, dihydroxybenzaldehyde, is known to react with catechin, albeit relatively slowly, to generate colourless dihydroxybenzene methine-linked catechin dimers (and also monomeric and dimeric catechin-derived yellow/red pigments (Lutter et al. 2007)). However, dihydroxybenzene methine-linked catechin dimers were not detected at day 8 in any of the samples, suggesting that its rate of formation was too slow to allow detectable amounts to be formed. Also, the presence of catechin in the binary sample did not lead to any significant difference in the concentration of dihydroxybenzaldehyde.

In the ternary mixture, only one new peak was detected in the 280-nm chromatogram, which is not present in the single-component chromatograms or the binary mixtures. This peak elutes at 38 min (data not shown), and the extracted UV spectrum, with a maximum at 265 nm and shoulder at 310 nm, indicates that it may contain a caffeic acid moiety. The time course of this peak showed that, similar to peaks 5 and 6, it reached a maximum intensity at day 8 and then decayed to be absent by day 12, indicating that it also required the presence of ascorbic acid in solution in order to accumulate. Even on day 8, the intensity of this peak was relatively small, being five times less than the maximum intensity observed for the dihydroxybenzaldehyde peak.

Coloured compounds. As discussed earlier, in samples containing ascorbic acid, the colour was completely due to ascorbic acid breakdown products. This was confirmed by the 440-nm chromatograms, which showed a single sharp peak eluting near the void volume for ascorbic acid alone, and the binary and ternary mixtures (peak 7 Figure 6b). In all samples, this peak reached a maximum at day 8, then decreased in intensity to day 17. The lack of influence of other components on the formation of this

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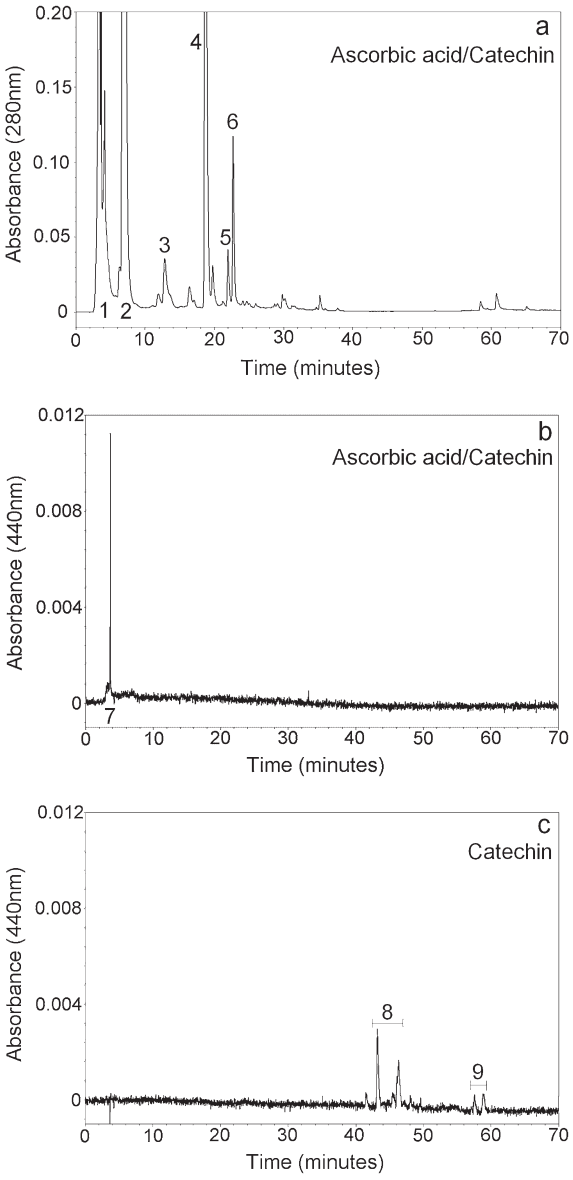


Figure 6. The day 8 chromatograms for catechin/ascorbic acid at (a) 280 nm and (b) 440 nm, and for catechin at (c) 440 nm.

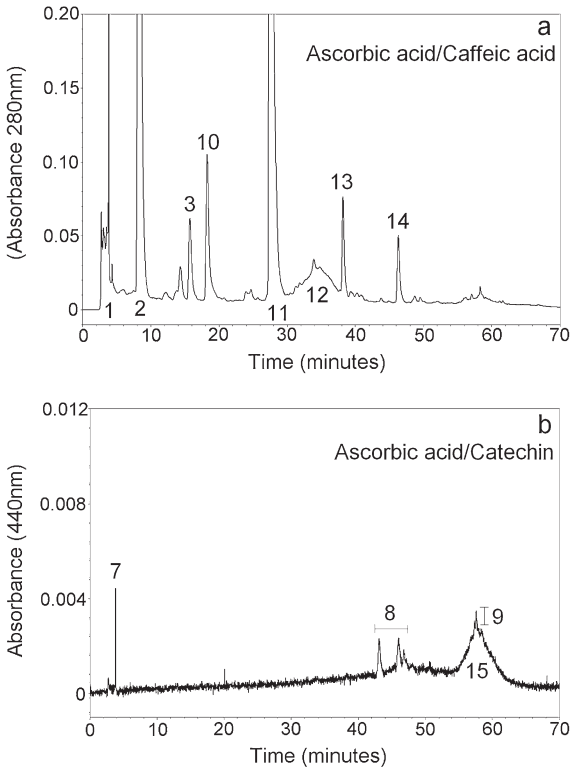


Figure 7. The day 17 chromatograms for (a) caffeic acid/ascorbic acid at 280 nm, and (b) catechin/ascorbic acid at 440 nm.

peak indicates that it was derived directly from ascorbic acid and not through reactions with other components in the mixture.

In the catechin sample (Figure 6c), xanthylium cations (peaks 8 and 9) accounted for the yellow colour as previously reported (Oszmianski et al. 1996, Fulcrand et al. 1997, Es-Safi et al. 1999, George et al. 2006). Caffeic acid was the least yellow of the samples at day 8 (Table 1) and the 440-nm chromatogram showed a single small peak that was due to an impurity in the starting material. This peak did not change intensity during the course of experiments and is not discussed further.

In the catechin/caffeic acid binary sample, the 440-nm chromatogram (data not shown) showed the presence of xanthylium cations, albeit at reduced levels compared with the catechin-only sample (Figure 6c), as previously reported (George et al. 2006). A broad peak, centred at 58 min (~5 min in width), appeared in the chromatogram of the binary mixture at day 8. The compound(s) responsible for this peak may be giving rise to the extra yellow colour, compared with catechin alone, detected in the CIELab results (Table 1). This broad peak continued to grow after day 8 to become the dominant peak in the chromatogram at day 17.

For the ternary mixture, the only coloured compounds detected in the 440-nm chromatogram at day 8 were those attributed to ascorbic acid breakdown (peak 7, Figure 6b). Thus, ascorbic acid has completely inhibited the coloured material formed in the catechin and catechin/caffeic acid mixtures. It was previously shown (Bradshaw et al. 2003) that ascorbic acid could inhibit the production of xanthylium cations from catechin, but now, we have evidence that ascorbic acid can inhibit other pigment formation – i.e. those derived from catechin/caffeic acid – providing there is sufficient unreacted ascorbic acid present in the solution.

Analysis of products at Day 17 by LC-DAD

Colourless compounds. In single component samples, no new peaks appeared in the 280-nm chromatograms that have not been reported elsewhere (see above). In binary

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mixtures, there was a broad increase in the baseline intensity of the 280-nm chromatograms in the catechin/ascorbic acid sample between days 8 and 17, indicating the production of polymeric/isomeric products. The extracted spectra of the broadened baseline had no absorbance in the visible region, indicating that the products were colourless. Therefore, the increased rate of catechin loss in this time is consistent with the production of these colourless compounds. Similarly, a number of new peaks in the caffeic acid/ascorbic acid chromatogram grew during this time. Figure 7a shows a very broad peak, 12, and two well-resolved peaks, 13 and 14. As these form in the samples containing ascorbic acid and caffeic acid, including the ternary mixture, it may be concluded that they are the products of the 'fast' reaction occurring over days 8 to 17, which was mentioned above. The spectra of these peaks were different (Figure 8) but were consistent with the products being derived from caffeic acid, as caffeic acid has similar dual maxima around 280 and 320 nm. It is significant that peaks 12–14 appear only after sufficient depletion of ascorbic acid. A possible explanation for this is that the reaction between ascorbic acid breakdown products and caffeic acid requires an oxidation step, which is inhibited until ascorbic acid falls below a critical level.

As noted above, the other colourless product identified in the caffeic acid/ascorbic acid sample was dihydroxybenzaldehyde (peak 10, Figure 7a). Its rate of production did not change during days 8–17.

In the ternary mixture, no new peaks were detected in the 280-nm chromatogram that have not been discussed. This is consistent with the binary mixture kinetic data which showed that while ascorbic acid accelerated reactions of catechin and caffeic acid over days 8–17, the kinetics of the ternary mixture were unaltered.

Coloured compounds. In single-component samples, no new peaks emerged in the 440-nm chromatograms. In fact, the main peak attributable to colour formation in the ascorbic acid sample (peak 7 Figure 6b) decreased in size, consistent with this sample being less intensely coloured by day 17 (Table 1). As noted previously, ascorbic acid inhibited xanthylium cation production in the catechin/ascorbic acid sample up to day 8. After day 8, colour formation was accelerated, and the 440-nm chromatogram showed the broad peak centred on 58 min (peak 15 Figure 7b), as well as peaks attributable to xanthylium cations (peaks 8 and 9 Figure 7b). Peak 15 grew in intensity from day 8 to day 17, indicating that the breakdown products of ascorbic acid (formed days 0–8) reacted rapidly with catechin to form coloured species, giving rise to peak 15. It is presumed that the species are isomeric and/or polymeric in nature, and thus do not give well-resolved peaks on a reverse-phase column. A similar chromatogram was obtained in the catechin/caffeic acid sample, as previously reported (George et al. 2006). Time course data for the formation of the broad peak 15 as well as the xanthylium cation peaks 8 are shown in Figure 9. Extracted UV-VIS spectra from peak 15 show absorbances tailing further into the visible region than the xanthylium cation spectra. This may account for the distinct brown colour observed in samples that have a significant peak 15 in their chromatograms (catechin/ascorbic acid, catechin/caffeic acid, catechin/caffeic acid/ascorbic acid, see also Figure 2). The data reinforce the critical role that the loss of ascorbic acid plays in colour development – i.e. that no significant colour development due to peak 15 or xanthylium cations (peaks 8) occurred until sufficient depletion of ascorbic acid.

In the caffeic acid/ascorbic acid sample, a broad peak with the same retention time as peak 15 was observed to emerge in the 440-nm chromatogram, concurrently with

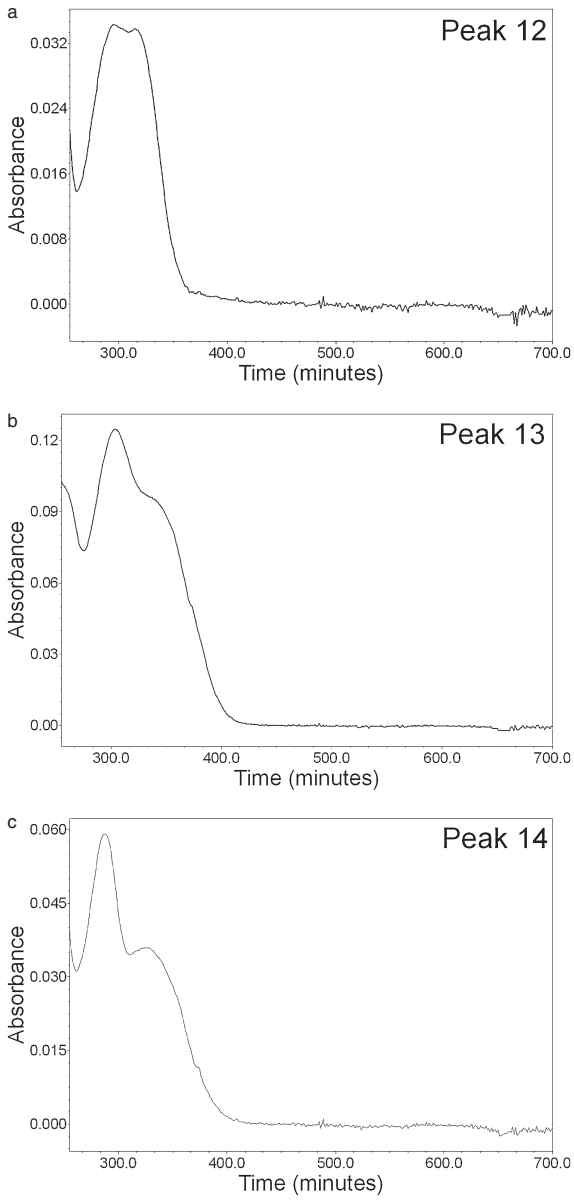


Figure 8. The UV/VIS spectra for (a) peak 12, (b) peak 13 and (c) peak 14 from Figure 7a.

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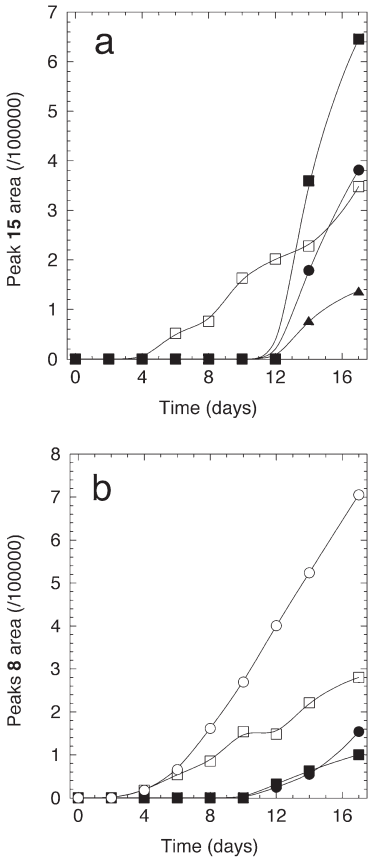


Figure 9. The change in intensity of (a) peak 15, and (b) peaks 8 throughout the experiment. The samples are: (■) caffeic acid/catechin/ascorbic acid, (□) caffeic acid/catechin, (●) catechin/ascorbic acid, (○) catechin and (▲) caffeic acid/ascorbic acid.

the decrease in peak 7. Presumably, this peak is not due to the same compounds as the one above, and this is consistent with the UV spectra derived from this peak. CEILab data (Table 1) indicated that this sample was the least yellow of the binary mixtures, and less yellow than the ternary mixture. This was also evident from the low intensity of the peaks in the 440-nm chromatogram.

The ternary mixture showed no new peaks in the 440-nm chromatogram that have not been discussed for the binary mixtures. Figure 9 shows that peak 15 was of higher intensity than in the chromatograms of the binary samples, consistent with the sample being the most intensely coloured (Figure 2 and Table 1). Understanding the chemical species giving rise to peak 15 will be important for understanding brown colour formation. However, this will be a challenging analytical exercise given that there are likely to be a very large number of isomeric and/or polymeric species contributing to the broadness of this peak.

Conclusion

The use of spectral data and CIELab allowed a more holistic measure of colour than monitoring at a single wavelength, especially when differentiating between yellow and brown colouration. Overall, this study has shown that although ascorbic acid promotes yellow colouration, from its degradation products, it inhibits the brown colouration by suppressing the formation of phenolic-derived yellow and red pigments. However, reactions that occur while ascorbic acid is breaking down 'prime' the system for rapid colour formation once the ascorbic acid concentration falls below a critical level. Catechin is particularly susceptible to these reactions, as demonstrated by its rapid decrease in concentration in the presence of ascorbic acid. Work is in progress to identify the new colourless and coloured species reported above. This study reinforces earlier research that suggested that ascorbic acid is an effective anti-browning agent, provided it persists in the wine after bottling.

Acknowledgements

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ORIGINAL PAPER

The production of yellow pigments from (+)-catechin and dihydroxyfumaric acid in a model wine system

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Abstract The oxidation of tartaric acid by hydroxyl radicals, produced by gamma-irradiation of aqueous solutions of tartaric acid, was shown to generate dihydroxyfumaric acid. To confirm the importance of this oxidative degradation pathway in wine oxidation, dihydroxyfumaric acid was itself reacted with (+)-catechin in a wine-like solution and the yellow pigments formed were identified by liquid chromatography mass spectrometry (LC-MS) studies as xanthylium cations. The concentration of these pigments increased if the wine-like system also contained 0.6 mg/L copper(II). The results clearly demonstrate a link between the production of yellow xanthylium cation pigments from (+)-catechin and the degradation of tartaric acid by hydroxyl radicals.

Keywords Xanthylium cations · Dihydroxyfumaric acid · Glyoxylic acid · Browning · Oxidation · White wine

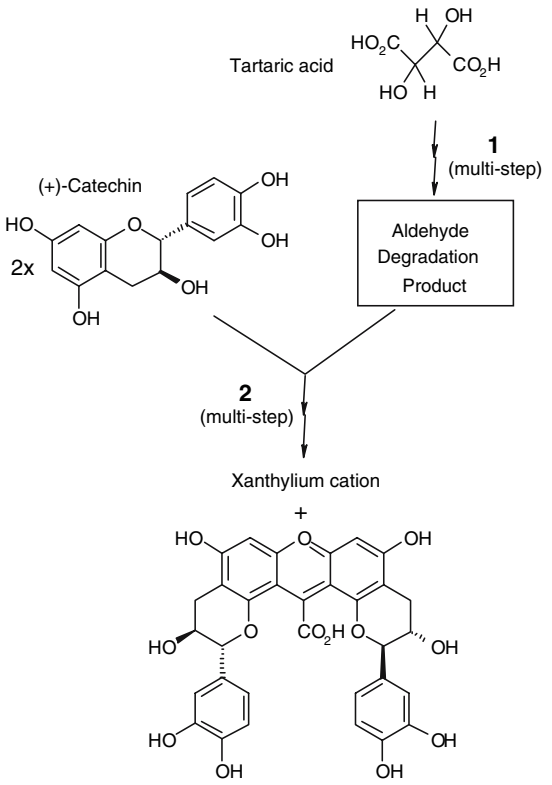
Introduction

During the oxidation of white wine a darkening of the wine colour often occurs that is traditionally termed 'browning' by the wine industry. The detrimental colour development is a consequence of absorbance increases at both yellow (∼420 nm) and red (∼500 nm) regions of the visible spectrum of the wine [1]. Past studies have demonstrated that the phenolic compounds are the main substrates responsible for the uptake of oxygen by wine [2], however the identity of the pigments generated by the oxidation of white wine has not been confirmed.

In wine-like solutions, used to model the oxidation of white wine, the reaction of dissolved molecular oxygen with phenolic compounds is a complex process, which is catalysed by the presence of trace amounts of metal ions [3, 4] and accelerated by higher temperatures or pH values [5]. An initial consequence of the oxidation of the phenolic compounds, and subsequent reduction of molecular oxygen, is the production of reactive oxygen species [6]. One such reactive oxygen species is hydrogen peroxide, and in the presence of trace metal ions, hydrogen peroxide may be transformed into the hydroxyl radical, a far more powerful oxidant. This radical can react in a near diffusion-controlled manner [7, 8]. That is, it can oxidise the component, excluding water, in closest vicinity to the radical. In wine, it is typically ethanol (∼2 M), glycerol (∼0.2 M) and tartaric acid (∼0.02 M) that are the most concentrated components in wine after water, and therefore are the most likely components to be oxidised by hydroxyl radicals.

It has been suggested [7, 8] that this oxidation mechanism is ultimately responsible for the production of yellow pigments from oxygenated wine-like solutions of (+)-catechin, iron and tartaric acid [4]. That is, hydroxyl radicals, generated from hydrogen peroxide and iron(II) as described above, oxidise tartaric acid to form an aldehyde (step 1, Scheme 1) that reacts with (+)-catechin, a wine phenolic compound, which leads subsequently to form yellow xanthylium cation pigments (step 2, Scheme 1). The reactivity of flavanols, such as (+)-catechin, towards aldehydes is well established [9–14]. In fact, both glyoxylic acid (HO_2C–CHO) and glyoxal (OHC–CHO) can react with (+)-catechin to provide the same xanthylium cation pigments as identified in the model wine solutions [13, 15]. These xanthylium cation pigments have been detected in red wine

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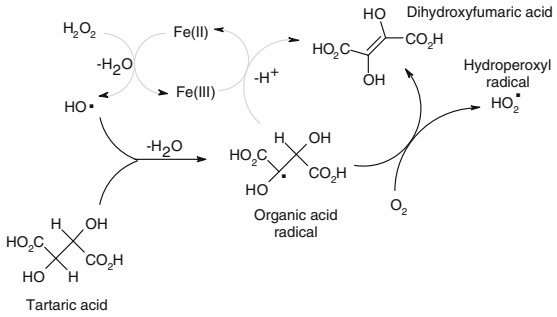


Scheme 1 Generalised scheme for the production of xanthylium cations from (+)-catechin and tartaric acid. Glyoxylic acid and glyoxal are aldehydes that are known precursors to the xanthylium cation shown [13, 15]

[16], and although they have not been reported in white wine, it has been suggested that they may evolve to other pigments yet to be identified [17].

Although presented in a simplified manner in Scheme 1, the mechanisms involved in steps 1 and 2 are complex multi-step reactions. Scheme 2 shows the initial mechanism postulated [7] for step 1 in Scheme 1. This involves the production of the hydroxyl radical from hydrogen peroxide and iron(II), subsequent production of an organic acid radical derived from tartaric acid and then oxidation of this organic acid radical to dihydroxyfumaric acid. The latter oxidation step is suspected to be performed by iron(III) or by molecular oxygen present in the reaction system, as depicted in Scheme 2. The hydroperoxyl radical generated in Scheme 2 may react with easily oxidisable species, such as certain phenolic compounds, or may undergo disproportionation reactions, to generate hydrogen peroxide [7, 8].

Previous studies have confirmed that dihydroxyfumaric acid is the initial product generated on the oxidation of tartaric acid by hydrogen peroxide and iron(II) [18, 19].



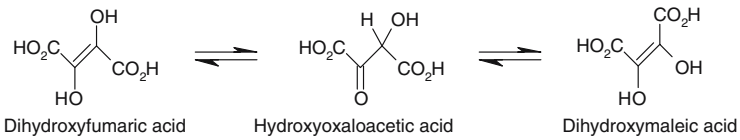
Scheme 2 The proposed production of dihydroxyfumaric acid from tartaric acid by iron(II) and hydrogen peroxide, and/or hydroxyl radicals [7]

However, the ability of hydroxyl radicals to directly generate dihydroxyfumaric acid from tartaric acid (solid arrows, Scheme 2) has not been demonstrated. Although hydroxyl radicals are the commonly proposed product of iron(II) and hydrogen peroxide, the formation of hydroxyl radicals from iron and hydrogen peroxide is suggested to be medium dependent and stem from an initial iron(II)-hydrogen peroxide complex [20, 21]. The composition of the solution, including pH and the presence of complexing agents for iron, can influence the degradation of the iron(II)-hydrogen peroxide complex into either the hydroxyl radical or into another other highly oxidising iron-species. For example, an iron(IV) species, rather than the hydroxyl radical, has been proposed to be the dominant oxidising agent in strongly acidic solutions of iron and hydrogen peroxide [21, 22]. Therefore, confirmation is required that dihydroxyfumaric acid can be generated from the direct reaction of hydroxyl radicals with tartaric acid.

The second step of Scheme 1, involving acid-catalysed condensation reactions, dehydration reactions and oxidation reactions, has been well established for the reaction of (+)-catechin with glyoxylic acid or glyoxal [13, 15, 16]. However, dihydroxyfumaric acid has the potential to generate alternate pigment precursors. Dihydroxyfumaric acid can readily undergo oxidative and/or decarboxylative degradation to generate a number of different three-carbon ketones and aldehydes, and two-carbon aldehydes, including glyoxylic acid and glyoxal [23]. Dihydroxyfumaric acid is also in tautomeric equilibrium with a ketone (Scheme 3) and may be further oxidised to a four-carbon diketone [23], diketosuccinic acid. Although reactions between (+)-catechin and ketones in wine-like media have not been reported, the possibility exists that ketones, of sufficient electrophilicity to react with (+)-catechin may be generated from dihydroxyfumaric acid.

This study was performed to confirm that dihydroxyfumaric acid could be generated from the oxidation of tartaric acid by hydroxyl radicals alone, rather than from the oxidation of

Scheme 3 The keto-enol relationship between dihydroxyfumaric acid, hydroxyoxaloacetic acid and dihydroxymaleic acid



tartaric acid by hydrogen peroxide and iron. Furthermore, the study was conducted to determine if yellow pigments could be generated in a wine-like solution containing both (+)-catechin and dihydroxyfumaric acid.

Materials and methods

Reagents and apparatus

All glassware and plastic-ware were soaked for at least 16 h in 10% (*v/v*) 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. Chemicals were obtained from Aldrich [glyoxylic acid monohydrate (98%)], Sigma [potassium hydrogen tartrate (>99%), L(+)-tartaric acid (>99.5%), and (+)-catechin monohydrate (98%)], Fluka [dihydroxyfumaric acid (95%)] and BDH [copper(II) sulfate pentahydrate (AnalaR)].

Ultraviolet/visible (UV/VIS) spectra of samples were recorded on a μQuant Universal Microplate Spectrophotometer that was run by KC4 software.

Reverse phase liquid chromatography with photodiode array detection (LC-DAD) experiments were performed with instrumentation as described previously [3]. The LC-DAD analyses were carried out with a sample injection of 100 μL and flow rate of 0.15 mL/min with solvent A, 0.5% (*v/v*) acetic acid in water, and solvent B, 0.5% (*v/v*) acetic acid in methanol. Gradient elution was performed with the following proportions of solvent A: 100% at 0 min, 95% at 1 min, 62% at 59 min, 56% at 74 min, 48% at 75 min, 45% at 88 min, 0% at 112 min, and 100% at 120 min. The column was left to equilibrate with solvent A for 10 min prior to the next injection. Liquid chromatography mass spectrometry (LC-MS) experiments were made with equipment and procedures as described in Clark and Scollary [3].

Ion exclusion chromatography with mass spectrometry (IEC-MS) detection was conducted using a Waters 2690 Separation Module and a Micromass ZMD mass spectrometer, both run by Mass Lynx software. The chromatography was performed on two 300 × 7.8 mm Aminex HPX-87H organic acid analysis cation exchange columns (Bio-Rad Laboratories) connected in series, with a guard column of the same stationary phase. The ion exclusion analyses were conducted with a sample injection volume of 10 μL and a flow rate of 0.5 mL/min with an isocratic elution of 0.5% (*v/v*) acetic acid in water. The flow into the mass spectrometer was decreased to around 0.05 mL/min with the use of a 'T' piece and a back flow regulator. The mass spectrometer was set up with a capillary voltage of −3.8 kV, cone voltage of −30 V and desolvation temperature of 140 °C.

Gamma-irradiations were performed at the Physics Division of the Australian Nuclear and Science Technology Organisation located in Menai, NSW. The sample irradiated consisted of 0.011 mol/L potassium hydrogen tartrate and 0.008 mol/L tartaric acid in water (pH 3.2). The sample (20 mL) was placed in a scintillation vial and positioned within the cavity of a cylinder that was then lowered into the source. The cobalt-60 source was enclosed in a cylindrical shield of lead. A gaseous mixture of 50% nitrous oxide and 50% molecular oxygen were bubbled through the sample. The dosage of the gamma irradiator was calibrated using the Fricke Dosimeter technique [24] and found to be 5.9 ± 0.5 Gy/min. Based on this dose rate, the 7 h irradiations that were performed were calculated to produce a molar ratio of hydroxyl radical to tartaric acid of 1:12.5. After irradiation the sample was immediately analysed by IEC-MS.

Reactions

The wine-like solution (2.5 L) was prepared by adding 0.011 mol/L potassium hydrogen tartrate and 0.008 mol/L tartaric acid to aqueous ethanol [12%(*v/v*), 2L] and stirring overnight at room temperature. The pH of the wine-like solution was 3.2 ± 0.1. (+)-Catechin (150 mg/L, 0.52 mmol/L) was added to this solution to form the model white wine. This concentration of (+)-catechin was adopted as it is at the upper levels of catechin-type phenolic compounds found in white wine, and would allow appreciable amount of pigment to be detected and identified. In experiments involving dihydroxyfumaric acid or glyoxylic acid, the concentration of these acids was 0.26 mmol/L and corresponded to a mole ratio of the respective acid to (+)-catechin of 1:2. These ratios were chosen as the xanthylium cation generated from glyoxylic acid and (+)-catechin was known to contain two phenolic units to each aldehyde-derived moiety. Copper(II) (0.6 mg/L) was also added to the glyoxylic acid and (+)-catechin samples as it was known to accelerate their reaction [25]. Each sample was prepared in triplicate with a 150 mL sample volume in a 250 mL Schott bottle with a screw top lid. The samples were held in darkness at 45 °C, and the sample bottles were only opened on measurement days. The quoted and plotted

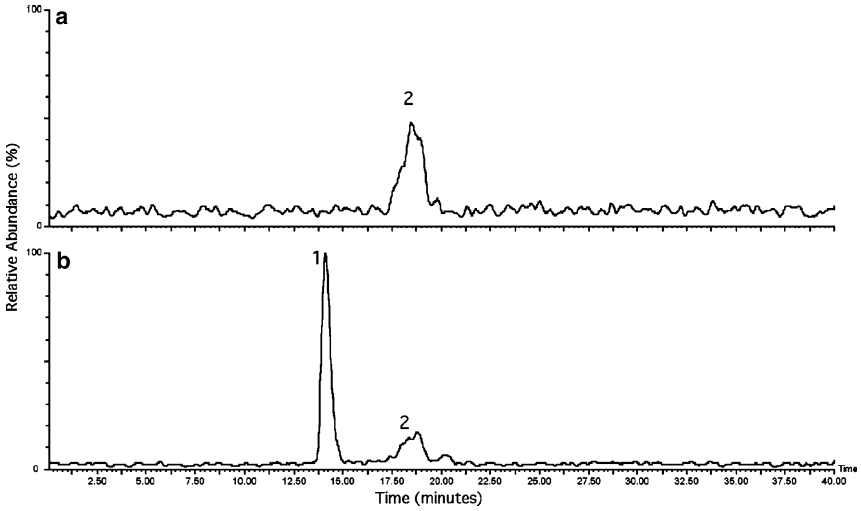


Fig. 1 The 147 m/z mass chromatogram for an aqueous solution of 0.011 M potassium hydrogen tartrate and 0.008 M tartaric acid (**a**) and the same solution after gamma-irradiation for 7 h (**b**). Peak 1 corresponds to dihydroxyfumaric acid while peak 2 is due to fragmentation of tartaric acid by the mass spectrometry detector

data are the mean of the replicates with the error limits (or error bars) representing the 95% confidence limits [26].

Results and discussion

An initial study was conducted to confirm that dihydroxyfumaric acid could be formed from the direct reaction of hydroxyl radicals with tartaric acid. The gamma-irradiation of aqueous solution saturated with nitrous oxide is known to produce a quantitative yield of hydroxyl radicals [24]. Therefore, an aqueous solution of tartaric acid (0.019 mol/L), saturated with 50% nitrous oxide and 50% molecular oxygen, was gamma-irradiated for 7 h to obtain a ratio of generated hydroxyl radicals to tartaric acid of 1:12.5. The molecular oxygen was present to allow the production of the hydroxyl radical in an oxygenated system. The tartaric acid solution was analysed by IEC-MS before and immediately after irradiation, and mass chromatograms at 147 m/z are shown in Fig. 1. Peak 1 was identified as dihydroxyfumaric acid, and/or one of its isomeric forms (Scheme 3), due to identical retention times (14.1 min), mass signals (147 m/z) and mass fragmentation pattern (decarboxylation, 103 m/z) to a standard of dihydroxyfumaric acid. Based on this data, no discrimination could be made between the isomeric forms of dihydroxyfumaric acid (Scheme 3), and it is likely that peak 1 represents a distribution between the different isomeric forms. This same distribution would also occur for the dihydroxyfumaric acid standard. Therefore, future references to dihydroxyfumaric acid will imply some distribution between the isomeric forms shown in Scheme 3.

These data confirmed that dihydroxyfumaric acid could be generated from tartaric acid by the direct attack of the hydroxyl radical. Peak 2 (Fig. 1) was due to the LCMS fragmentation of tartaric acid. When the gamma-irradiated solution, stored at room temperature, was analysed 2 h after being irradiated, the dihydroxyfumaric acid was no longer detected in the sample (data not shown). This was consistent with the reported ability of dihydroxyfumaric acid to readily undergo oxidative decarboxylation degradation [19, 23], a characteristic typical of many *alpha*-keto acids [27].

The influence of dihydroxyfumaric acid on the colouration of a model white wine, containing 150 mg/L of catechin, was investigated. Triplicate samples, with and without dihydroxyfumaric acid, were stored at 45 °C in darkness and throughout the storage period the yellow colour in both samples increased in intensity. The UV/VIS spectra of the samples all showed absorbance maxima around 440 nm in the visible region. The samples containing dihydroxyfumaric acid were observed visually to be significantly more yellow in colour and, as followed by spectroscopic measurements, had an overall 440 nm absorbance increase of 0.0070 absorbance units per day (a.u./day), compared to 0.0016 a.u./day for the control. The loss of (+)-catechin during the experiment was linear in the sample containing dihydroxyfumaric acid and the overall rate of loss was 4 ± 1 μmol/(L.day) [or 1.1 ± 0.3 mg/(L.day)]. This overall rate of (+)-catechin loss was higher than the sample without added dihydroxyfumaric acid [<2 μmol/(L.day) or <0.4 mg/(L.day)]. The results suggested that the presence of dihydroxyfumaric acid in the reaction system was enhancing the production of yellow pigments from (+)-catechin.

Analysis of the samples by LC-DAD, with detection at 440 nm, showed that the yellow coloured compounds in the sample containing dihydroxyfumaric acid had identical retention times (peaks 3–6, Fig. 2a) to the xanthylium cat-

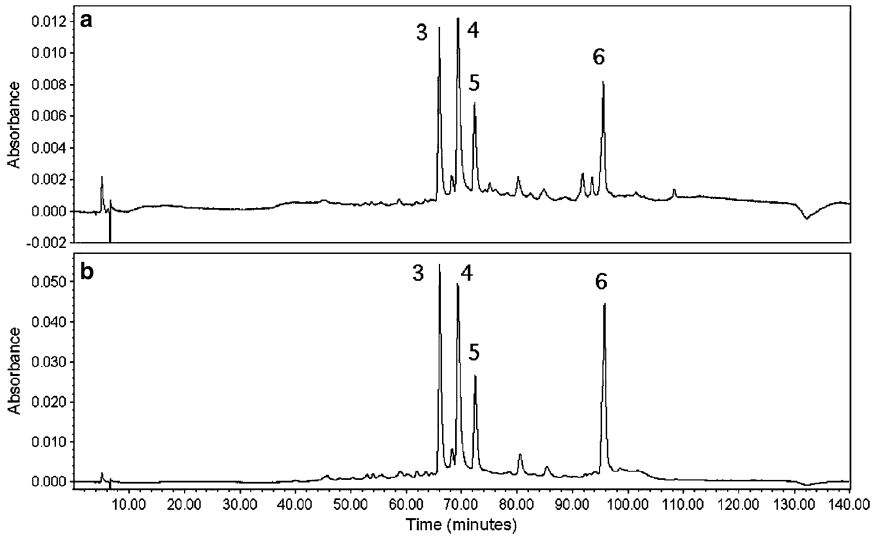


Fig. 2 LC-DAD chromatograms at 440 nm after the 10-day induced oxidation process of the wine-like solution with 0.26 mM dihydroxyfumaric acid and 0.50 mM (+)-catechin (**a**), and the wine-like solution with 0.26 mM glyoxylic acid, 0.50 mM (+)-catechin and 0.6 mg/L copper(II) (**b**). Different absorbance scales are used in order to aid comparison

ions generated from the reaction of glyoxylic acid with (+)-catechin (peaks 3–6, Fig. 2b) in the wine-like solution. Furthermore, all peaks 3–5 had extracted UV/VIS spectra with shoulders at 310 nm and maxima at 280 and 440 nm, and corresponded to 617 m/z by LC-MS (positive ion mode). Peak 6 had UV/VIS spectra with a shoulder at 310 nm and maxima at 280 and 460 nm, and corresponded to 645 m/z by LC-MS (positive ion mode). Peaks 3–5 corresponded to various isomers of the xanthylium cation shown in Scheme 1, as described by Es-Safi et al. [16], while peak 6 corresponded to the ethyl ester of these xanthylium cations [16]. These results clearly demonstrate that the major pigments generated from a solution containing both dihydroxyfumaric acid and (+)-catechin were the same xanthylium cation pigments generated from the reaction of glyoxylic acid or glyoxal with (+)-catechin. Importantly, the concentration of these pigments was enhanced by the presence of dihydroxyfumaric acid (Fig. 3).

The experiment was repeated with 0.6 mg/L copper(II) added to the samples. This was performed as it was known that metal ions can influence the oxidation and/or decarboxylation of keto acids [27, 28], such as the tautomeric form of dihydroxyfumaric acid (Scheme 2), and also that copper(II) can accelerate the reaction between glyoxylic acid and (+)-catechin [25]. The loss of (+)-catechin was again found to be linear and the overall rate of the loss at 4 ± 1 µmol/(L.day) [or 1.1 ± 0.2 mg/(L.day)], not significantly different ($P = 0.001$) to the same sample without copper(II). The identical xanthylium cations were identified as in the sample without copper(II) (data not shown), however the production of the xanthylium cations (peaks 3–6, Fig. 2) was found to increase substantially in the sample containing dihydroxyfumaric acid and copper(II) compared to the sample without copper(II) (Fig. 3).

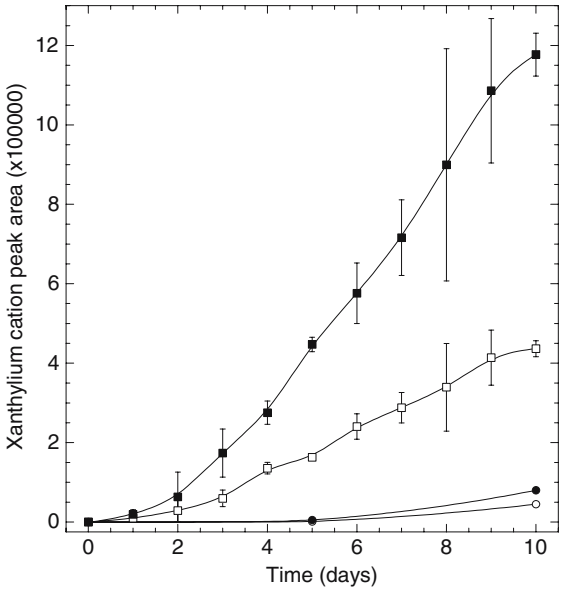


Fig. 3 The increase in xanthylium cation peak area during the induced oxidation of the wine-like solution with 0.50 mM (+)-catechin (*open circle*), and the same solution but with 0.6 mg/L copper(II) (*filled circle*), 0.25 mM dihydroxyfumaric acid (*open square*), or both 0.6 mg/L copper(II) and 0.25 mM dihydroxyfumaric acid (*filled square*)

These results suggest that the presence of copper favours the production of the xanthylium cation pigments over some other competing mechanism in which (+)-catechin is involved. This is confirmed by the presence of an extra peak, labelled peak 7 in Fig. 4, in the 280 nm chromatogram of the dihydroxyfumaric acid/(+)-catechin sample compared to the equivalent sample with added copper(II).

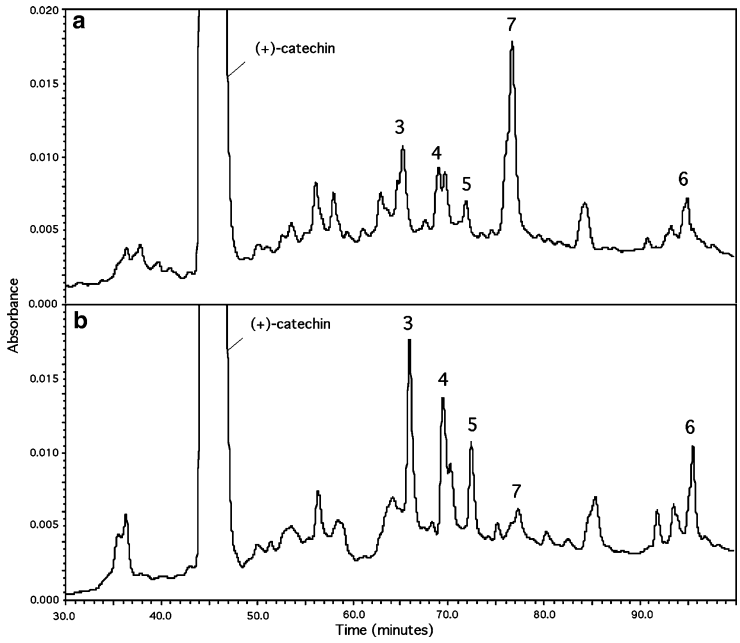


Fig. 4 LC-DAD chromatograms at 280 nm after the 10-day induced oxidation process of the wine-like solution with 0.26 mM dihydroxyfumaric acid and 0.50 mM (+)-catechin (**a**), and the same solution but with 0.6 mg/L copper(II) (**b**). Peaks 3–6 are due to the xanthylium cations that absorb at both 280 and 440 nm (Fig. 2) and peak 7 was unidentified

Other than the peak due to (+)-catechin, the main peaks in the 280 nm chromatograms from the sample containing copper(II) (peaks 3–6, Fig. 4b) are due to xanthylium cations, as these pigments have absorbance maxima at 440–460 and 280 nm.

Peak 7 increased in a linear manner throughout the experiment for the dihydroxyfumaric acid/(+)-catechin sample. The extracted spectrum of this peak had a maximum at 295 nm and a shoulder at 340 nm, indicating that the compound was colourless, while its elution after (+)-catechin (Fig. 4a) demonstrated that is was less polar than (+)-catechin. The LC-MS did not provide any clear mass signal corresponding to peak 7 which suggested that the species corresponding to this peak was not sufficiently ionisable to be detected under the conditions adopted. These results demonstrate that the presence of copper(II) in the dihydroxyfumaric acid/(+)-catechin reaction system was able to limit the production at least one colourless compound, responsible for peak 7 (Fig. 4a), and enhance the formation of xanthylium cations.

A link between hydroxyl radical attack on tartaric acid and the production of yellow pigments from (+)-catechin has been clearly demonstrated by this study. The results show that the reaction of the hydroxyl radical with tartaric acid (solid arrows, Scheme 2) can generate dihydroxyfumaric acid, whose subsequent incubation with (+)-catechin in wine-like conditions can generate the same xanthylium cations as those generated from the either glyoxylic acid or glyoxal and (+)-catechin. Copper was able to enhance the production of the pigments without accelerating the loss of (+)-catechin. The mechanism by which copper achieves this increase in pigment production will be the subject of future work.

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PUBLICATION 4

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Formation of Pigment Precursor (+)-1″-Methylene-6″-hydroxy-2*H*-furan-5″-one-catechin Isomers from (+)-Catechin and a Degradation Product of Ascorbic Acid in a Model Wine System

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The present study investigates the contribution of ascorbic acid to the formation of pigment precursors in model white wine systems containing (+)-catechin as the oxidizable phenolic substrate. The two main colorless products in these systems were structurally characterized as isomers of (+)-catechin substituted at either C6 or C8 on the A ring with a furan-type unit, namely, (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-6-catechin and (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-8-catechin. A known degradation product of ascorbic acid, L-xylosone, was separately prepared and, when reacted with (+)-catechin, generated the same (+)-furanone-catechin isomers as in model white wine systems. Incubation of these isomers in wine-like conditions yielded yellow xanthylium cation pigments. This study has shown that undesirable spoilage reactions (yellow coloration) can occur from a breakdown product of ascorbic acid−L-xylosone.

KEYWORDS: Ascorbic acid; (+)-catechin; L-xylosone; model white wine; oxidation; xanthylium cation; biomimetic synthesis

INTRODUCTION

Flavanols in wine, such as (+)-catechin (**Figure 1**), are well-known to react with aldehydes (*1*) formed or extracted during the winemaking process and aging. The most commonly occurring aldehyde in wine production is acetaldehyde, formed mainly by yeast during fermentation or by the oxidation of ethanol (*2*). Other compounds formed in wine that also contain an aldehyde group include glyceraldehyde, glyoxylic acid, and furfural. The first two may be generated under oxidative storage conditions of wine (*3*−*5*), whereas furfural can be extracted during the aging of wine in oak (*6*). Of these aldehydes, only glyoxylic acid (*7*) and furfural (*6*) have been shown to participate in xanthylium cation pigment formation after their reaction with (+)-catechin in model systems. However, pigments containing glyoxylic acid have been reported for red wine (*8*) but have not yet been observed in white wine.

Another wine constituent capable of producing electrophilic carbonyl compounds, despite its primarily antioxidant properties, is ascorbic acid (**Figure 1**). Ascorbic acid is naturally present in grapes but is usually rapidly consumed after crushing, typically due to ascorbic acid either scavenging oxygen or reducing *o*-quinone compounds formed from the enzymatic oxidation of phenolic compounds (*9*, *10*). The ascorbic acid present in white wine is mostly due to exogenous additions, often just before the bottling of white wine, although it may be used at various stages in the wine production process. The levels of ascorbic acid added may vary considerably, but it is typically added at rates ranging from 50 to 150 mg/L (*9*−*12*).

Ascorbic acid is added to white wine due to its ability to scavenge molecular oxygen efficiently, but in the process it is initially converted to dehydroascorbic acid and hydrogen peroxide. The dehydroascorbic acid then undergoes rapid degradation into a variety of species including numerous carboxylic acid, ketone, and aldehyde compounds (*13*−*16*) (**Figure 1**). Recent studies have shown the increased production of phenolic pigments from model wine systems of ascorbic acid and (+)-catechin and demonstrated that a degradation product emanating from ascorbic acid was able to react with (+)-catechin and form colored xanthylium cations (*17*, *18*). In general, phenolic pigments formed during the storage of white wine are undesirable as they are perceived as indicative of wine spoilage.

The behavior of (+)-catechin solutions in the presence of relatively high concentrations of ascorbic acid has been the focus of a recent study by Clark et al. (*19*). In this study, two colorless species were observed using HPLC-PDA in model wine samples containing iron(II), ascorbic acid, and (+)-catechin. The compounds accumulated while ascorbic acid was still present in solution and then underwent rapid degradation once the ascorbic acid was near depletion. Their degradation also coincided with the production of pigmented compounds in the model wine system. These colorless compounds thus appear to be key intermediates in the formation of pigments in model white wine systems. Despite intense effort, these compounds have until

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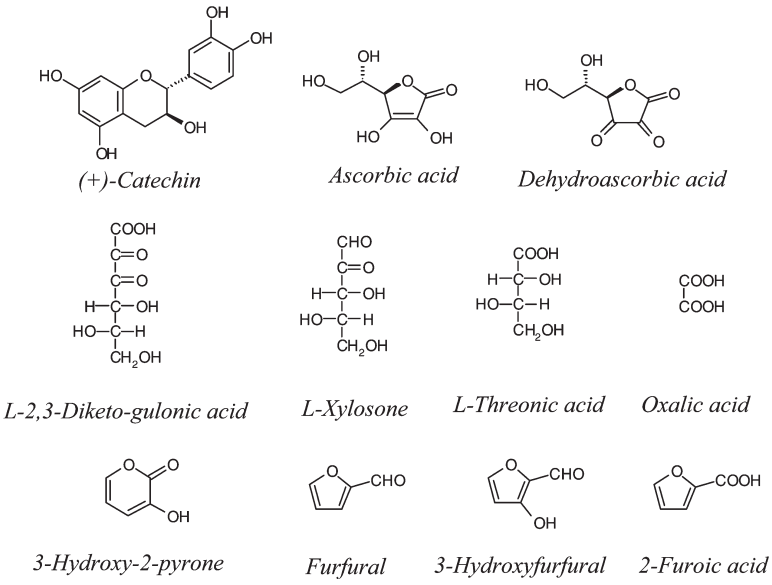


Figure 1. Molecular structures of (+)-catechin, ascorbic acid, and some reported degradation products of ascorbic acid.

now eluded identification. Furthermore, direct evidence that these compounds are indeed intermediates in pigment formation has not been available. This study addresses these two issues by identifying the colorless compounds as (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-6-catechin and (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-8-catechin and showing that these compounds react to give pigments. The study goes further to directly implicate a breakdown product of ascorbic acid, L-xylosone, as capable of reacting with (+)-catechin to generate the two (+)-furan-catechin isomers observed in model wine systems. Finally, the pigments formed by reaction of the (+)-furanone-catechin isomers were shown to be xanthylium cations, which have been reported in other model wine systems.

MATERIALS AND METHODS

General. Water purified through a Milli-Q (Millipore) water system (ISO 9001) was used for all solution preparations and dilutions. All glassware was soaked overnight in a 10% nitric acid (AnalR, BDH, Poole, U.K.) and then rinsed with copious amounts of water. L-Ascorbic acid (99%), L-(+)-tartaric acid (>99.5%), (+)-catechin hydrate (98%), potassium bitartrate (99%), d_6-DMSO (99.98%), L-(−)-xylose (99%), glyoxylic acid monohydrate (98%), and copper(II) acetate (98%) were purchased from Sigma-Aldrich (USA). Potassium hydroxide (AR grade, >85%) was obtained from BDH/Merck (Australia), and sulfuric acid (AR grade, >95%) and iron(II) sulfate heptahydrate (>98%) were purchased from Ajax Fine Chemicals (Australia). Ethanol (AR grade, >99.5%, Ajax Fine Chemicals), methanol (AR grade, >99.9%, Mallinckrodt Chemicals, USA), glacial acetic acid (AR grade, >99.7%, APS Ajax Fine Chemicals), hydrochloric acid (31.5%, AnalR, BDH, Australia), formic acid (98%, Fluka, Germany), and acetonitrile (HPLC grade, >99.9%, Ajax Fine Chemicals) were used without further purification.

Analytical LC analyses were conducted on an Ultra Performance Liquid Chromatography (UPLC) system consisting of a Waters Acquity binary solvent manager connected to a sample manager and a PDA detector all run by Empower^2 chromatography manager software. The column was a Waters Acquity BEH C18 (2.1 × 50 mm) with 1.7 μm particle diameter. Injection volume was 7.5 μL, column temperature 35 °C, and flow rate 0.45 mL/min; the elution gradient consisted of solvent A, 0.5% acetic acid in water, and solvent B, 0.5% acetic acid in methanol, as follows (expressed in solvent A followed by cumulative time): 100%, 0 min; 100%, 1 min; 95%, 1.31 min; 62%, 5.25 min; 56%, 6.27 min; 48%, 6.34 min; 45%, 7.22 min; 0%, 8.85 min; 0%, 9.40 min; 100%, 8.74 min; and 100%, 9.76 min.

LC-MS studies on the colorless compounds were conducted as described by Lutter et al. (*20*). LC-MS studies conducted on the xanthylium cations were conducted on an Agilent 1200 series triple-quadrapole (6410) HPLC-MS. The column and LC conditions were as described for the UPLC (above), except for an injection volume of 20 μL. The MS was operated with drying gas temperature at 350 °C, gas flow of 9 L/min, nebulizer pressure at 40 psi, and capillary voltage at 4 kV. MS analyses for the xanthylium cations were carried out in the positive ion mode with the fragmentor at both 50 and 150 V, the former providing parent ion signals and the latter inducing fragmentation.

Preparative HPLC separations were conducted on a Perkin-Elmer 250 binary LC pump connected to a Varian 320 Pro Star UV−visible detector (280 nm) controlled by a Varian Star (v 6.41) chromatography workstation. The column was a semipreparative 4 μm Phenomenex Synergy Hydro-RP C18 column (250 × 10 mm) coupled to a Phenomenex ODS Octadecyl C18 (10 × 10 mm) security guard column. Injection volume was 2 mL and flow rate 2 mL/min; the elution gradient consisted of solvent A, 2% formic acid in water, and solvent B, 2% formic acid in 80% acetonitrile, as follows (expressed in solvent A followed by cumulative time): 100%, 0 min; 85%, 5 min; 71%, 35 min; 0%, 40 min; 0%, 50 min; 100%, 55 min; 100%, 60 min. Lyophilization was performed on a Christ-Alpha 2-4D freeze-dryer (Biotech International, Germany).

High-resolution MS (HRMS) data were obtained on a 4.7 T FT-ICR Bruker Apex 3 mass spectrometer, running Bruker X-Mass software (v 7.0.2). The instrument was calibrated using sodium iodide in methanol. The samples were dissolved in methanol and infused at a flow rate of 150 μL/min in negative ionization mode (ESI^-). The mass range scanned was 45−1000 amu. Capillary exit voltage was −50 V, and drying gas temperature was 120 °C. Elemental analysis was run on a VG Opus 3.6 data station running VG elemental program.

NMR data were acquired on a Bruker DRX600 NMR spectrometer operating at a frequency of 600.18 MHz at 25 °C using TopSpin (version 1.3, Bruker GMBH). Samples were prepared in d_6-DMSO in Shigemi tubes (Sigma-Aldrich) and degassed (argon) before sealing. Spectral widths were set to allow at least 0.5 ppm either side of observed resonances. 1D NMR spectra were recorded (6009.62 Hz) with 32K data points zero filled to 64K and resolution enhanced using a Gaussian multiplication of the raw FID (Gaussian broadening = 0.2, line broadening = −2 Hz) before Fourier transformation. All 2D NMR experiments were run with quadrature

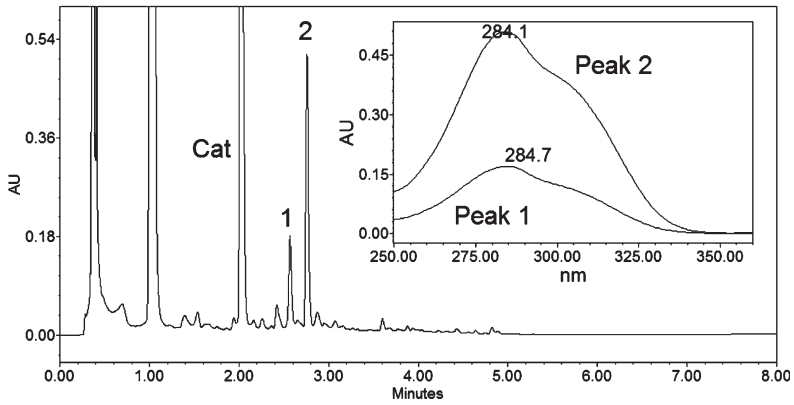


Figure 2. Liquid chromatogram at 280 nm of an ascorbic acid−(+)-catechin solution after 4 days at 45 °C with spectra of compounds **1** and **2** (inset). The model wine system consisted of a tartaric acid-buffered 12% ethanol solution (pH 3.2) with 1000 mg/L ascorbic acid, 500 mg/L catechin, and 1.5 mg/L iron(II). Peaks eluting before (+)-catechin are ascorbic acid and its degradation products.

detection with a ^{1}H spectral width of 6009 Hz and a recycle delay of 2 s. Chemical shifts were referenced to the residual d_5-DMSO (δ_H 2.49 ppm; δ_C 39.8 ppm). High-power ^{1}H $\pi/2$ pulses were determined to be 11.2 μs and low power (for MLEV spin lock), 30 ms. The ^{13}C high-power $\pi/2$ pulse was 11.0 μs, and a low-power pulse of 65 μs was used for GARP4 decoupling. Gradient pulses were delivered along the z-axis using a 100 step sine program.

Homonuclear proton−proton correlation was achieved with the eCOSY sequence (*21*) using gradient pulses for selection. The data were recorded in 4K data points in t_1 and in 512K data points in t_2. Points were predicted out to 8K data points in t_2 and 1K data points in t_1 (maximum entropy) and zero filled to 8K and 4K data points, respectively. The data were processed using a $\pi/2$ sine-bell shifted apodization in both dimensions and carefully phase corrected. TOCSY experiments were recorded over 2K data points in t_2 and 512 in t_1 using the X_M16 sequence (*22*) for the mixing time (30 ms; P29 = 50 μs, SP0 = 8.86 dB). The data were processed using a $\pi/2$ sine-bell shifted apodization in both dimensions. HSQC spectra were recorded over 2K data points in t_2 and 512 data points in t_1 (10−170 ppm) using the sensitivity-enhanced double INEPT transfer with no trim pulses and adiabatic pulses for ^{13}C decoupling in $f2$ (*23*). The spectra were made phase sensitive using echo−antiecho gradient selection (80:20:11:−5). Long-range ^{1}H−^{13}C correlation (HMBC) spectra were recorded with 2K data points in t_1 (10−210 ppm) and t_2 and processed with magnitude calculation in $f1$ to destroy all phase information. Spectra were obtained with a low-pass J-filter (145 Hz) to suppress one-bond correlations. No decoupling was used during the acquisition, and gradient pulses (50:30:40:1) were used for selection. Two experiments were run, optimized for a 20 and 2 Hz long-range coupling, respectively. Through-space connectivities were obtained using 2D homonuclear correlation via dipolar coupling (ROESY) using phase sensitive echo−antiecho gradient selection with a 250 ms mixing delay (2K × 512 data points).

Reactions under Various Model Wine Conditions. The composition of the model wine system was varied to establish the components essential for the formation of the colorless products. Ascorbic acid (1000 mg/L, 5.6 mM), (+)-catechin (500 mg/L, 1.6 mM), and iron(II) (1.5 mg/L, 0.027 mM) solutions were prepared in a tartaric acid-buffered model wine system (pH 3.2), with and without ethanol (12% v/v) present, as per Clark et al. (*19*). Tartaric acid- and formic acid-buffered model white wine (pH 3.2), ascorbic acid (500 mg/L, 2.8 mM), and (+)-catechin (250 mg/L, 0.8 mM) solutions, with and without ethanol (12% v/v) present, were prepared as per Barril et al. (*17*). The concentrations of the reactants are higher than those generally found in white wine but were required to generate sufficient amounts of oxidation products. It has been shown that the high concentration did not affect the species formed but only their rate of formation (*18*). All samples were prepared in triplicate, stored at 45 °C in a covered water bath (i.e., darkness), aerated daily (with rapid stirring for 2 min), and sampled every 2 or 4 days for chromatographic analysis. The uncertainties quoted are the 95% confidence limits (n = 3, Student's t test).

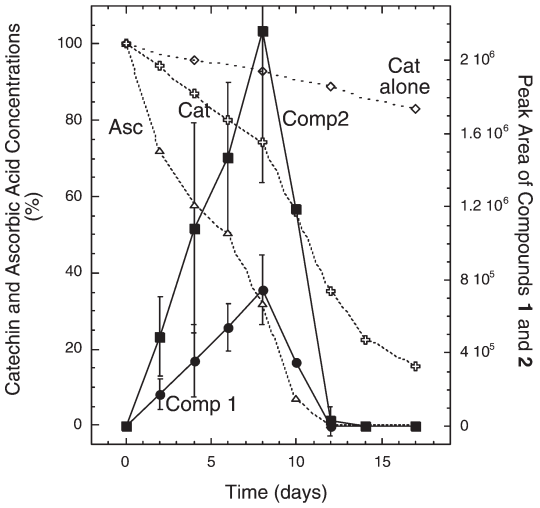


Figure 3. Formation of compounds **1** and **2** in a tartaric acid-buffered 12% ethanol solution with (+)-catechin (250 mg/L), ascorbic acid (500 mg/L), and 1.5 mg/L iron. Error bars indicate the 95% confidence limits (n = 3, Student's t test) and were omitted on day 10 for clarity. The peak areas and 95% confidence limits (× 10^6) for compounds **1** and **2** at day 10 were 0.3 ± 0.9 and 1 ± 9, respectively. The large uncertainty at day 10 was due to the rapid decrease in concentration of the compounds at this time.

Isolation of Reaction Products. A pH 3.2 solution was prepared by dissolving potassium bitartrate (2.4 g) and tartaric acid (1.2 g) in water (1 L). Ascorbic acid (1.0 g) and (+)-catechin (500 mg) were added to the solution, followed by 1.5 mg/L iron(II) (in the form of iron(II) sulfate heptahydrate), which was maintained in darkness at 45 °C, with daily aeration, until the colorless products formed reached a maximum concentration (8 days as determined by UPLC-PDA). At this time ascorbic acid (1.0 g) was added, to slow the otherwise rapid degradation of the accumulated colorless products, and the 1 L sample was stored at 4 °C.

The 1 L solution was then passed through a solid-phase extraction cartridge (Strata C18-E, 70 g/150 mL, Phenomenex, USA) to concentrate and provide a crude purification of the isomers. The cartridge was first conditioned with 80% aqueous methanol containing 2% formic acid (200 mL), followed by 2% formic acid (300 mL). The sample was absorbed and washed with 2% formic acid (300 mL) and eluted with 80% aqueous methanol with 2% formic acid (200 mL). The eluent was concentrated

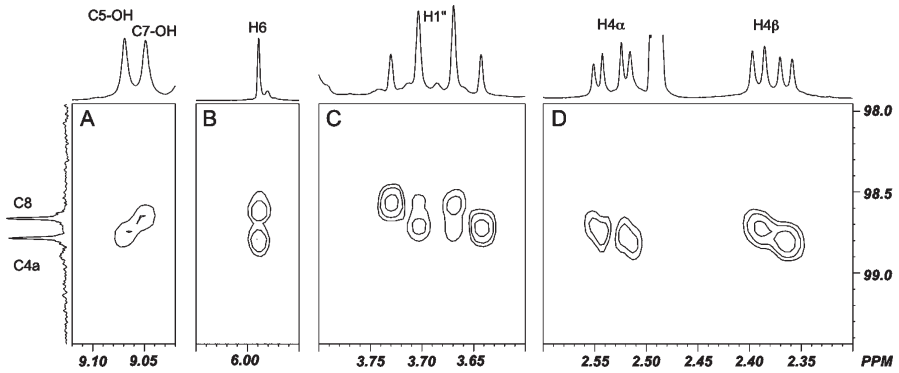


Figure 4. Heteronuclear multibond correlation spectrum of compound **2** showing correlations between C5OH and C4a and between C7OH and C8 (**A**); of H6 with both C8 and C4a (**B**); of $(H1'')_2$ and C8 (**C**); and $(H4)_2$ and C4a (**D**).

3-fold under a stream of nitrogen and injected directly onto preparative scale HPLC. Similar fractions were combined and blown down with a stream of nitrogen, and the aqueous residue was freeze-dried to yield the first eluting isomer as a pale yellow powder (15.0 mg; 2%): HRMS found, $[M - H]^-$, 401.088482, $C_{20}H_{17}O_9$ requires 401.087257; λ_{max} (0.5/24.8/74.7% (v/v) of acetic acid/methanol/water) 284 (300) nm; ^{1}H NMR (600 MHz, d_6-DMSO) δ 6.71 (d, J = 2.1 Hz, H2′), 6.68 (d, J = 8.1 Hz, H5′), 6.58 (dd, J = 8.2, 2.1 Hz, H6′), 5.87 (s, H8), 4.50 (d, J = 7.5 Hz, H2), 4.41 (s, $(H4'')_2$), 3.83 (m, H3), 3.78 (s, $(H1'')_2$), 2.64 (dd, J = 16.0, 5.5 Hz, H4α), 2.40 (dd, J = 16.0, 7.5 Hz, H4β); ^{13}C NMR (150.9 MHz, d_6-DMSO) δ 195.2 (C5″), 176.1 (C6″), 154.8 (C7), 154.2 (C5), 153.5 (C8a), 144.9 (C4′), 144.9 (C3′), 133.6 (C2″), 130.6 (C1′), 118.3 (C6′), 115.1 (C5′), 114.5 (C2′), 101.5 (C6), 99.9 (C4a), 94.5 (C8), 80.7 (C2), 72.3 (C4″), 66.3 (C3), 28.3 (C4), 21.6 (C1″).

The second (major) isomer to elute was similarly isolated as a pale yellow powder (25.0 mg; 4%): HRMS found, $[M - H]^-$, 401.086017, $C_{20}H_{17}O_9$ requires 401.087257; λ_{max} (0.5/24.8/74.7% (v/v) of acetic acid/methanol/water) 284 (300) nm; ^{1}H NMR (600 MHz, d_6-DMSO) δ 9.07 (bs, C5−OH), 9.05 (bs, C7−OH), 8.80 (bs, C4′−OH), 8.74 (bs, C3′−OH), 7.98 (bs, C6″−OH), 6.64 (d, J = 8.1 Hz, H5′), 6.64 (d, J = 2.1 Hz, H2′), 6.53 (dd, J = 8.2, 2.1 Hz, H6′), 5.99 (s, H6), 4.88 (bd, J = 5 Hz, C3−OH), 4.60 (d, J = 6.56 Hz, H2), 4.37 (AB, $W_{h/2}$ = 18.4 Hz, $(H4'')_2$), 3.82 (m, H3), 3.68 (AB, J = 16.05 Hz, $(H1'')_2$), 2.54 (dd, J = 16.05, 5.14 Hz, H4α), 2.38 (dd, J = 16.05, 7.10 Hz, H4β); ^{13}C NMR (150.9 MHz, d_6-DMSO) δ 195.1 (C5″), 176.0 (C6″), 154.5 (C5), 154.4 (C7), 153.3 (C8a), 144.7 (C4′), 144.6 (C3′), 133.7 (C2″), 130.9 (C1′), 117.6 (C6′), 115.1 (C5′), 114.1 (C2′), 98.8 (C4a), 98.7 (C8), 94.7 (C6), 80.7 (C2), 72.2 (C4″), 66.3 (C3), 27.0 (C4), 21.3 (C1″).

Synthesis of L-Xylosone and Subsequent Reaction with (+)-Catechin. The preparation of L-xylosone was conducted as described by Salomon et al. (*24*), following a method originating from Weidenhagen in 1937 using copper(II) acetate (*25*). Briefly, L-xylose (1 g, 6.7 mmol) was dissolved in aqueous methanol (95%; 60 mL), and the solution was boiled under reflux until the xylose was fully dissolved. Copper(II) acetate (4 g, 21.6 mmol) was then added in one portion, and the solution was boiled under reflux for a further 20 min. After cooling, the copper(I) oxide was removed by filtration, and the concentrated filtrate was percolated through a Chelex 100 ion-exchange resin (2.5 × 30 cm, Na^+ form; BioRad, USA) previously converted to the H^+ form. L-Xylosone was eluted with methanol to yield a pale yellow syrup (0.4 g, 40%).

L-Xylosone (1 g) was dissolved in a tartaric acid-buffered model wine system containing ethanol (50 mL) as prepared per Barril et al. (*17*). (+)-Catechin (500 mg) was added, and the solution was left to react in darkness at 45 °C. The formation of compounds **1** and **2** was monitored by UPLC-PDA every 12 h.

RESULTS AND DISCUSSION

Formation of Colorless Products in Various Model White Wine Systems. In model wine solutions containing ascorbic acid and (+)-catechin, colorless compounds giving rise to peaks 1 and 2 (**Figure 2**) were detected as previously reported (*19*). Initial studies were conducted to ensure that the colorless compounds emanated specifically from ascorbic acid and (+)-catechin and were not dependent on other components of the model wine system. For instance, an oxidative degradation product of tartaric acid, used to buffer wine-like media, is known to contribute to the formation of (+)-catechin oligomers through glyoxylic acid production (*5*, *7*).

The model wine systems were prepared at several concentrations of ascorbic acid, (+)-catechin, and iron(II), with the presence or absence of ethanol and with different supporting buffer systems of either tartaric acid or formic acid. **Figure 3** provides an example of the production and decay of compounds **1** and **2** in a model wine system containing (+)-catechin and ascorbic acid. In this case, ascorbic acid was almost depleted by day 10. As reported previously (*19*), compounds **1** and **2** in the model wine samples increased to a maximum concentration and then began to decrease in concentration once ascorbic acid was near depletion. The formation of compounds **1** and **2** is consistent with the initial degradation of (+)-catechin, as the polyphenol decay was immediate and faster than in the absence of ascorbic acid ((+)-catechin alone in **Figure 3**).

In all combinations of the model wine system, the formation of compounds **1** and **2** was reliant upon only the presence of both (+)-catechin and ascorbic acid. Other components of the model wine system, particularly iron (0−1.5 mg/L) and/or ethanol (0−12% (v/v)), had some impact on the rate of formation of compounds **1** and **2** but were not essential for the formation of these colorless compounds.

Identification of Compounds 1 and 2. Compounds **1** and **2** yielded identical ESI-MS spectra with peaks at m/z 401 and 403 in negative and positive ionization mode, respectively. High-resolution MS data established a molecular formula of $C_{20}H_{18}O_9$ (±3 ppm) for both compounds, indicating 12 degrees of unsaturation. These facts coupled with identical UV−visible spectra (**Figure 2**, inset) suggested that the compounds were isomers and contained the (+)-catechin chromophore (λ_{max} = 278 nm), albeit slightly bathochromically shifted to 284 nm. Loss of 113 amu from the compounds gave ions with the same mass as catechin-H^+ (m/z 289 (negative ionization mode); $C_{15}H_{13}O_6$), suggesting that the two compounds were both substituted with a $C_5H_5O_3$ fragment, composed, as determined by NMR, of a ketone (δ_C 195.1) and an enol (δ_C 176.0, 133.7), a methylene next to oxygen (δ_C 72.2), and an aliphatic methylene (δ_C 21.3). With 3 degrees of unsaturation for the substitution unit, this suggested a 5-methylene-4-hydroxy-2*H*-furan-3-one structure. Similar structures have been reported as products of acid treatment of xylitol (*26*). Two-dimensional

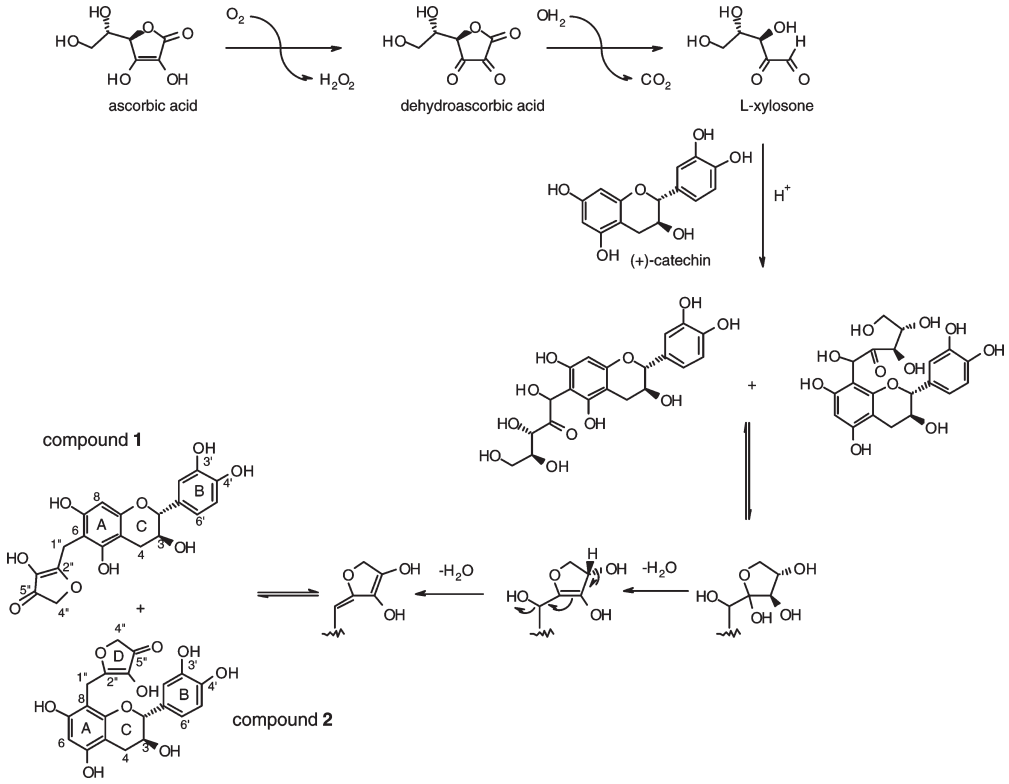


Figure 5. Scheme for the hypothetical formation of compounds **1** and **2**.

NMR evidence supported this structure for the substitution fragment. In particular, the two methylenes both showed $^{3}J_{CH}$ couplings to the enol carbon at 176.0 ppm but no couplings to each other. The methylene bonded to an oxygen (δ_C 72.2) was coupled to the ketone (δ_C 195.1) in the HMBC spectrum, whereas the aliphatic methylene (δ_C 21.3) was coupled to the enol (δ_C 133.7), indicating that this was the attachment point to the (+)-catechin. In the ^{1}H NMR spectrum of compound **2**, all of the (+)-catechin signals could be identified except one singlet for one of the A ring protons, suggesting that the fragment was linked to C6 or C8. This type of substitution is well-known for flavanols (*5*, *27*).

For compound **2**, the HSQC spectrum showed three methylenes, one was clearly that of the (+)-catechin C ring ($(H4)_2$ 2.54/2.38 ppm) as it showed long-range J_{CH} couplings to C5 (δ_C 154.5), C8a (δ_C 153.3), C4a (δ_C 98.8), C3 (δ_C 66.3), and C2 (δ_C 80.7) in the HMBC spectrum. C5 was differentiated from C8a on the basis of the former's $^{2}J_{CH}$ coupling to an exchangeable proton at 9.07 ppm. This exchangeable proton (9.07 ppm) was also coupled to C4a and C6 ($^{3}J_{CH}$), indicating that the attachment point of the −OH was C5. The other exchangeable proton on ring A (C7OH) was characteristically coupled to C8 (**Figure 4**). H6 was coupled to C4a and C8, with the latter also coupled to $(H1'')_2$ (**Figure 4**). $(H1'')_2$ was also coupled to C8a and C7 in the HMBC spectrum, unequivocally indicating that the furanone was attached at C8. As expected, H6 showed equally intense ROE correlation to C5−OH and C7−OH, the latter also showing a correlation to $(H1'')_2$. All other spectral data were consistent with structure **2** for the later eluting isomer (**Figure 5**).

Compound **1** was postulated to be the C6-attached isomer as has been reported for other aldehydes (*28*). In this case the point of attachment was confirmed by the fact that in compound **2**, both $(H4)_2$ and $(H1'')_2$ were coupled to C8a, whereas in compound **1** both were coupled to C5 (δ_C 154.2) in the HMBC spectrum. As in compound **2**, $(H4)_2$ was also coupled to C8a and $(H1'')_2$ was coupled to C7. Finally, the attachment point at C6 was confirmed by HMBC correlations between H8 (5.87 ppm) and C6 (101.5 ppm) and C4a (99.9 ppm) as expected but also C7 (154.8 ppm) and C8a (153.5 ppm).

Compounds **1** and **2** (**Figure 5**) are the first reported products to form between a carbon-based degradation product of ascorbic acid and (+)-catechin. Their formation explains the accelerated degradation of (+)-catechin that has been reported (*19*) in model wine solutions containing ascorbic acid. The formation of compounds **1** and **2** also confirms that (+)-catechin is not being directly oxidized in the presence of ascorbic acid but is rather forming an adduct with a degradation compound of ascorbic acid. These results highlight the multifaceted role that ascorbic acid may have in wine or other food systems. On the one hand, ascorbic acid undoubtedly acts as an antioxidant; on the other,

Table 1. 440 nm Absorbance of Samples during Storage at 45 °C in Darkness[a]

sample	day 0	day 14
(+)-catechin	0.001 ± 0.002	0.031 ± 0.002
compound **1**	0.005 ± 0.003	0.09 ± 0.02
(+)-catechin + compound **1**	0.010 ± 0.004	0.154 ± 0.006
compound **2**	0.009 ± 0.001	0.13 ± 0.02
(+)-catechin + compound **2**	0.011 ± 0.001	0.29 ± 0.01

[a] All samples were in a formic acid-buffered 12% aqueous ethanol solution. (+)-Catechin, 0.25 mM; compound **1** 0.02 mM; and compound **2**, 0.1 mM. Uncertainties are expressed as the 95% confidence limits (n = 3, Student's t-test).

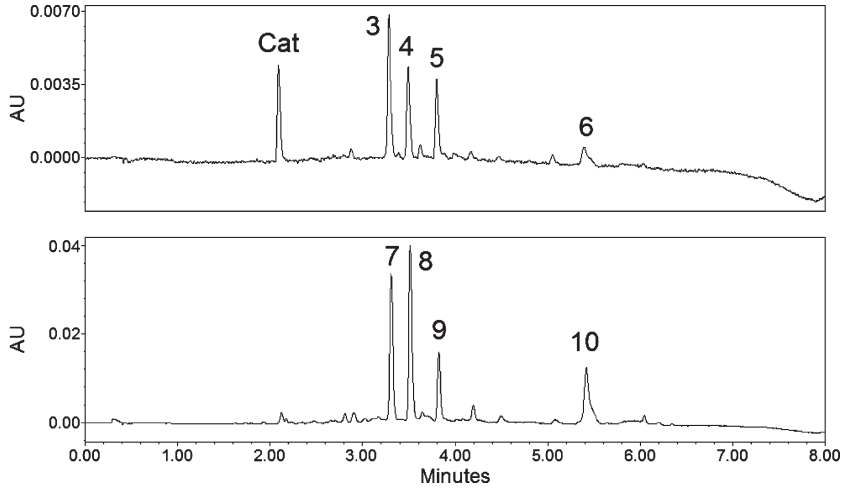


Figure 6. Liquid chromatograms at 440 nm of a solution of (+)-catechin and compound **1** (upper trace) and of (+)-catechin and glyoxylic acid (lower trace) after 14 days at 45 °C. The solutions consisted of a formic acid-buffered 12% ethanol solution.

breakdown products of ascorbic acid are capable of further reactions. In wines, those reactions may be with flavanols (e.g., (+)-catechin), which are the compounds most correlated to the browning of white wine.

Compounds **1** and **2** are stable substituted (+)-catechin monomers, provided some ascorbic acid remains in the model wine system. Other examples of stable (+)-catechin−adduct monomers are formaldehyde-substituted (+)-catechins generated as minor products in the reaction between glyoxylic acid and (+)-catechin (*29*) and certain oak-derived aldehydes that also form pyrylium-chromophores on addition to single (+)-catechin units (*30*). This is in contrast to many other (+)-catechin addition products that readily undergo further addition and form bridged-(+)-catechin dimers. The reliance on ascorbic acid for the stability of compounds **1** and **2** suggests that an oxidation step is required for their conversion to some other species (see below).

Formation and Fate of the Isomers as Model Wine System Components. Of the many degradation products of ascorbic acid that are reported in the literature (*13*−*16*)), L-xylosone (**Figure 1** appeared to be a reasonable candidate as a precursor to compounds **1** and **2**. Indeed, a retro-synthetic analysis (**Figure 5**) suggested that xylosone could be involved in the formation of **1** and **2**. Indeed, no other known degradation product of ascorbic acid allowed such feasible formation.

L-Xylosone, an aldehyde, is formed from the hydrolysis and subsequent decarboxylation of dehydroascorbic acid (*14*), but rapidly degrades to other aldehydes and ketones (*13*, *15*). Its lack an appropriate chromophore to allow detection above 250 nm, and its relatively polar character and expected elution near the injection front under the chromatographic conditions used here means that its detection by the system utilized in this experiment was not possible. Consequently, L-xylosone was synthesized as per the method described by Salomon et al. (*24*) and reacted with (+)-catechin in a model wine system consisting of a tartaric acid-buffered 12% ethanol solution. Colorless compounds were subsequently generated that had identical retention times, UV−visible spectra, and MS data as compounds **1** and **2** (as reported above). This confirmed that L-xylosone was the precursor to compounds **1** and **2** in model wine solutions of (+)-catechin and ascorbic acid.

Given that past work showed a link between the degradation of compounds **1** and **2** with the accelerated production of color in wine-like solutions (*19*), experiments were conducted to confirm the ability of compounds **1** and **2** to form colored compounds. Indeed, when left at room temperature after NMR analyses, the solution of both isolated isomers developed deep golden-brown coloration. When 0.02 mM compound **1** and 0.1 mM compound **2** were prepared ($n = 3$) at (+)-catechin concentrations of 0 and 0.25 mM in the model wine solution (formic buffer), they initially afforded 440 nm absorbances (a measure of yellow color) of < 0.012 absorbance unit. However, after incubation at 45 °C in darkness, the samples all increased in 440 nm absorbance to reach the values indicated in **Table 1**. It is evident that both compounds **1** and **2** were precursors to colored compounds and that the presence of 0.25 mM (+)-catechin enhanced the formation of colored compounds by compounds **1** and **2**.

Analysis by UPLC at day 14 revealed that all samples with added compound **1** or **2**, regardless of catechin addition, had three major peaks (3−5, **Figure 6**) in the 440 nm chromatograms. The UV−visible spectra extracted from the UPLC-PDA demonstrated that these major peaks (3−5, **Figure 6**), in all of the samples with compound **1** or **2** present, showed identical absorbance maxima at 280 and 440 nm with a shoulder at 310 nm. LC-MS data associated with these peaks all showed m/z signals at 617 in the positive ionization mode and the major fragmentation ion at m/z 465.

Such MS and UV−visible data were consistent with the xanthylium cation pigments derived from (+)-catechin and glyoxylic acid (**Figure 7**). The reaction of glyoxylic acid with (+)-catechin can generate six isomeric forms of the yellow xanthylium cations (*8*), albeit at different rates of formation, one of which is shown in **Figure 7**. These yellow pigments have UV−visible maxima at 280 and 440 nm with a shoulder at 310 nm (*7*) and a molecular mass of 617 amu (*7*) as well as the characteristic retro-Diels−Alder degradation of the dihydropyran moiety to form the m/z 465 fragment (positive ionization mode) (*31*).

To confirm that compounds **1** and **2** indeed form xanthylium cations, it was necessary to compare retention times of the purported xanthylium cations (as reported above) with those of genuine xanthylium cations synthesized from (+)-catechin and glyoxylic acid (*7*). Thus, a solution of xanthylium cations was prepared from 0.50 mM (+)-catechin and 0.25 mM glyoxylic acid by incubation at 45 °C in a tartaric acid-buffered model wine solution. The solution was analyzed by UPLC-PDA, and the

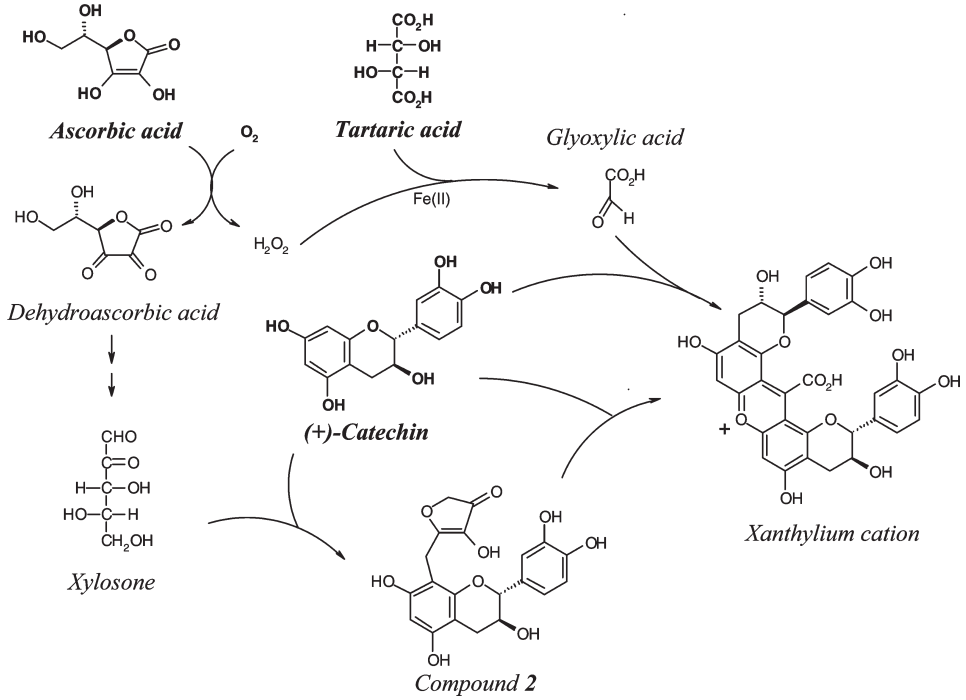


Figure 7. Formation of xanthylium cations from (+)-catechin and ascorbic acid in a tartaric acid-buffered model wine system.

peaks (7−9, **Figure 6**) corresponding to the xanthylium cations were confirmed by their UV−visible spectra and HPLC-MS analysis (data as described earlier). The remaining major peak in this solution (peak 10, **Figure 6**) corresponded to the ethyl ester of the xanthylium cation, which has also been described previously (*29*). The retention times of the xanthylium cations generated from glyoxylic acid with (+)-catechin (peaks 7−9, **Figure 6**) were at retention times identical to those of the major pigments in all of the samples containing compound **1** or **2** (peaks 3−5, **Figure 6**). The matching retention times were confirmed with the use of three different HPLC gradient systems and two different chromatography columns (data not shown). Consequently, the results demonstrate that compounds **1** and **2** form the same xanthylium cations as those generated by (+)-catechin and glyoxylic acid. Intriguingly, this was the case whether compound **1** or **2** was incubated with (+)-catechin or not, but more xanthylium cations were generated if (+)-catechin was present (**Table 1**).

The actual mechanism for the conversion of compounds **1** and **2** to xanthylium cations is not certain and is the subject of ongoing investigations. However, the results do establish a mechanistic link between (+)-catechin, a degradation product of ascorbic acid, and the production of xanthylium cations (**Figure 7**) that has previously been postulated (*17*, *18*). The results also add xylosone to the list of aldehyde compounds (i.e., glyoxylic acid (*7*) and glyoxal (*32*)) that can react with (+)-catechin to generate the xanthylium cation pigments such as that shown in **Figure 7**. Such a mechanism will be relevant to all beverages that contain flavanols and ascorbic acid, such as fruit juices. Furthermore, it will also be particularly relevant to wines with relatively high xylosone concentration, such as those produced from grapes infected with *Botrytis cinerea* (*33*).

In conclusion, the results of this study show a unique outcome from the reactions of two "antioxidants" in wine-like conditions, ascorbic acid and (+)-catechin. The process is far more complex than the anticipated and simple transfer of two electrons and two protons and demonstrates that ascorbic acid is a highly unpredictable molecule capable of inducing a variety of reaction processes. From a model white wine system, two new "natural" products derived from (+)-catechin and ascorbic acid were isolated. The two compounds were unequivocally identified on the basis of spectral data, primarily NMR spectroscopy. A retrosynthetic study indicated that the compounds could be formed from L-xylosone, a known degradation product of ascorbic acid, and (+)-catechin. Both isomers were independently synthesized from L-xylosone to establish this compound as a key precursor to compounds **1** and **2**. The accumulation of the isomers (**1** and **2**) depends on the presence of ascorbic acid, and they eventually react further to give colored xanthylium cation pigments.

ABBREVIATIONS USED

HPLC-DAD, high-performance liquid chromatography with diode array detection; DMSO, dimethyl sulfoxide; UV, ultraviolet; HRMS, high-resolution mass spectrometry; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; ESI, electrospray ionization; NMR, nuclear magnetic resonance; FID, free induction decay; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; INEPT, insensitive nuclei enhanced by polarization transfer; HMBC, heteronuclear multibond correlation; ROESY, rotating frame Overhauser effect spectroscopy.

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PUBLICATION 5

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The influence of stereochemistry of antioxidants and flavanols on oxidation processes in a model wine system:

ascorbic acid, erythorbic acid, (+)-catechin and (-)-epicatechin.

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ARTICLE

The Influence of Stereochemistry of Antioxidants and Flavanols on Oxidation Processes in a Model Wine System: Ascorbic Acid, Erythorbic Acid, (+)-Catechin and (−)-Epicatechin

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The stereochemical influence of antioxidant and flavanol compounds on oxidation processes in a model wine system was studied. The diastereoisomers, ascorbic acid and erythorbic acid, were used as antioxidants in a model wine system containing either (+)-catechin or (−)-epicatechin as the oxidizable flavanol compound. Samples were stored at 45 °C for a period of 14 days and analyzed by UV/visible spectrometry, CIELab, UPLC-PDA, and LC-MS. The results showed that less brown oxidative coloration occurred for samples with erythorbic acid for a given flavanol compound, while (+)-catechin provided less yellow coloration for a given antioxidant. Although erythorbic acid was degraded faster than ascorbic acid, it was associated with less decay in the accompanying flavanol compound. Xanthylium cation pigments were identified as the major contributor to color development. Furthermore, the production of pigment precursors, previously identified as furanone-substituted flavanols, was confirmed in all cases and their corresponding xanthylium cation pigments were lower in the presence of erythorbic acid than ascorbic acid. The results demonstrate that erythorbic acid is more efficient at minimizing oxidative color development than ascorbic acid in the model wine system.

KEYWORDS: Browning; white wine; (+)-catechin; xanthylium cation; (−)-epicatechin; model wine; ascorbic acid; erythorbic acid

INTRODUCTION

The oxidation of white wine is a well-known spoilage phenomenon. With sufficient oxygen ingress, a white wine will eventually change from a faint yellow color to a brown color (*1*, *2*), and will also have a dramatic change toward negative aroma and palate notes (*3*, *4*). The change in color is linked to the presence of skin and seed-derived flavanol-type phenolic compounds in the white wine, such as (+)-catechin or (−)-epicatechin (**Figure 1**) (*2*). The total flavanol concentration in white wines is highly variable, ranging from trace levels (< 5 mg/L) to levels upward of 200 mg/L, and depends on the grape variety, production techniques adopted and measurement techniques utilized (*5*−*10*). The majority of dry table white wines have flavanol levels in the lower ranges (i.e. < 30−50 mg/L), and those with more than 50 mg/L flavanols are often prepared from juice with extended skin and seed contact.

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Despite the common occurrence of white wine oxidation, the actual pigments responsible for the brown coloration in white wine have not yet been fully characterized. This is mainly due to the diverse range of phenolic compounds present in white wine (*11*), their variable and often low concentrations, and the complexity involved in both oxidation and the subsequent polymerization reactions (*12*−*15*).

For this reason, model wine systems have commonly been used to study oxidation reactions that have some relevance to white wine (*12*−*14*, *16*). This generally involves ethanol concentrations in the range of 10−14% (v/v), pH buffering between 3.0 and 3.7, and the presence of at least one phenolic compound. Such a model wine system, with saturated oxygen levels, has led to the identification of yellow xanthylium cation pigments (**Figure 1**) that are formed from the reaction of (+)-catechin with a degradation product of tartaric acid, one of the two major organic acids of grapes (*17*). It has been subsequently shown that similar xanthylium cations can be generated from the diastereoisomer of (+)-catechin, namely, (−)-epicatechin (**Figure 1**), albeit with molar absorptivity coefficients approximately twice that of the (+)-catechin-derived xanthylium cations (*18*). This means that, for a

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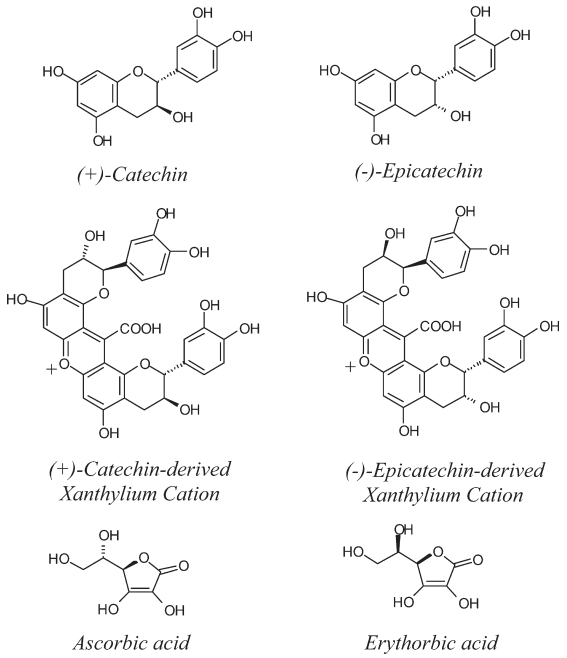


Figure 1. Structures of (+)-catechin, (−)-epicatechin, their respective xanthylium cations, ascorbic acid, and erythorbic acid.

similar initial flavanol concentration in a model wine system, (−)-epicatechin is capable of producing a color that is about twice as intense as that observed with (+)-catechin in the final oxidized model wine. This highlights the importance of stereochemistry in determining the molar absorptivity coefficients of xanthylium cations. The presence of these same yellow xanthylium cation pigments has been confirmed in white wines with elevated flavanol levels and after oxidative storage conditions (*19*).

In order to prevent the oxidative spoilage of white wine, the traditional antioxidant utilized is sulfur dioxide (*15*, *20*). However, sulfur dioxide is not efficient at preventing the oxidation of phenolic compounds in the presence of dissolved oxygen, especially at the sulfur dioxide concentrations typically adopted in white wine (i.e., 20−35 mg/L free sulfur dioxide) (*20*). Instead, the main antioxidant role of sulfur dioxide is to react with the products formed from the oxidation of phenolic compounds and the reduced molecular oxygen. This mainly includes *o*-quinone compounds and hydrogen peroxide (*15*), but may also include acetaldehyde if the reaction between sulfur dioxide and hydrogen peroxide is not sufficiently rapid (*21*). For this reason, the complementary use of the antioxidant ascorbic acid has added benefits due to its efficient ability to react directly with molecular oxygen, albeit mediated by metal ions, and thereby prevent the oxidation reactions of wine components at the earliest possible stage. The timing and concentration of ascorbic acid addition are variable, but it is typically added at rates ranging from 50 mg/L to 150 mg/L (*22*−*25*). Wine trials have clearly shown that the addition of ascorbic acid as well as sulfur dioxide to Chardonnay and Riesling at bottling resulted in wines able to maintain their color and flavor longer than those wines that only adopted a sulfur dioxide addition (*26*). On the other hand, the use of ascorbic acid without hydrogen sulfite has been demonstrated to be detrimental (*27*, *28*), with its degradation products inducing oxidative coloration after the ascorbic acid is near depleted (i.e., > 90% consumed). In this sense, ascorbic acid is still acting as an antioxidant while present, but its degradation products exert their pro-oxidative effects after the sufficient decay of ascorbic acid.

However, ascorbic acid is not the only complementary antioxidant to sulfur dioxide on offer, as erythorbic acid is also a permissible wine additive in many countries. Given that erythorbic acid and ascorbic acid are diastereoisomers, it is reasonable to expect that they should both have antioxidant activities. Erythorbic acid is commonly described as having identical antioxidant capability to ascorbic acid in white wine (*21*, *29*), while in other foods or biological systems, their comparative antioxidant capability is variable (*30*−*32*) but in some instances erythorbic acid shows superior activity to ascorbic acid (*33*−*35*). There are also several anecdotal observations in the Australian wine industry that erythorbic acid may have some improved abilities over ascorbic acid to prevent the production of dominant red tints, or pinking, in white wine. However, despite this, ascorbic acid is still the main complementary antioxidant to sulfur dioxide used in white wine.

Based on potential for improvement in antioxidant capabilities, this study was undertaken to compare the role of erythorbic acid and ascorbic acid as antioxidants using a model wine system containing the isomeric flavanol compounds (+)-catechin and (−)-epicatechin. The work was conducted in the presence of elevated oxygen and the absence of sulfur dioxide in order to determine the role of the antioxidants in a worst-case scenario for white wine. The impact of the antioxidant and flavanol compound combinations on the oxidative coloration, degradation kinetics and products were followed by UV/visible spectrophotometry, CIELab, UPLC-PDA and LC−MS.

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. Potassium hydrogen L-(+)-tartrate (> 99%) was obtained from Aldrich (USA) and L-(+)-tartaric acid (> 99.5%) from Sigma-Aldrich (USA). L-Ascorbic acid (> 99%, Sigma, USA), erythorbic acid (98%, Aldrich, USA), (+)-catechin monohydrate (98%, Sigma, USA) and (−)-epicatechin (95%, Sigma, USA) were used without further purification. To determine the concentration of metal contamination in the flavanol and antioxidant reagents, concentrated solutions (130 g/L) of each reagent, as well as a blank, were acid-digested with concentrated nitric acid and analyzed by inductively coupled plasma with optical emission spectroscopy (ICP-OES). The only contaminant found greater than the detection levels of the ICP-OES method was 1.2 ± 0.1 ng/kg iron (n = 4, 95% confidence limits) in the ascorbic acid standard, corresponding to an iron concentration of 0.48 ± 0.04 μg/L in the final model wine system. For this reason, 100.0 μg/L iron(II) was added to all samples in the form of iron(II) sulfate heptahydrate (98%, Ajax Chemicals, Australia) to limit the impact of the iron contamination on oxidation reaction rates.

Absorbance measurements and spectra were recorded on a μQuant Universal Microplate Spectrophotometer (Biotek Instruments, New York, NY) with the software KC4 v3.0 (Biotek Instruments). The absorbance measurements were recorded at 440 nm and spectra recorded from 200 to 600 nm. CIELab measurements were conducted on a Shimadzu (Kyoto, Japan) UV-1700 UV/visible spectrophotometer with UVPC Color Analysis software (version 3.00). The L^*, a^* and b^* CIELab values were calculated at the daylight illuminant D65 and with a 10° observer angle. The transmission was scanned over the range 380−780 nm with samples in 10 mm quartz cuvettes. The model wine solution without additives was used as the blank solution.

Liquid chromatography was conducted with an ultraperformance liquid chromatography (UPLC) system consisting of a Waters (Milford, MA) Acquity binary solvent manager connected to a sample manager and a PDA detector all run by Empower^2 chromatography manager software. The column was a Waters Acquity BEH C18 (2.1 × 50 mm) with 1.7 μm particle diameter. Injection volume was 7.5 μL, and the elution gradient consisted of solvent A (0.5% acetic acid in water) and B (0.5% acetic acid

in methanol), as follows (expressed in solvent A and cumulative time): 100% at 0 min, 100% at 1 min, 95% at 1.31 min, 62% at 5.25 min, 56% at 6.27 min, 48% at 6.34 min, 45% at 7.22 min, 0% at 8.85 min, 0% at 9.4 min, 100% at 9.74 min, and 100% at 10.76 min. The flow rate was 0.45 mL/min. Chromatograms and UV/visible spectra were recorded over the range 200−700 nm.

LC−MS experiments were conducted on a SpectraSYSTEM Thermo Scientific-LC (Waltham, MA) run by Xcalibur software (Waltham, MA) with a P4000 sample pump, UV6000LP UV detector and a Finnigan AQA quadrapole MS with an electrospray source. The column used was a reverse phase Wakosil C18RS (5 μm packing, 250 mm × 2 mm) from SGE (Ringwood, Australia) with a guard column of the same type. The flow rate, solvent composition, solvent gradient and injection volume were the same as described previously (*14*). The LC−MS experiments were carried out in the positive ion mode, with an ion spray voltage of +3 kV and orifice voltage of +10 V, and in the negative ion mode, with an ion spray voltage of −3 kV and orifice voltage of −30 V. In experiments investigating fragmentation products, the orifice voltages were increased to +80 and −80 V in the positive and negative ion modes respectively. Simultaneous wavelength detection at 278 and 440 nm was performed.

The ratio of 280 nm molar absorptivity coefficients for the (+)-1″-methylene-6″-hydroxy-2H-furan-5″-one-flavanol isomers was determined by LC−MS according to a variation of the method of Labrouche et al. (*18*), and outlined further below (see Calculation of Relative 280 nm Molar Absorptivity Coefficients). The MS ion chromatogram, corresponding to the parent ion for each isomer (403 m/z in the positive ion mode, 401 m/z in the negative ion mode), was obtained in duplicate at each cone voltage of −30 V, +30 V and +60 V, and the 280 nm chromatogram was obtained (n = 6) from the photodiode array detector.

Reactions. The model wine solution 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 stirred overnight at room temperature. The pH of the model wine solution was 3.2 ± 0.1. The majority of the studies were conducted with (+)-catechin or (−)-epicatechin (0.69 mM, 0.20 g/L), and either ascorbic acid or erythorbic acid (2.3 mM, 0.40 g/L) which were added to this solution and stirred until dissolved. Iron(II) (100 μg/L), in the form of iron(II) sulfate heptahydrate, was added immediately prior to the commencement of an experiment. Samples (100 mL) were placed in "250 mL" Schott Duran reagent bottles and had a measured headspace volume of 220 mL. Samples were stirred vigorously to vortex for 3.0 min at the start of the experiment and during sampling to replenish the oxygen in the bottles. The total available oxygen at the start of the experiment and after sampling was calculated to be around 19×10^{-4} mol, composed of 8.0 mg/L (0.25×10^{-4} mol) dissolved oxygen in the 100 mL sample, assuming saturation (*1*), and 18.8×10^{-4} mol in the 220 mL headspace. Given that the maximum drop in ascorbic acid was 120 mg/L (or 0.68×10^{-4} mol) between sampling, and the reaction between oxygen and ascorbic acid is 1:1, the amount of oxygen available to the sample was in excess of that consumed by ascorbic acid or erythorbic acid. The samples were held in darkness at 45 °C, and the sample bottles were only opened on measurement days. All samples were prepared in triplicate and the plotted data are the means of the replicates with the error bars representing the 95% confidence limits.

Calculation of Relative 280 nm Molar Absorptivity Coefficients. The ratios of the 280 nm molar absorptivity coefficients for (−)-MHF-8-epicatechin to (+)-MHF-8-catechin, and for (−)-MHF-6-epicatechin to (+)-MHF-6-catechin, were determined from LC−MS data using a modified method of Labrouche et al. (*18*). Labrouche et al. (*18*) calculated the overall ratio of molar absorptivity coefficients for xanthylium cations, such as those shown in **Figure 1**, in the positive ion mode at a single cone voltage. For the MHF-flavanols in this current study, the ratio of 280 nm molar absorptivity coefficients were calculated at three different cone voltages (−30 V, +30 V and +60 V), covering both positive and negative voltages, in order to limit any impact of stereochemical structural differences on ionization efficiency.

The ratio of the relative molar absorptivity coefficients of the (+)-catechin-derived and (−)-epicatechin-derived MHF-flavanols were calculated using the Beer−Lambert Law as

$$\varepsilon_e/\varepsilon_c = (A_e \times c_c)/(A_c \times c_e)$$

where A, ε and c refer to the absorbance, molar absorptivity coefficients and concentration, and the subscripts c and e refer to (+)-catechin-derived and (−)-epicatechin-derived MHF-flavanols respectively. From the mass chromatograms, the concentration of MHF-flavanol, c, will be proportional to the total ion current, I,

$$c = r \times I$$

where r is a constant; then,

$$\varepsilon_e/\varepsilon_c = (A_e \times I_c)/(A_c \times I_e)$$

The ratio of molar absorptivity coefficients for MHF-8-catechin to MHF-8-epicatechin was found to be 1.08 ± 0.09. The ratio of molar absorptivity coefficients for MHF-6-catechin to MHF-6-epicatechin was found to be 1.1 ± 0.2.

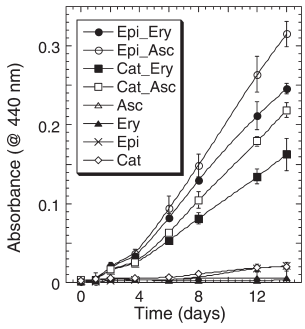


Figure 2. Change in 440 nm absorbance during the storage of samples at 45 °C in darkness. Error bars indicate the 95% confidence limits (n = 3).

RESULTS AND DISCUSSION

Oxidative Coloration of Model Wine Systems. The model white wine system was prepared with the different combinations of flavanol and antioxidant compounds, namely, (+)-catechin/(−)-epicatechin and ascorbic/erythorbic acids, respectively. The concentrations of antioxidant (400 mg/L) and flavanol (200 mg/L) were at high levels for white wine, but allowed sufficient time to analyze the kinetics of reagent decay and for appreciable concentrations of reaction products to form. It has been previously shown that higher concentrations of ascorbic acid/(+)-catechin did not affect the species formed but only their rate of formation (*27*).

All the samples were initially a similar pale yellow color and consequently of low 440 nm absorbance (day 0, **Figure 2**). After 14 days of incubation in darkness at 45 °C the binary combinations of flavanol and antioxidant constituents increased in 440 nm absorbance intensity, much more rapidly than for the samples with a single component (**Figure 2**). This was consistent with past reports for the ascorbic acid and (+)-catechin combination in model wine systems (*27*, *28*) but demonstrated that erythorbic acid also had the potential to induce coloration. Of the binary systems, those with ascorbic acid as the antioxidant were of higher 440 nm absorbance, as were those with the flavanol compound, (−)-epicatechin. Consequently, the ascorbic acid/(−)-epicatechin pair resulted in the highest 440 nm absorbance after 14 days while the erythorbic acid/(+)-catechin combination gave the lowest 440 nm absorbance. The presence of a lag period before the accelerated coloration was evident in all the binary samples (**Figure 2**), and duration of the lag period appeared similar in length regardless of the antioxidant present.

Although the data in **Figure 2** provide an insight into the 440 nm absorbance change in the samples, it does not provide a measure of the overall color of the samples. To achieve this, the samples were also analyzed by CIELab, which provides an analytical description of sample color with respect to three

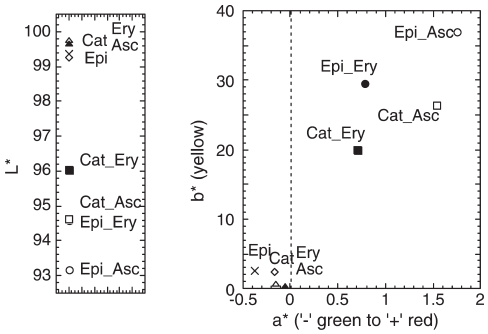


Figure 3. CIELab analysis of samples after 14 days of storage at 45 °C in darkness. The results are significantly different ($P = 0.05$) for any samples whose symbols are not in contact for a given parameter. Error bars indicate the 95% confidence limits ($n = 3$).

parameters: a^* indicating green to red coloration, b^* indicating blue to yellow coloration, and L^* indicating transparent (100) to opaque (0) coloration. At day 0, the samples were of either little or no difference to each other as described by the CIELab parameters (data not shown). However, by day 14 a divergence in the CIELab data for the samples was apparent (**Figure 3**). The description of the samples by the b^* and L^* parameters provided an ordering of the samples consistent with the 440 nm data (**Figure 2**): epicatechin/ascorbic > epicatechin/erythorbic > catechin/ascorbic > catechin/erythorbic. Intriguingly, the CIELab analysis also showed that the samples with ascorbic acid had higher a^* values than those with erythorbic acid. Therefore, these CIELab data demonstrate that not only can erythorbic acid provide lower intensity of yellow color in the oxidizing samples compared to ascorbic acid but it also can limit the amount of red coloration. Such an outcome is consistent with the anecdotal observations by winemakers that erythorbic acid is more effective than ascorbic acid against pinking, or development of red tints, in white wines. In the samples with ascorbic acid, the increased production of the primary yellow and red colors meant that the samples appeared more orange than the samples with erythorbic acid, which was in agreement with the visual assessment of the samples (data not shown).

Change in Flavanol and Antioxidant Concentrations. During the oxidative coloration of the samples, the flavanol and antioxidant concentrations were also monitored. Consistent with past studies (*28*), the decay of ascorbic acid was not influenced by the presence of flavanol compounds. The same was observed in this study for erythorbic acid. Consequently, the data shown for ascorbic acid and erythorbic acid in **Figure 4a** are the average ($n = 9$) of all samples containing either ascorbic acid or erythorbic acid regardless of the presence of the supporting flavanol compound. The rate of erythorbic acid degradation, 11.9 mg/h from 0 to 32 h, was significantly greater ($P = 0.05$) than that of ascorbic acid, 9.6 mg/h over the same period of time. The degradation of these antioxidants was repeated at room temperature (20 °C) at which the difference in degradation rate became more apparent (**Figure 4b**). The time for depletion of the antioxidant is important as the protective effect of such antioxidants in wine often only occurs while at least some of the antioxidant still remains in solution (*28*).

The oxidation rate of ascorbic acid (and presumably erythorbic acid) is known to be critically dependent on trace metal concentrations (*36*). In the model wine system adopted here, iron ions at a concentration of 100 μg/L are most probably chelated by the chiral pH buffer L-tartrate (*37*). An explanation as to the different

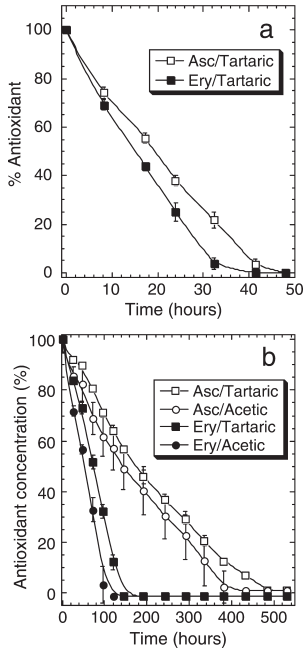


Figure 4. Decay in antioxidant during the storage of samples at (**a**) 45 °C or (**b**) room temperature. The samples contain either ascorbic acid or erythorbic acid in tartaric acid or acetic acid buffer. Error bars indicate the 95% confidence limits ($n = 9$ in (**a**) and $n = 3$ in (**b**)).

rate of antioxidant degradation may be that it was due to the chirality of the metal−buffer complex. To test this hypothesis, L-tartaric acid was substituted by acetic acid, an achiral organic acid, and the antioxidant decay monitored during storage at 20 °C. Despite the change in supporting buffer, the decay rates for ascorbic acid and erythorbic acid were still significantly different from each other at 1.6 mg/h and 3.9 mg/h respectively from 0 to 96 h, and only slightly different from those with the tartrate buffer (**Figure 4b**). Based on these results, it can be concluded that the rates of decay of the antioxidants are not related to the chirality of the supporting buffer.

Consideration also must be given to the ability of both ascorbic acid and erythorbic acid to undergo nonoxidative degradation. This degradation is often termed "anaerobic degradation" despite still occurring during the oxidative degradation of the antioxidants (*38*). However, based on the kinetic and activation energy data of Anmo et al. (*39*), the first order nonoxidative decay constants (45 °C) for ascorbic acid and erythorbic acid were calculated to be 2.6×10^{-4} h^{-1} and 9.6×10^{-4} h^{-1}, respectively. This corresponds to negligible antioxidant decay (i.e., 1.3% and 3.8% of the initial concentration, respectively), in the time scale of **Figure 4a**. At 20 °C the rate constants are 10-fold lower (i.e., 0.28×10^{-4} h^{-1} and 0.60×10^{-4} h^{-1}, respectively) and correspond to a loss of less than 1.5% the initial concentration of the antioxidants. Based on these low rates of anaerobic degradation, it was apparent that nonoxidative decay mechanisms were not responsible for the different rates of antioxidant decay between antioxidants in **Figure 4**.

Consequently, it seems that erythorbic acid is intrinsically capable of reacting more rapidly, compared to ascorbic acid, with either molecular oxygen, hydrogen peroxide and/or some other degradation product of the antioxidant. Therefore, although ascorbic acid may protect the model wine for a longer

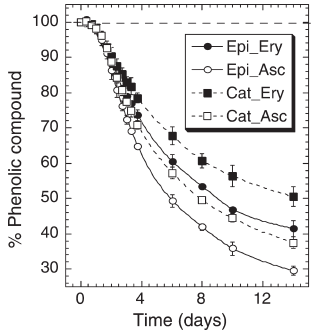


Figure 5. Decay in flavanol compound concentration during the storage of samples at 45 °C. The concentrations for (+)-catechin or (−)-epicatechin alone did not significantly ($P = 0.05$) deviate from their starting concentration. Error bars indicate the 95% confidence limits ($n = 3$).

period of time, due to its slower reaction in the presence of oxygen, erythorbic acid may offer a greater capacity for protection, and less possibility of oxidative side-reactions, due to its faster reaction in the presence of oxygen.

A comparison of the rate of loss of each flavanol compound (**Figure 5**) demonstrates that erythorbic acid, like ascorbic acid (*28*), accelerates the consumption of the accompanying flavanol. However, at day 14, the loss of the flavanol compound is significantly less ($P = 0.05$) in the samples with erythorbic acid compared to those with ascorbic acid, and the difference is evident well after the depletion of both antioxidants in the samples (i.e., 48 h, **Figure 4a**). Such results support the above proposition that erythorbic may offer a greater capacity for protection of wine components, such as flavanols, against oxygen than ascorbic acid. As far as the flavanol compounds are concerned, the loss of (−)-epicatechin is significantly faster than (+)-catechin, and this is consistent with the respective reaction rates of these compounds with aldehydes such as acetaldehyde and glyoxylic acid (*18*, *40*).

Production of Colorless Pigment Precursors. During the incubation of a flavanol with an antioxidant, the major products generated in the first two days were peaks a to h (**Figure 6**) in the 280 nm chromatograms. Samples containing (+)-catechin with either ascorbic acid or erythorbic acid showed colorless products with the same retention times (peaks a with c, and b with d, **Figure 6**), UV/visible spectra (λ_{max} 284 nm), and LC−MS data (403 m/z, positive ion mode), and hence were identical products. These colorless products have already been identified in the (+)-catechin and ascorbic acid system (peaks a and b, **Figure 6a**) as (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one-6-catechin ((+)-MHF-6-catechin, **1** in **Figure 7**) and (+)-MHF-8-catechin (**2** in **Figure 7**) isomers respectively (*12*), and both have the ability to evolve into yellow xanthylium cation pigments (*12*). Their formation involves the degradation of ascorbic acid to form the aldehyde, L-xylosone, which then undergoes an electrophilic addition to the phloroglucinol ring of (+)-catechin at either carbons-8 or -6, followed by rearrangement to form the (+)-MHF-catechin. Given that the MHF moiety has no chiral carbons, and that erythorbic acid presumably can form either identical degradation products to ascorbic acid or related stereoisomers (*41*, *42*), it is understandable that erythorbic acid and ascorbic acid provide the same substituted-(+)-catechin compounds.

Equivalent products (peaks e to h in **Figure 6**, **3** and **4** in **Figure 7**) were also identified for (−)-epicatechin during its incubation with either ascorbic acid or erythorbic acid. This identification was based on the identical UV/visible spectra (λ_{max} 284 nm), LC−MS parent ion data (403 m/z, positive ion mode) and LC−MS fragmentation data as for the (+)-MHF-catechin isomers. The fragmentation ions included the retro Diels−Alder cleavage of the dihydropyran moiety in the isomers to provide an ion at 251 m/z, the loss of the (+)-catechin or (−)-epicatechin unit to leave the methylenehydroxyfuranone unit at 113 m/z, or a combination of both fragmentation mechanisms to yield an ion with m/z of 139. Another fragment ion at 151 m/z appeared to be the 139 m/z ion with a $-CH_2^+$ group attached, hence the cleavage of the hydroxyfuranone unit with the methylene group remaining behind.

During the reaction of (+)-catechin and ascorbic acid, the concentration of MHF-addition isomers increases to a maximum and then begins to decrease upon depletion of ascorbic acid in solution (*12*). The results in **Figure 8** confirm that this is also the case for the (−)-MHF-epicatechin isomers. In fact, the decrease in the erythorbic acid-derived MHF-addition products (**Figure 8**) occurs earlier than for the equivalent ascorbic acid products, coinciding with the different decay rates for the respective antioxidants (**Figure 4a**).

The 280 nm chromatogram peak areas for the MHF-addition products generated by erythorbic acid are substantially lower in intensity than those generated by ascorbic acid. In order to convert chromatographic peak areas into concentrations of the MHF-addition products, it is necessary to know the molar absorptivity coefficients of these species. Although the molar absorptivity coefficients for these compounds are yet to be reported, relative concentrations of the various MHF-addition products (**1**−**4**, **Figure 7**) can be calculated based on the ratio of their 280 nm molar absorptivity coefficients. Using the method reported by Labrouche et al. (*18*) (see Material and Methods), the ratio of 280 nm molar absorptivity coefficients for MHF-8-catechin (**1**) to MHF-8-epicatechin (**3**) was 1.08 ± 0.09. For the 6-substituted isomers **2** and **4** the ratio was 1.1 ± 0.2. Therefore, the molar absorptivity coefficient between the different MHF-flavanols is not significantly different ($P = 0.05$) and hence the peak areas shown in **Figure 8** allow the relative concentrations of the different species to be compared. That is, erythorbic acid produces a maximum concentration for the MHF-flavanols that is around half that of those generated from ascorbic acid. Furthermore, the type of flavanol appeared to have little influence on the maximum MHF-flavanol concentration reached.

Production of Colored Products. In all model wines, the main colored compounds detected in the solutions were the xanthylium cations, in both their acid and ester forms, as reported by Labrouche et al. (*18*). The xanthylium cations were identified by matching their UPLC retention times with xanthylium cations synthesized by published methods (*17*), UV/visible spectra (λ_{max} 280, 440, or 460 nm, with shoulder at 310 nm), and LC−MS parent ion data (617 m/z acid form, 645 m/z ester form, positive ion mode). Therefore, regardless of whether ascorbic or erythorbic acid is present in solution, identical xanthylium cations were formed for a given flavanol. However, peak areas from the 440 nm chromatograms (**Figure 9**) show that the (−)-epicatechin-derived xanthylium cations provide over twice the intensity of the corresponding (+)-catechin-derived pigments. This is consistent with the known extinction coefficient data for the corresponding xanthylium cations (*18*), as well as the increased rate of loss of (−)-epicatechin compared to (+)-catechin (**Figure 5**). Furthermore, there is good consistency between the 440 nm absorbance data in **Figure 2** and the xanthylium peak area data in **Figure 9**, indicating that xanthylium cations make the predominant contribution to absorbance at 440 nm. In the samples with ascorbic acid, the higher concentration of the yellow xanthylium cations led to increased tailing of their absorbance into the 500 nm region

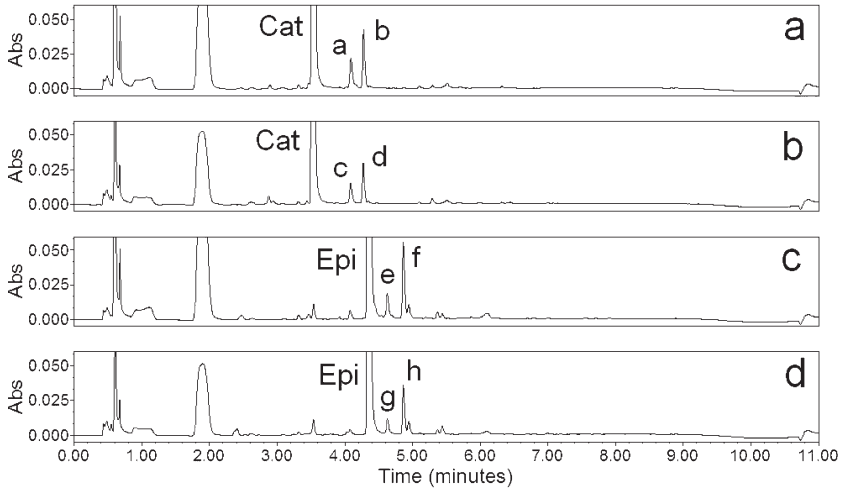


Figure 6. 280 nm chromatograms of the (**a**) (+)-catechin/ascorbic acid, (**b**) (+)-catechin/erythorbic acid, (**c**) (−)-epicatechin/ascorbic acid, and (**d**) (−)-epicatechin/erythorbic acid samples after 32 h of storage at 45 °C. The peaks eluting before the flavanol are due to the antioxidant and its degradation products.

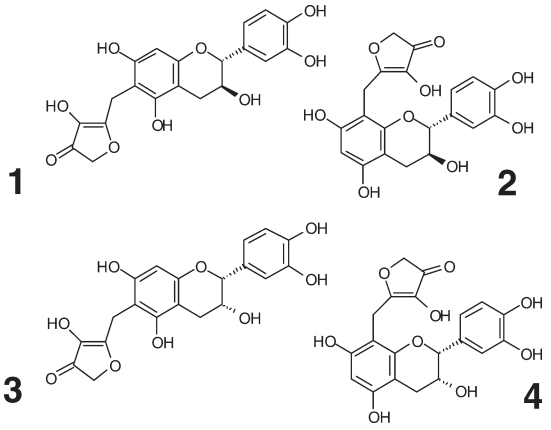


Figure 7. MHF-flavanol compounds identified in the model wine systems.

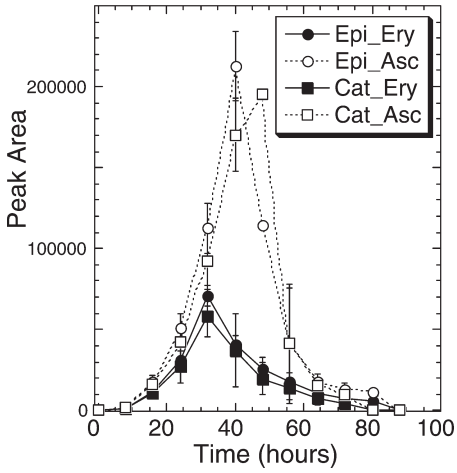


Figure 8. Peak area of the MHF-8-flavanols during the storage of samples at 45 °C. Error bars indicate the 95% confidence limits (n = 3).

of the spectrum and this accounted for the increased yellow and red coloration in the samples with ascorbic acid. This is consistent with the CIELab data reported above. Given that the xanthylium cations were the main pigments generated in all samples, it is apparent that the (−)-MHF-epicatechin isomers were also precursors to xanthylium cation pigments, as is known to be the case for (+)-MHF-catechin (*12*).

After day 7, it is evident that the rate of xanthylium cation production for the erythorbic acid-derived xanthylium cations has slowed compared to that of the ascorbic-acid derived xanthylium cations (**Figure 9**). This is consistent with the lower maximum concentrations reached for the MHF-flavanols in the case of erythorbic acid compared to ascorbic acid (**Figure 8**). It must be noted that the MHF-flavanols are not the only precursors for the xanthylium cation production as these pigments can also form via the oxidative degradation of tartaric acid in the presence of hydrogen peroxide and metal ions, or via exposure to light (*12*, *43*). The source of hydrogen peroxide in the model wine system may be from the oxidation of flavanol compounds and/or the oxidation of the antioxidant compounds (*37*). However, based on the studies of Barril et al. (*12*, *44*), the MHF-flavanol pathway would appear to make the more significant contribution to xanthylium cation production in samples containing ascorbic acid, and therefore by implication, also samples containing erythorbic acid.

The results of this study have shown that on deliberate oxidation of a model wine system the stereochemical impacts of antioxidants and flavanols are significant. In the presence of either of the flavanols (+)-catechin or (−)-epicatechin, erythorbic acid produces less oxidative coloration, at wavelengths corresponding to yellow and red, than the equivalent solutions containing ascorbic acid. Stereochemistry was also important in the oxidative degradation of both antioxidants and flavanols. Erythorbic acid was oxidized faster than ascorbic acid, and (−)-epicatechin was oxidized faster than (+)-catechin. This difference in reaction rates has significant implications in binary mixtures. First, the decomposition of flavanols did not occur as rapidly in the presence of erythorbic acid as it did in the presence of ascorbic acid. Second, this translated into lower production of colorless

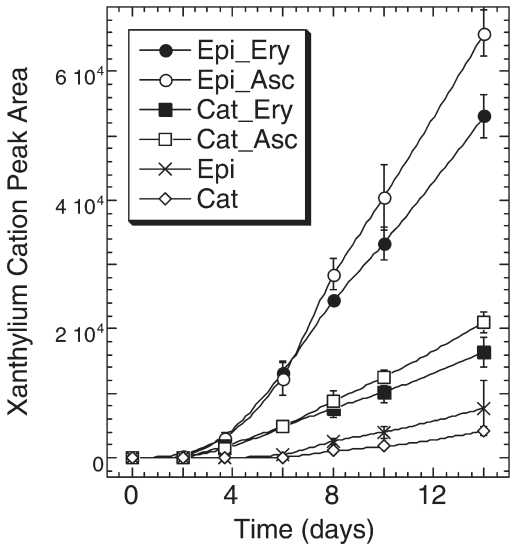


Figure 9. Comparison of the xanthylium cation peak areas, in the 440 nm chromatograms, formed during the storage of samples at 45 °C. Error bars indicate the 95% confidence limits (n = 3).

MHF-flavanol adducts, which, although not yet detected in white wine, are known precursors to yellow xanthylium cation pigments. Hence solutions containing erythorbic acid develop less color than those of ascorbic acid for a given flavanol. As reported previously (*18*) xanthylium cations derived from (−)-epicatechin are more intensely colored than those derived from (+)-catechin because the molar absorptivity coefficient is greater. Based on these results, erythorbic acid has certain advantages over ascorbic acid as an antioxidant and further studies are required to assess whether such advantages still exist during the complementary role of erythorbic acid to sulfur dioxide in wine media and under less oxidative storage conditions.

ABBREVIATIONS USED

UPLC-PDA, ultraperformance liquid chromatography with photodiode array detector; LC−MS, liquid chromatography with mass spectrometry; UV, ultraviolet; CIE, International Commission on Illumination; ICP-OES, inductively coupled plasma with optical emission spectroscopy; MHF, (+)-1″-methylene-6″-hydroxy-2*H*-furan-5″-one.

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