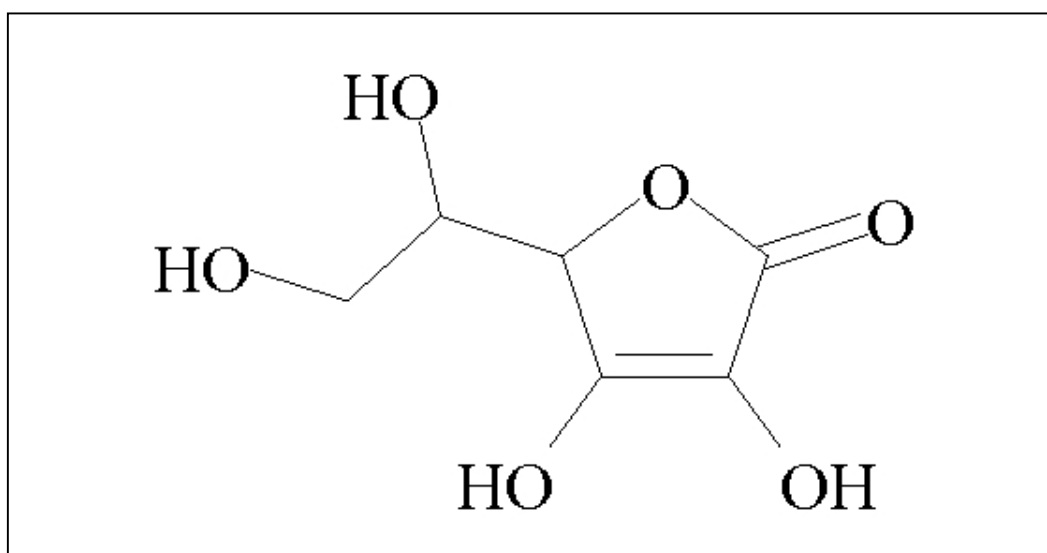


FACTORS DETERMINING WINE OXIDATION: THE CRITICAL ROLE OF ASCORBIC ACID



FINAL REPORT to
GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: UM 96/1

Principal Investigator: **G. R. SCOLLARY**

Research Organisation:
National Wine & Grape Industry Centre

Date: **31 May 2002**

EXECUTIVE SUMMARY

1. An extensive examination of the chemistry of ascorbic acid in a model white wine system has been completed. Considerable knowledge with respect to the dual role of ascorbic acid as an anti-oxidant and a pre-oxidation has been obtained.
2. A review of the literature (94 research papers, technical articles and oenology texts) has been completed. This review addressed the general chemistry of ascorbic acid and its use in winemaking. From the review, it became apparent that there is considerable confusion regarding the behaviour of ascorbic acid, particularly in wine-like systems. This review provided a useful base on which to design experiments to overcome the existing confusion.
3. A model white wine system was used to study the behaviour of ascorbic acid. This model system was composed of 12% (v/v) aqueous ethanol containing tartaric acid and set to a pH of 3.2. (+)-Catechin (100 mg/L) was used as the oxidisable substrate. Ascorbic acid concentrations were generally set at 200 mg/Litre. An accelerated "browning" reaction scheme was employed. This involved heating the samples at 45°C in the dark. Sufficient head space was employed to ensure that an adequate amount of oxygen was available to support the oxidative process.
4. This model system allowed careful control of the variables impacting on the rate and extent of oxidation of (+)-catechin. The oxidation reaction was accompanied by an increase in absorbance at 440nm, adjacent to the "wine browning" oxidation wavelength of 420nm.
5. The on-set of ascorbic acid induced oxidation of (+)-catechin was preceded by a lag period during which all ascorbic acid decayed. That is, an oxidation product of ascorbic acid, and not ascorbic acid itself, induces the (+)-catechin oxidation. Experiments with different head space volumes demonstrated that the different lag period was oxygen dependent with a longer lag period observed for samples with smaller head space volumes. On the other hand, similar increases of absorbance at 440nm were observed post the lag period (that is, independent of head space volume) implying that the browning reaction post lag period was independent of oxygen concentration.
6. Oxidation of ascorbic acid itself led to the initial formation of dehydroascorbic acid and hydrogen peroxide, the latter being formed at significantly less than the stoichiometric 1:1 ratio. Further, dehydroascorbic acid degraded to smaller, unidentified molecules. Several attempts using liquid chromatography-mass spectrometry were made in an attempt to identify the degradation products of dehydroascorbic acid. Although it did not prove possible to identify the compound, tentative evidence was obtained to suggest that hydrogen peroxide itself may well catalyse the further breakdown of dehydroascorbic acid.
7. Sulfur dioxide did not prevent the ascorbic acid induced oxidation of (+)-catechin, although it did delay the onset of browning measured at 440nm.
8. Sulfur dioxide consumption increased dramatically in the presence of ascorbic acid, and consequently, the lag period for the break down of ascorbic acid was increased. That is, sulfur dioxide is consumed rapidly at the beginning of the reaction process and protects ascorbic acid from breaking down itself until all sulfur dioxide is consumed. Following the loss of sulfur dioxide, ascorbic acid degraded and (+)-catechin oxidation ensued. Sulfur dioxide alone (no ascorbic acid) was found to inhibit the oxidation of (+)-catechin.
9. The (+)-catechin oxidation products absorbing at 440nm were identified as xanthylum cations. The formation of xanthylum cations has been described in the literature and involves a four reaction sequence commencing with the cleavage of tartaric acid to give glyoxylic acid.

This glyoxylic acid bridges two (+)-catechin molecules. The bridged catechin dimer undergoes dehydration to form a xanthenone and further oxidation of the xanthenone generates the xanthylium salt. All pre-cursors of the xanthylium salts were found to be colourless, confirming that the increase in absorbance at 440nm is in fact due to the production of xanthylium cations.

10. The xanthylium cations were formed only after all ascorbic acid had degraded. That is, the loss of ascorbic acid involved a cross-over from anti-oxidant conditions to pro-oxidant status. Ascorbic acid can be classified as an anti-oxidant whereas its breakdown products have a pro-oxidant function.
11. The implication of these project outcomes for white wine production are that, if ascorbic acid is used, its concentration must be sufficiently high to ensure that it does not break down over the life of the wine. This concentration must take into consideration all factors including random oxygen ingress that might occur when the wine is in the bottle.

SIGNIFICANT OUTCOMES

1. Identification of the conditions underlying the cross-over from anti-oxidant to pro-oxidant for ascorbic acid (Objective 2).
2. Recognition that a degradation product of ascorbic acid is responsible for inducing (+)-catechin oxidation (Objective 1).
3. Recognition that sulfur dioxide only delays and does not inhibit the ascorbic acid induced oxidation of (+)-catechin (Objective 4).
4. A detailed understanding of the chemistry of ascorbic acid in a wine-like system and the implications of ascorbic acid chemistry for its safe use in white wine making (Objectives 1, 2 and 5).

COMMUNICATION OUTCOMES

1. PhD thesis submitted and examined. Dr Bradshaw graduated on April 17, 2002.
2. Two published refereed scientific publications.
3. One review and two refereed scientific publications in preparation.
4. One technical article for the Australian Grapegrower and Winemaker to be published (July 2002).
5. One oral, one poster and two workshop presentations at the 11th Australian Wine Industry Technical Conference.
6. One oral and four poster presentations at scientific and wine industry conferences.

PROJECT OVERVIEW

Preamble

This report summarises research activities on the investigation into the critical role of ascorbic acid as a factor contributing to oxidation of white wine.

Research venues

- National Wine and Grape Industry Centre, Wagga Wagga
- Institut des Produits de la Vigne, Institut National de la Recherche Agronomique (INRA), Montpellier, France

Research staff and PhD student

Professor Geoffrey R. Scollary (NWGIC, Wagga Wagga)

Dr. Paul D. Prenzler (NWGIC, Wagga Wagga)

Mark P. Bradshaw (PhD student, NWGIC, Wagga Wagga)

Dr. Veronique Cheynier (INRA, Montpellier)

Project Dates and Funding

Project commencement	November, 1997
Completion date	December, 2001

Funds provided by the GWRDC were

1996/97	\$10,007
1997/98	\$34,417
1998/99	\$10,762
1999/2000	\$26,635
2000/01	\$25,745

Additional funding was also provided through CSU00/4 for Mr Bradshaw to spend five weeks at INRA, Montpellier, France. A separate report has been submitted for this work.

Projective Objectives

1. To establish the critical role of ascorbic acid in wine oxidation.
2. To establish the conditions under which ascorbic acid acts as an antioxidant and as a promoter of wine oxidation.
3. To determine the wine components most susceptible to oxidation in the presence of ascorbic acid.
4. To study the influence of winemaking additives such as copper sulfate and sulfur dioxide on the ability of ascorbic acid to act as either an antioxidant or a pro-oxidant.
5. To propose criteria for the safe use of ascorbic acid in winemaking.

OUTPUTS

Scientific Publications

Published

Bradshaw, M. P., Prenzler, P. D. and Scollary, G. R. 2001, Ascorbic Acid-Induced Browning of (+)-Catechin In a Model Wine System. *J Agric. Food Chem.*, **49**, pp. 934-939.

Bradshaw, M. P., Prenzler, P. D. and Scollary, G. R. 2002, Square Wave Voltammetric Determination of Hydrogen Peroxide Generated from the Oxidation of Ascorbic Acid in a Model Wine Base. *Electroanalysis* **14**, pp. 546-550.

In preparation

Effect of Sulfur Dioxide on the Ascorbic Acid Induced Oxidation of (+)-Catechin in a Model White Wine. For submission to *Australian Journal of Grape and Wine Research*.

Ascorbic Acid: a review of its Chemistry and Reactivity. For submission to *Australian Journal of Grape and Wine Research*

Identification of the Ascorbic Acid Crossover from Antioxidant to Prooxidant in a Model White Wine Containing (+)-Catechin. For submission to *J. Agric. Food Chem.*

Technical Paper

Bradshaw, M. P. 2002, Ascorbic Acid – An Oxymoron of Antioxidants? accepted in *The Australian and New Zealand Grapegrower and Winemaker (Australian Technical Issue)*.

Conference Presentations

oral presentation

- Bradshaw, M. P., Prenzler, P. D., Scollary, G. R. 2000, Ascorbic Acid: Is it helpful for wine quality. In *Proceedings for the 5th International Symposium on Cool Climate Viticulture and Oenology*. Melbourne, Australia, 16-20 January.

poster presentation

- Bradshaw, M. P., Prenzler, P. D., Cheynier, V., Scollary, G. R. 2001, Ascorbic acid-induced browning of catechin in a model wine. In *Proceedings for the 9th RACI Research and Development Topics Conference*. Melbourne, Australia, 3-6 December, pp. PS2.12.
- Bradshaw, M. P., Prenzler, P. D., Cheynier, V., Scollary, G. R. 2001, Ascorbic acid-induced browning of catechin in a model wine. In *Proceedings for the 2nd Symposium In Vino Analytica Scientia*. Bordeaux, France, 14-16 June, pp. 113.
- Bradshaw, M. P., Prenzler, P. D., Scollary, G. R. 2000, Ascorbic acid-induced browning of catechin in wine. In *Proceedings for the XXth International Conference on Polyphenolics*. Freising-Weihenstephan, Germany, 10-15 September, pp. 523-524.

- Bradshaw, M. P., Prenzler, P. D., Scollary, G. R. 1999, Investigations of Ascorbic Acid as a Promoter of Wine Spoilage. In *Proceedings for the Australian International Symposium on Analytical Science*. Melbourne, Australia, 4-9 July, pp. 285.

11th Australian Wine Industry Technical Conference

oral presentation

Scollary, G. R., Bradshaw, M. P., Clark, A. C. Prenzler, P. D. New insights into the phenomenon of random oxidation: the role of ascorbic acid and metal ions. To be published in conference proceedings (expect August 2002)

poster presentation

Mark Bradshaw, Paul Prenzler, Veronique Cheynier and Geoffrey Scollary, "Ascorbic acid induced-browning of catechin in a model wine"

workshop

G R Scollary, M P Bradshaw and A C Clark, "Oxidation (copper and ascorbic acid)", W36 and W65.

DETAILED PROJECT REPORT

BACKGROUND

Ascorbic acid has been widely used in winemaking, especially white wine production, for more than twenty five years. Its use remains high in some countries including Australia compared to Europe and the USA: in these latter two countries, a significant reduction in its application has occurred. The claimed basis for the application of ascorbic acid in winemaking lies in its ability to scavenge molecular oxygen, thereby preventing the onset of oxidative spoilage (browning) of white wines. The browning of the wine is the result of chemical reactions (sometimes mediated by an enzyme) that lead ultimately to a change in colour of the wine. This colour alteration can be assessed by a change in absorbance at 420 nm and is often accompanied by the development of an off-flavour as well as a loss of freshness of the nose of the wine.

Browning reactions can be either enzymic or non-enzymic. The enzymic process, as illustrated in Figure 1, is a consequence of the oxidative reaction of an *ortho*-dihydroxyphenolic compound to an *ortho*-quinone that can undergo further reaction to form brown products. This process is catalysed by the polyphenoloxidase enzyme (PPO) *tyrosinase*. Non-enzymic browning of fruits and vegetables has generally been attributed to the Maillard process although the full significance of this process for wine browning is not clear. It has been argued that non-enzymic browning of wine can also occur through direct aerobic oxidation of the polyphenol compound with the possible involvement of metal ions such as copper, iron, tin and aluminium. The anti-oxidant capabilities of ascorbic acid supposedly prevent the onset of browning by reducing the *ortho*-quinone to the original phenolic compound.

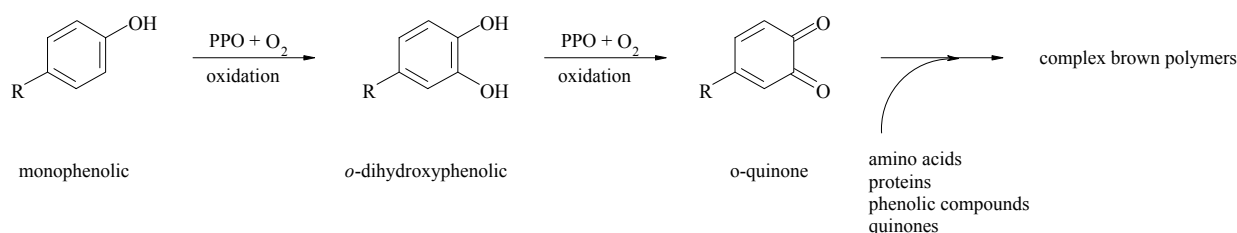


Figure 1. Enzymic oxidation of a phenolic compound

There are now increasing concerns that the addition of ascorbic acid to white wine may not always prevent spoilage but rather may enhance the browning process. It was these concerns that led to the creation of this GWRDC-funded project. The negative (or pro-oxidative) effect of ascorbic acid has been described by some authors to be the result of the oxidising abilities of hydrogen peroxide and/or radical species generated from the oxidation of ascorbic acid. It is therefore always recommended in oenology texts that, when ascorbic acid is used in white wine, it be used in combination with sulfur dioxide. However, there appears to be a lack of evidence as to whether there is any benefit to be gained from the addition of ascorbic acid to wines that already contain sulfur dioxide.

Ascorbic acid is a fascinating molecule with a clear anti-oxidant capacity but with increasing evidence that under some (not defined) conditions it can also act as a pro-oxidant. In fact, ascorbic acid has on some occasions been referred to as an "oxymoron of anti-oxidants".

It is within this context of the potential for ascorbic acid to contribute to the enhancement of white wine oxidation that this project has been set. The basis of the project has been to understand the fundamental behaviour of ascorbic acid in a model white wine system and to apply this knowledge of ascorbic acid chemistry to its potential to act as both an anti-oxidant and pro-oxidant of phenolic compounds that are found in white wine. The model system that was used in this work was designed to provide a well defined medium in which the variables impacting on ascorbic acid chemistry could

be well controlled. Knowledge of this chemistry was regarded as an essential first step in the guidelines for the use of ascorbic acid in white winemaking.

LITERATURE REVIEW

A review of ninety eight research papers, technical articles and oenology texts was carried out as a preliminary to the establishment of the experimental component of this research project. This review addressed the general chemistry of ascorbic acid and its use in winemaking. The critical points developed as part of this review were:

- Ascorbic acid is widely used as an anti-oxidant in winemaking and other chemical, biological and physiological systems
- There is inconclusive evidence for the role of ascorbic acid in initiating oxidative browning of white wine
- There is tentative evidence to suggest that ascorbic acid, especially if amino acids are present, could enhance browning by the Maillard pathway.
- When oxidised, ascorbic acid is converted to dehydroascorbic acid and this in turn is converted to 2,3-diketogulonic acid.
- There are many reports on the further reaction of 2,3-diketogulonic acid and there are reports of some 50 compounds having been identified following the degradation of ascorbic acid.
- Although there is a lack of certainty as to the nature of the ascorbic acid degradation products, it does appear that most are smaller than ascorbic acid and that those containing an aldehyde functional group could be involved in bridging phenolic compounds, similar to the established acetaldehyde bridging process.
- There are several postulations regarding free radical and activated oxygen species linked to the oxidation of ascorbic acid. There is, however, a lack of decisive evidence to confirm these claims.

From this review, it became apparent that there is considerable confusion in the literature regarding the behaviour of ascorbic acid, particularly in wine-like systems. In fact, there have been very few experiments carried out to understand the actual chemistry of ascorbic acid in systems approximating the wine medium. Rather, most of the published research has used systems that bear little or no relationship to the wine medium but have drawn conclusions for wine without any evidence that the non-wine medium chemistry has any relevance to that which may occur in a wine-like medium. The review provided a useful base on which to design experiments that could overcome the confusion existing in the literature regarding the general anti-oxidative behaviour of ascorbic acid.

NON-ENZYMIC OXIDATION

Figure 2 sets out the non-enzymic process for the oxidation of phenolic compounds as frequently found in oenology texts. It is essentially the same as enzymic oxidation (Figure 1), except that a catalyst (eg: a metal ion) is required in place of the enzyme. The direct interaction between molecular oxygen and organic molecules is 'spin forbidden' due to the arrangement of electrons in the oxygen molecule. Conversion of molecular oxygen from its lowest energy (ground) state to a higher energy (excited) state is required before a reaction can occur. The catalyst is one means by which this activation of molecular oxygen can occur.

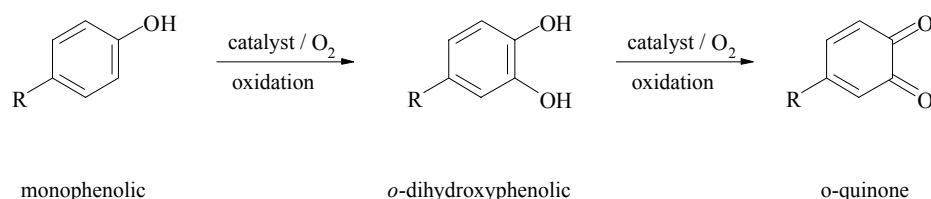


Figure 2. Non-enzymic oxidation of a phenolic compound

Ascorbic acid is used to counter the process described in Figure 2 as it becomes the sacrificial substrate for oxidation. That is, ascorbic acid can be oxidised in preference to the phenolic compound or, if a phenolic compound has been oxidised to a quinone, ascorbic acid can re-generate the phenolic compound. The oxidation of ascorbic acid to dehydroascorbic acid is shown in Figure 3 and the coupled reaction involving the phenolic/quinone and ascorbic acid/dehydroascorbic acid redox pairs in given in Figure 4.

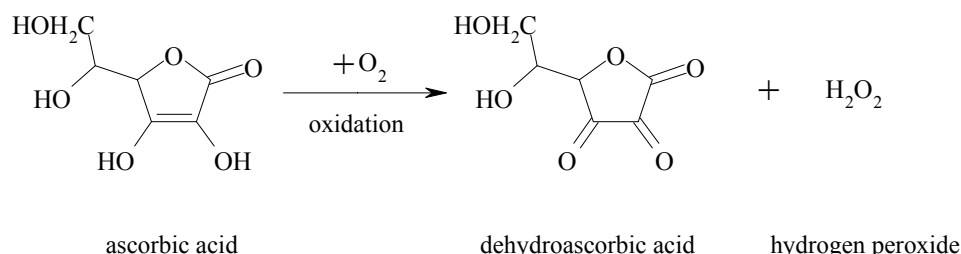


Figure 3. Oxidation of ascorbic acid to dehydroascorbic acid

The reaction of ascorbic acid with molecular oxygen has the same spin restriction mentioned above for phenolic compounds. That is, activation of molecular oxygen requires some form of catalyst and it is generally assumed that a metal ion is required as the catalyst. Only a trace amount of the metal ion is required.

From the process shown in Figure 3, it can be seen that:

- 1 mole of ascorbic acid produces 1 mole of dehydroascorbic acid
- 1 mole of ascorbic acid produces 1 mole of hydrogen peroxide.

This simple stoichiometry will be re-examined later in this report. It should also be noted that the generation of hydrogen peroxide is the basis for the frequently stated winemaking requirement for the use of sulfur dioxide in combination with ascorbic acid, the sulfur dioxide acting as a scavenger for the hydrogen peroxide.

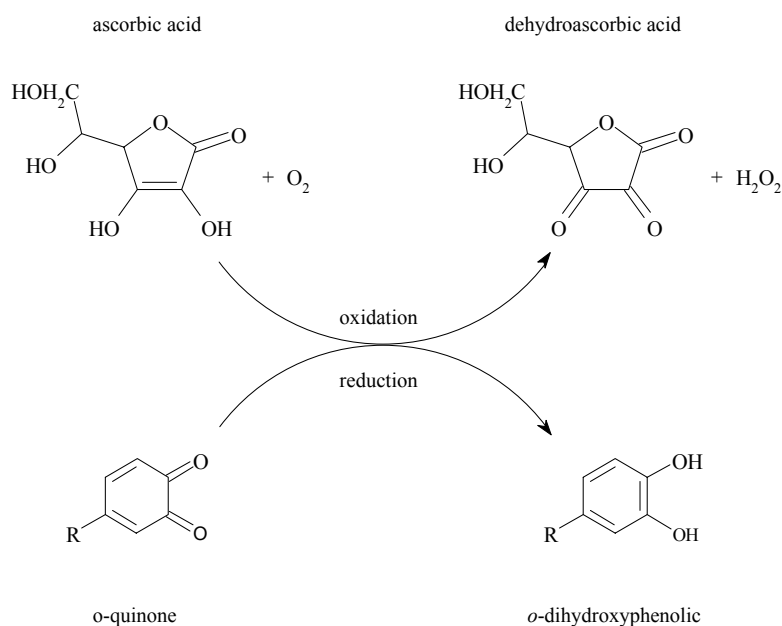


Figure 4. Coupled reaction between the phenolic/quinone and ascorbic acid/dehydroascorbic acid redox pairs

THE MODEL WINE SYSTEM

The model wine system was prepared by dissolving 2.11 g of potassium hydrogen tartrate and 0.806 g of potassium hydroxide in 1 litre of 12% (v/v) aqueous ethanol. This resulted in a total tartrate concentration of 0.011 M and a total potassium concentration of 0.026 M. The pH of the model wine base was just 3.2 with 10% (v/v) sulfuric acid. (+)-Catechin (100mg/L) was used as the oxidisable substrate in the model wine system. Generally, an ascorbic acid concentration of 200 mg/L was used.

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

In some experiments the head space volume was varied to obtain some insight into the role played by molecular oxygen in the browning process.

The increase in browning was assessed by measuring the absorbance of the sample in 10 mm quartz cells at 440 nm. The model wine base (unheated) was used as the blank in these absorbance measurements. The wavelength of 440 nm was used as separate experiments had indicated that it is the wavelength corresponding to the absorbance maximum in this model system that has undergone extensive browning. It should be noted at this point that the term "browning" is perhaps not the most appropriate descriptor for the change in the system as 440 nm corresponds to a system which is more yellow than brown in colour. However, browning is used widely in the oenology literature to describe the process that results from both enzymic and non-enzymic oxidative processes. For this reason, the term browning is used throughout this report although it is recognised that it is perhaps not the most appropriate term to use.

STUDIES OF THE ASCORBIC ACID INDUCED BROWNING PROCESS

When the model wine system containing both ascorbic acid and (+)-catechin was subjected to the induced browning process, a significant increase in the absorbance at 440 nm was observed over a fourteen day period. For the model wine system containing both ascorbic acid and (+)-catechin, the absorbance reached 0.055 after fourteen days compared to 0.015 for the model wine system containing (+)-catechin only. Clearly ascorbic acid is capable of significantly enhancing the extent of browning. This induced browning process was preceded by an initial drop in absorbance, termed the "lag period", that lasted one to two days. After the lag period, the system containing ascorbic acid showed a rapid increase in absorbance at 440 nm. Intriguingly, the addition of (+)-catechin to a model wine system containing pre-oxidised ascorbic acid did not exhibit this lag period, suggesting that an oxidation product of ascorbic acid was responsible for inducing the browning of catechin and not ascorbic acid itself. These results also suggest that molecular oxygen is critical for the initiation of the browning process.

The head space volume, that is the volume above the model wine system that reflects the amount of available molecular oxygen, was also found to influence the lag period. Table 1 compares the absorbance after fourteen days and the rate of increase of browning (as the slope of the absorbance versus time plot) from the end of the lag period to day fourteen for solutions containing 1, 3, 5 and 250 mL head space volumes. Increasing the head space volume shortens the lag period, with a consequent higher absorbance at day fourteen. The rate of browning, calculated as the change in absorbance as a function of time after the lag period, was similar for the 3, 5 and 250 mL head space volume experiments. The rate for the 1 mL head space is only about 60% of that for the 250 mL head space.

In a second head space experiment, two solutions containing equal volumes of the model wine system were subjected to the induced browning process with one solution being aerated daily and the second being aerated daily only until day seven (post lag period) and then sealed until day fourteen. The gradients of the absorbance versus time curves from day seven to day fourteen were 0.0040 for daily aeration and 0.0037 (no aeration post day seven). The similarity of these two gradients indicates that

molecular oxygen appears to have a critical role in the initiation of the browning process. That is, once the browning process has commenced molecular oxygen is no longer required to sustain that process.

Table 1. Effect of headspace volume on the extent of browning of catechin as induced by ascorbic acid

Headspace volume (mL)	Lag period (days)	A _{440nm} at Day 14	Browning rate (day ⁻¹)
1	7	0.021	0.0025
3	4	0.042	0.0040
5	3	0.045	0.0040
250	1	0.053	0.0041

The oxidation of ascorbic acid produces dehydroascorbic acid. Both compounds absorb in the UV-region with wavelengths of maximum absorbance being 245 nm for ascorbic acid and 300 nm for dehydroascorbic acid. An examination of the decay of ascorbic acid in the model wine base showed the expected decrease in absorbance at 245 nm but the increase at 300 nm (the wavelength for dehydroascorbic acid) was considerably higher than expected for a direct 1:1 conversion of ascorbic acid to dehydroascorbic acid. This higher than expected absorbance value at 300 nm suggests that the dehydroascorbic acid is reacting in the model wine base.

Hydrogen peroxide is also produced in the aerobic oxidation of ascorbic acid and it is this hydrogen peroxide that is frequently regarded as a critical component in any pro-oxidative reaction based on ascorbic acid. When hydrogen peroxide was added to the catechin/model wine base solution, the extent of browning as measured at 440 nm did increase but significantly less than the increase produced by ascorbic acid itself. After a fourteen day reaction period the ascorbic acid/catechin/model wine base solution showed an absorbance at 440 nm of 0.056 whereas the hydrogen peroxide/catechin/wine base solution had an absorbance value at 0.024 and the catechin/wine base solution showed an absorbance value of 0.015. Clearly hydrogen peroxide is not capable of inducing the same degree of browning of catechin as is ascorbic acid. This suggests that the mechanism for the ascorbic acid induced browning process is not only dependent on the generation of hydrogen peroxide.

Detailed results from these preliminary experiments are set out in Appendix 1.

This preliminary work has demonstrated that ascorbic acid has a clear role in inducing the browning of (+)-catechin and that the production of hydrogen peroxide from ascorbic acid is not sufficient to explain the degree of ascorbic acid induced browning. The next phase of the work involved an examination of the production of hydrogen peroxide from ascorbic acid and this was followed by an examination of the behaviour of the decay of the ascorbic acid in the model wine base system.

DETERMINATION OF HYDROGEN PEROXIDE FROM ASCORBIC ACID

The technique of square wave voltammetry was used to develop a procedure for the determination of both ascorbic acid and hydrogen peroxide in the one solution. Square wave voltammetry (SWV) on a hanging mercury drop electrode involves measuring the current flowing for an oxidation or reduction process as the potential is scanned in either the positive (anodic) or negative (cathodic) direction. Ascorbic acid could be analysed by employing an anodic scan and by resetting the potential and scanning in the cathodic direction hydrogen peroxide could then be determined. That is, SWV provided an ideal system for determining the relationship between the loss of ascorbic acid and the generation of hydrogen peroxide in the one sample solution. The technique was refined as an analytical technique for both components and the work was accepted for publication in "Electroanalysis", a journal dedicated to analytical methods based on electrochemistry.

A copy of the publication is attached as Appendix 2. This Appendix and also Appendix 3 sets out in more detail the results summarised here.

The significant observations of these experiments were:

- Over a 12-hour reaction period, only a small amount (13%) of hydrogen peroxide decayed when left in the model wine base containing ethanol and tartaric acid, indicating that in the absence of some catalyst or activator, hydrogen peroxide is not capable of oxidising ethanol.
- SWV provides an efficient and effective method for the simultaneous analysis of ascorbic acid through an oxidative scan and hydrogen peroxide through a cathodic scan.
- Separate calibration processes were established for the quantitation of both ascorbic acid and hydrogen peroxide, with linear graphs for both analytes over the concentration range relevant to the model wine system.
- Oxidation of ascorbic acid results in the concurrent generation of hydrogen peroxide as expected.
- However, after 12 hours reaction time, the ratio of ascorbic acid lost to hydrogen peroxide produced is 4.67:1, significantly greater than the expected 1:1 ratio (Figure 3).
- The concentration of hydrogen peroxide generated through the oxidation of ascorbic acid was observed to decrease after 12 hours (interpretation of cathodic scan voltammograms).
- This loss of hydrogen peroxide coincided with the appearance of a number of new peaks in the cathodic voltammogram, suggesting the formation of new compounds.

The higher than expected ratio for the amount of ascorbic acid reacted to hydrogen peroxide produced, coupled with the loss of the hydrogen peroxide signal and the detection of several new peaks in the cathodic scan implies further reactions involving hydrogen peroxide. In the system used for these experiments, the only components are the model wine base (containing ethanol and tartaric acid) and ascorbic acid. As mentioned above, it was observed that hydrogen peroxide does not react with the wine base components. This in turn implies that the generated hydrogen peroxide must react with a breakdown product of ascorbic acid.

Identification of the compounds that gave rise to the peaks in the cathodic SWV scan following the decrease in the hydrogen peroxide peaks is not possible by SWV alone. However, the reduction potentials at which the peaks were observed suggest that the species contain aldehyde and/or ketone functional groups. Several of the published degradation products of ascorbic acid contain the appropriate functional groups that could be reduced in the potential range at which peaks were observed in the voltammograms. Other techniques, particularly chromatographic techniques are required to assist in elucidating the identity of these ascorbic acid degradation products as is discussed in a later section of this report.

SULFUR DIOXIDE AND ASCORBIC ACID

The production of hydrogen peroxide from the oxidation of ascorbic acid (Figure 3) provides the reason for the winemaking practice of using sulfur dioxide as a complement to ascorbic acid. It has been argued that, if ascorbic acid is used on its own, the generated hydrogen peroxide will lead to rapid oxidation of both ethanol and phenolic compounds. Sulfur dioxide, with its ability to react rapidly with hydrogen peroxide, ensures its ready removal from the system thereby minimising any hydrogen peroxide induced oxidative process.

This study has shown that the amount of hydrogen peroxide generated from the oxidation of ascorbic acid is considerably less than expected as the generated hydrogen peroxide appears to react with a breakdown product of ascorbic acid. Further, hydrogen peroxide itself was not capable of oxidising ethanol or inducing the same degree of browning of (+)-catechin as could ascorbic acid.

These observations question whether hydrogen peroxide is the major cause of the elevated browning of (+)-catechin observed in the presence of ascorbic acid. Irrespective, it was considered to be of value to examine the effect of sulfur dioxide on the ascorbic acid-induced browning process.

Initial experiments used 50 mg/L sulfur dioxide, in approximate mole ratio of 0.8:1.0 with ascorbic acid. It was observed that this concentration of sulfur dioxide only delayed the onset of ascorbic acid induced browning of (+)-catechin. Further, the rate of sulfur dioxide consumption was increased in the presence of ascorbic acid. As shown in Table 2, there was no sulfur dioxide remaining at Day 14 in the system containing the combination of ascorbic acid and sulfur dioxide with (+)-catechin compared with about 40% for a “blank” (sulfur dioxide alone) and 43% for sulfur dioxide with (+)-catechin

It was found that the presence of this concentration of sulfur dioxide (50 mg/L; mole ratio 0.8:1.0 with ascorbic acid) increased the lag period for the on-set of browning. It has been described above how browning is preceded by a lag period corresponding to the decay of ascorbic acid. The lag period in the presence of 50 mg/L sulfur dioxide was extended to 4 days compared with 2 days with ascorbic acid alone. Further, it was observed that all sulfur dioxide was consumed by Day 3 and, with the decay of ascorbic acid occurring at Day 4, the onset of ascorbic acid induced browning of (=)-catechin ensued.

Intriguingly, if only sulfur dioxide was added to (+)-catechin in the model wine base, the rate of loss of sulfur dioxide was essentially the same as the blank system. Further, the degree of browning of the sulfur dioxide/(+)-catechin system was negligible, implying that sulfur dioxide alone can inhibit browning of (+)-catechin.

Table 2. Consumption of sulfur dioxide (initial concentration 50 mg/L) during the ascorbic acid (200 mg/L) induced browning of (+)-catechin (100 mg/L)

Sample	Residual SO ₂ after 14 days	%loss of SO ₂ after 14 days
SO ₂	30 mg/L	40%
SO ₂ + catechin	28 mg/L	43%
SO ₂ + catechin + ascorbic acid	0 mg/L	100%

When the concentration of sulfur dioxide was increased to 200 mg/L (mole ratio 3.0:1.0 with ascorbic acid), the ascorbic acid induced browning was inhibited. However, this inhibition was coupled with a rapid loss of sulfur dioxide (73%; Table 3). Again, sulfur dioxide alone inhibited any browning process with the loss of sulfur dioxide being the same as the blank system. When sulfur dioxide was added to the browning ascorbic acid/(+)-catechin system at Day 7, browning ceased but rapid loss of sulfur dioxide ensued (Table 3).

Appendix 3 sets out detailed experimental results for the summary presented here.

These results are inconsistent with the viewpoints expressed in many oenology texts. In fact, these results challenge the long-held view of a chemically symbiotic relationship between ascorbic acid and sulfur dioxide. Our results suggest that the use of ascorbic acid is counterproductive with respect to maintaining appropriate levels of sulfur dioxide. That is, the oxidation of sulfur dioxide is enhanced in the presence of ascorbic acid. The chemical basis for this enhanced loss of sulfur dioxide requires more detailed investigation as it is a project in its own right.

Table 3. Consumption of sulfur dioxide (initial concentration 200 mg/L) during the ascorbic acid (200 mg/L) induced browning of (+)-catechin (100 mg/L).

Sample	Residual SO ₂ after 14 days	%loss of SO ₂ after 14 days
SO ₂	152 mg/L	24%
SO ₂ + catechin	147 mg/L	26%
SO ₂ + catechin + ascorbic acid	54 mg/L	73%
SO ₂ + catechin + ascorbic acid (SO ₂ added at Day 7)	79 mg/L	61%

OXIDATION OF ASCORBIC ACID

It has been noted several times already in this report that the browning of (+)-catechin occurs after the ascorbic acid has degraded. Further, there was sufficient evidence from the early work of this project to implicate a breakdown product of ascorbic acid as a major contributor to the induced browning process. The study summarised in this section of the report was designed to examine the ascorbic acid degradation process and to attempt to identify the oxidation products. Higher concentrations of ascorbic acid were used (up to 1000 mg/L) with the intention of being able to separate, isolate and identify the breakdown products.

Appendix 4 sets out a detailed description of the experiments summarised here.

When the absorbance of a model wine base containing either 500 mg/L or 1000 mg/L ascorbic acid was monitored at 440 nm, there was an initial increase in absorbance followed by a decrease. A rise in absorbance was expected, as dehydroascorbic acid absorbs at 300 nm and, at the concentrations of ascorbic acid used here, some tailing of the absorbance peak for dehydroascorbic acid into the visible region occurred (in fact, a solution of dehydroascorbic acid is pale yellow while that for ascorbic acid at the equivalent concentration is colourless).

Equation 3 above shows that 1 mole of ascorbic acid produces 1 mole of dehydroascorbic acid. If the actual process was as simple as this, then the observed increase in absorbance when solutions containing ascorbic acid are allowed to oxidise should have remained constant rather than decrease. The decrease in absorbance implies that a further reaction must be occurring.

Square wave voltammetry (SWV) was used to follow the decay in the ascorbic acid concentration and to compare its concentration variation with the absorbance data. For the model wine base solutions containing 500 mg/L and 1000 mg/L ascorbic acid, the time to reach maximum absorbance at 440 nm matched the time taken for 95% of the ascorbic acid to be oxidised (4 and 8 days respectively). This in turn implies that the decrease in absorbance must be due to a breakdown of the coloured species formed from ascorbic acid.

That is, two reactions would appear to be taking place

- the oxidation of ascorbic acid to yield coloured species, of which one must be dehydroascorbic acid;
- the breakdown of the coloured species to give less coloured and/or colourless species.

These two reactions occur simultaneously, with the dominant process being determined by the concentration of available ascorbic acid. At higher concentrations of ascorbic acid in the reaction systems, the formation of coloured species dominates and hence an increase in absorbance at 440 nm is observed. However, as the concentration of ascorbic acid falls through oxidation, the second reaction generating the species of less or no colour then dominates leading to a decrease in absorbance.

The reaction processes were monitored by LC/DAD and it became apparent that the changes were highly complex. An analysis of the DAD chromatograms showed the height of some peaks increasing and then decreasing with time and other peaks would appear at different stages over the 14 day reaction period.

Several attempts were made to identify the peaks by LC/MS but without success. The only useful data obtained indicated that the main peaks in the chromatograms had masses less than that for ascorbic acid, suggesting that the peaks were due to degradation products. LC/MS has its limitations when applied to small molecules and unfortunately it did not yield any useful information in this instance. GC/MS was also tried but without success also. To identify the degradation products, it will be necessary to use other techniques more suited to small molecule analysis. This was outside the scope of this project.

ASCORBIC ACID CROSSOVER FROM ANTI-OXIDANT TO PRO-OXIDANT

The final section of this project examined in more detail the effect of ascorbic acid on the browning of (+)-catechin. Concentrations of both ascorbic acid and (+)-catechin were increased for the same reason described in the section above: higher concentrations provide a greater opportunity to isolate and identify the reaction products.

Appendix 5 describes the experiments summarised here in more detail.

Chromatographic analysis using LC/DAD and LC/MS showed that the major brown product formed in the ascorbic acid induced oxidation of (+)-catechin was in fact due to the formation of xanthylum cations. The xanthylum cations result from the bridging of two catechin units by glyoxylic acid. The xanthylum cations obtained in this work were identical in chromatographic and mass spectral analysis to those reported elsewhere from both copper(II) and iron(II) mediated oxidation of (+)-catechin. It is intriguing that these xanthylum cations can be formed in reasonable amounts in the absence of a metal ion. This implies that ascorbic acid itself is able to generate a considerable amount of glyoxylic acid from tartaric acid.

The formation of xanthylum cations involves four steps, two of which involve oxidation:

- Cleavage of tartaric acid to generate glyoxylic acid (oxidation 1)
- Bridging of two (+)-catechin molecules by glyoxylic acid
- Dehydration of the bridged species to give a xanthene
- Conversion of the xanthene to the xanthylum cation (oxidation 2)

Only the xanthylum cation is capable of absorbing around 400 – 450 nm and is therefore the only species formed in this four-step sequence that will contribute to the absorbance at 440 nm, the wavelength used to monitor the browning species. The bridged species and the xanthene can both be detected at 278 nm.

In the section above, it was discussed that as ascorbic acid is oxidised, there is an increase in absorbance at 440 nm. The time of maximum absorbance corresponds to the time required for 95% oxidation of the ascorbic acid. When both ascorbic acid and (+)-catechin were present in the model wine system, an increase in the 440 nm absorbance was observed and after a time dependent on the ascorbic acid concentration, a breakpoint occurred after which the 440 nm absorbance increased at an enhanced rate. Square wave voltammetry confirmed that 95% of the ascorbic acid was oxidised prior to the breakpoint in the absorbance/time plot. Table 4 summarises the relevant observations. Clearly, the loss of ascorbic acid corresponds to the “lag” period discussed earlier in this report

The increase in absorbance observed post the lag period was shown by LC/DAD and LC/MS analyses to be due to the formation of xanthylum cations. Importantly, xanthylum cations were not detected until after the oxidation of ascorbic acid implying that ascorbic acid inhibits their formation. The bridged catechin species and the xanthene were detected during the lag period in the 278 nm chromatogram.

Table 4. Comparison of time required to oxidise ascorbic acid (95%) with the lag period preceding the browning of (+)-catechin

Ascorbic acid (mg/L)	(+)-Catechin (mg/L)	Time to breakpoint in A_{440} plot	Time of oxidation of 95% ascorbic acid	Lag period for (+)-catechin browning
200	100	-	2	1
500	250	4	4	4
1000	500	8	8	7

There is therefore a well-defined sequence of events leading to the ascorbic acid induced browning of (+)-catechin. Step 1 involves

- Oxidation of ascorbic acid to at least 95% of its concentration
- Formation of degradation products (not yet identified) of ascorbic acid
- Formation of glyoxylic acid or a species capable of generating glyoxylic acid
- Formation of the bridged catechin species and the xanthene species.

Step 2 involves the formation of the xanthylum cations that absorb at 440 nm.

The inability to detect any xanthylum cations prior to the decay of ascorbic acid demonstrates the anti-oxidant capacity of ascorbic acid. Ascorbic acid either inhibits the formation of xanthylum cations or, if they are formed, reduces them to the colourless xanthene precursor. Once the ascorbic acid has been oxidised, the accumulation of xanthylum cations is permitted to proceed.

These results clearly show the crossover from anti-oxidant to pro-oxidant by ascorbic acid. While ascorbic acid remains an intact molecule, it clearly acts as an anti-oxidant. However, once fully oxidised, its breakdown products induce oxidation and the term ‘pro-oxidant’ is then appropriate.

These results are significant as they clarify the confusion regarding the anti-oxidant or pro-oxidant behaviour of ascorbic acid. An “oxymoron of antioxidants” is then an apt description for ascorbic acid that, through its own complex chemistry, can play both anti-oxidant and pro-oxidant roles.

WINEMAKING IMPLICATIONS OF THIS WORK

There are significant implications of the work described in this report for white wine production. It is clear from the results of this work that once ascorbic acid is close to fully oxidised, an enhancement of the browning of (+)-catechin, the polyphenolic compound used in this study, occurs.

If ascorbic acid is used in the vinification process, then its concentration must be high enough to ensure that it does not break down over the life of the wine. This concentration must take into consideration all factors that can impinge on the life of the wine. This includes random oxygen access that might occur once the wine is in bottle. Once ascorbic acid has been oxidised, its pro-oxidant role will dominate leading to elevated browning.

The sulfur dioxide regime must also be considered if ascorbic acid is used. Rather than being able to decrease the concentration of sulfur dioxide when ascorbic acid is used, the results of this project clearly show that enhanced consumption of sulfur dioxide occurs as of ascorbic acid is oxidised. That is, the sulfur dioxide concentrations should be increased to two to three times the molar concentration of ascorbic acid to provide additional protection against the onset of ascorbic acid-induced browning.

It must be stressed that ascorbic acid itself is an anti-oxidant and it is only when the ascorbic acid is oxidised that the pro-oxidant effect is observed. Thus, if ideal conditions can be maintained so that there is no oxygen ingress to the bottled wine, then the ascorbic acid will retain its anti-oxidant status.

On the other hand, as sulfur dioxide alone is capable of minimising any browning of (+)-catechin even in highly oxidising conditions, it becomes necessary to question what value can be gained from the addition of ascorbic acid.