Ascorbic Acid-Induced Browning of (+)-Catechin In a Model Wine System

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The ability of ascorbic acid to induce browning of (+)-catechin in a model wine system has been studied. A significant increase in absorbance at 440 nm was observed over 14 days when ascorbic acid was incubated at 45 °C with (+)-catechin in a model wine base. The onset of browning was delayed for about 2 days, although the length of the lag period was dependent on the amount of molecular oxygen in the headspace of the reaction system. The lag period was not observed when a preoxidized solution of ascorbic acid was used, suggesting that a product of ascorbic acid oxidation is responsible for the onset of browning. Hydrogen peroxide, when added directly to (+)-catechin in the model system, was not capable of producing the same degree of browning as that generated by ascorbic acid. Liquid chromotography evidence is presented to show that different reaction products are produced by ascorbic acid and hydrogen peroxide.

Keywords: *(+)-Catechin; ascorbic acid; hydrogen peroxide; browning; model wine system; winemaking*

INTRODUCTION

Ascorbic acid has been widely used in winemaking, especially white wine production, for more than 25 years. Its use remains high in some countries (eg., Australia), whereas in Europe and the US a significant decline in its application has occurred. The basis for the application of ascorbic acid in winemaking lies in its ability to scavenge molecular oxygen (*1*−*4*). This antioxidant capacity was seen to be advantageous as it has been argued that ascorbic acid minimizes the oxidative spoilage (browning) of white wine (*2*−*4*). Browning is a quality defect in wine as it can affect the aesthetic appeal and have a negative influence on the wine's flavor and nose.

Browning reactions can be either enzymic or nonenzymic. The enzymic process is a consequence of the oxidative reaction of an *ortho*-dihydroxyphenolic compound to an *ortho*-quinone which can undergo further reaction to form brown products. This process is catalyzed by the polyphenoloxidase enzyme, tyrosinase (*4* and *5*). Ascorbic acid is capable of reducing the *ortho*-quinone to the original phenolic compound (*2*, *6*−*8*). Nonenzymic browning of fruits and vegetables has generally been attributed to the Maillard process (*5*, *9*, *10*), although the full significance of this process for wine browning is not clear (*11*). It has been argued that nonenzymic browning of wine can occur through direct aerobic oxidation of the polyphenolic compound (*12*) with the possible involvement of metal ions such as copper, iron, tin, and aluminum (*8* and *12*).

Ascorbic acid additions in white wine production were seen to have the beneficial role (*1*, *2*, *4*) of molecular oxygen scavenging with the consequent advantage of a reduction in the amount of sulfur dioxide, the latter being not particularly efficient as an oxygen scavenger (*6* and *12*). On the other hand, it has always been argued that some sulfur dioxide is necessary in combination with ascorbic acid as the oxidation of the latter produces dehydroascorbic acid and hydrogen peroxide (*1*, *2*, *6*, *7*, *13*−*15*). The oxidative capacity of hydrogen peroxide is well established, but it is readily removed by reaction with the added sulfur dioxide (*3*, *4*, *16*).

There is growing evidence that ascorbic acid may have a pro-oxidant role, rather than antioxidant, under some situations (*14* and *17*). In white wine, Peng et al. (*2* and *3*) have reported that ascorbic acid led to elevated levels of browning in wines which had been bottled with molecular oxygen in the headspace. Sulfur dioxide, when added in combination with ascorbic acid, did not minimize the degree of browning (*18*). There is anecdotal evidence that, in the US and Europe, the application of ascorbic acid as an "oxygen mop" has decreased markedly, although it is used in wines from Germany which show "untypical aging" (*19*).

The chemistry of ascorbic acid and its breakdown products in wine is poorly understood. Much of the knowledge about ascorbic acid has been generated through studies in the absence of a solvent, or in neutral or alkaline conditions (*20*−*23*). Extensive studies on beer oxidation have also questioned the proposed antioxidant status of ascorbic acid (*7* and *13*). Although the first step in the aerobic oxidation of ascorbic acid, to produce dehydroascorbic acid and hydrogen peroxide, is well established, there is now considerable evidence to suggest that the reaction sequence continues. Jung and Wells (*24*) have shown that dehydroascorbic acid spontaneously decomposed at neutral to high pH values to 3 products, one of which was ascorbic acid. Deutsch (*25*) has demonstrated interconversion reactions in the ascorbic acid/dehydroascorbic acid system and Niemela (*21*) has reported the identification of over 50 products

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from the aerobic oxidation of ascorbic acid under alkaline conditions.

White wine is a highly complex matrix with a range of potentially oxidizable compounds of which the flavonoids (eg., dihydroxyphenolic compounds) and nonflavonoids (eg., caffeic acid) can lead to brown products (*12*). Given the complexities of both the wine matrix and the ascorbic acid reactivity, it was decided to use a model wine in this study to ensure better control of the variables in the system. The model consisted of an artificial wine base of known ethanol and tartaric acid concentrations at pH 3.2. Catechin was used as the oxidizable substrate as this is the dihydroxyphenolic compound which is best correlated with white wine browning (*26*).

This well-defined model system has allowed the identification of the critical role played by molecular oxygen and hydrogen peroxide in the browning process. These results are reported here along with a liquid chromatography (LC) study of the browning reactions which has allowed considerable insight into the chemistry of the ascorbic acid/wine matrix system.

MATERIALS AND METHODS

Reagents. Grade 1 water (ISO3696) purified through a Milli-Q (Millipore, Bedford, MA) water system was used for all solution preparation and dilutions. (+)-Catechin and potassium hydrogen tartrate were purchased from Sigma (St. Louis, MO). Ascorbic acid was obtained from Prolabo. Nitric acid (AR grade, 70%) and hydrogen peroxide (AR grade, 100 volume) were purchased from Univar, and potassium hydroxide (AR grade) came from BDH. Glacial acetic acid (Ajax Univar) and methanol (Ajax Unichrom) were used without further purification.

Model Wine Base. The model wine base was prepared by dissolving 2.11 g of potassium hydrogen tartrate and 0.806 g of potassium hydroxide in 1 L of 12% (v/v) aqueous ethanol, resulting in a total tartrate concentration of 0.011 M and a total potassium concentration of 0.026 M (1 g/L). The pH of the model wine base was adjusted to 3.2 with 10% (v/v) sulfuric acid.

Browning Reactions. (+)-Catechin (100 mg/L; 3.4×10^{-3} M) and ascorbic acid (200 mg/L; 1.1×10^{-3} M) were dissolved with stirring in 100 mL of model wine base in a 250-mL Schott bottle. Until required for analysis, the samples were maintained in the dark in a water bath at 45 °C. Flasks were exposed to the air on a daily basis to replenish the molecular oxygen content.

In experiments with hydrogen peroxide, appropriate volumes of a working solution of hydrogen peroxide (0.15%) were added to the (+)-catechin/ascorbic acid system in model wine base as required.

Headspace Experiments. Wine base (64.3 mL) was placed in each of three 100-mL Schott bottles, and glass beads (of known volume) were added to generate headspace volumes of 1, 3, and 5 mL. Another, larger (250-mL), headspace-volume was investigated by using the same volume of wine base (64.3 mL) in a "250-mL" Schott bottle. A constant volume of solution was found to be critical in yielding reproducible results. Browning reactions were carried out as described above.

Absorbance Measurements. Absorbance measurements at 440 nm using a Unicam 8625 UV−visible spectrometer were recorded daily for 14 days. Quartz cells (10 mm or 2 mm) were used. The model wine base (unheated) was used as the blank.

Analytical HPLC Analyses. HPLC analyses were performed using a Waters 2690 separation module, a Waters 996 photodiode array detector, and Millenium 32 chromatography manager software. The column was a SGE Wakosil C18RS glass-lined column (250 × 2 mm) with 5-μm particle diameter. Elution conditions were 0.15 mL/min flow rate; Solvent A, 0.5% (v/v) acetic acid/water; Solvent B, 0.5% (v/v) acetic acid/methanol. The gradient varied from 100% A to 100% B over a 112 min period, followed by an 8 min flush of 100% A to clean the column (*27*).

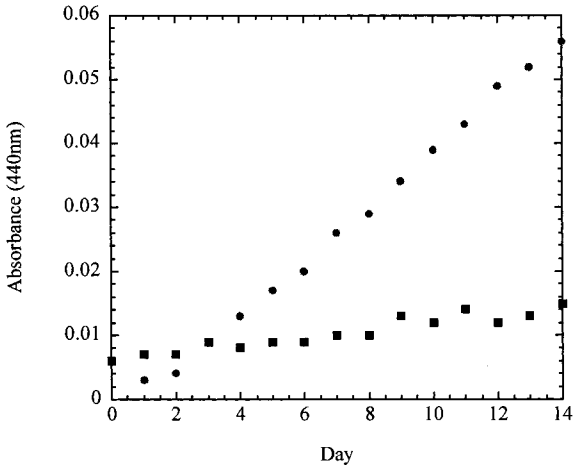

Figure 1. Ascorbic acid effect on the browning of catechin in a model wine base:. ■, catechin; ●, catechin plus ascorbic acid (see text for concentrations).

RESULTS

Traditionally, wine browning measurements are recorded at 420 nm (*11* and *28*). In the catechin/wine base model, the onset of the browning reaction was coupled with an increase in absorbance in the 400−450 nm region, with the maximum absorbance occurring at 440 nm. Consequently, all absorbance values in this work were recorded at this wavelength.

The increase in absorbance at 440 nm of catechin in model wine base solutions in the presence and absence of ascorbic acid is shown in Figure 1. Clearly, the inclusion of ascorbic acid produces a marked enhancement in the extent of browning. After 14 days, the absorbance of the ascorbic acid containing catechin/wine base sample was found to be more than three times that of the sample to which ascorbic acid had not been added. For 10 repeat experiments, the RSD of the Day 14 absorbance values was 5.4%, demonstrating the reproducibility of the experimental procedure.

It is also apparent in Figure 1 that, when ascorbic acid is present, the commencement of browning (as reflected by an increase in absorbance) is preceded by a decrease in absorbance. The nonascorbic-acid-containing solution does not show this decrease. The period prior to the absorbance increase was termed the "lag period" and generally lasted between 1 and 2 days.

To investigate the basis for this lag period, catechin was added to a sample containing preoxidized ascorbic acid, achieved by maintaining the ascorbic acid/model wine base solution at 45 °C for 2 days prior to the addition of catechin. The rate of browning was compared to a catechin/model wine base system containing fresh ascorbic acid. Figure 2 shows that for the sample containing preoxidized ascorbic acid, browning commences immediately after mixing with catechin. That is, there is no lag period as is observed when fresh ascorbic acid is used. On the other hand, the rates of browning are very similar when the slopes of the absorbance/time plots in Figure 2 post Day 2 are compared.

The headspace volume, and hence the amount of available molecular oxygen, also influences the lag period. Changes in absorbance for solutions with 1, 3,

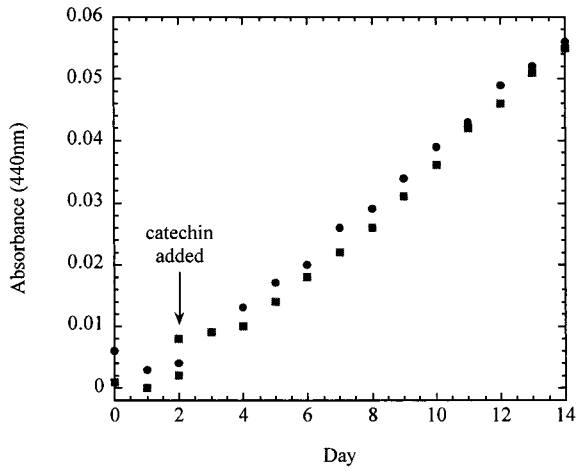

Figure 2. Comparison of catechin browning rates: ■, pre-oxidized ascorbic acid; ●, fresh ascorbic acid.

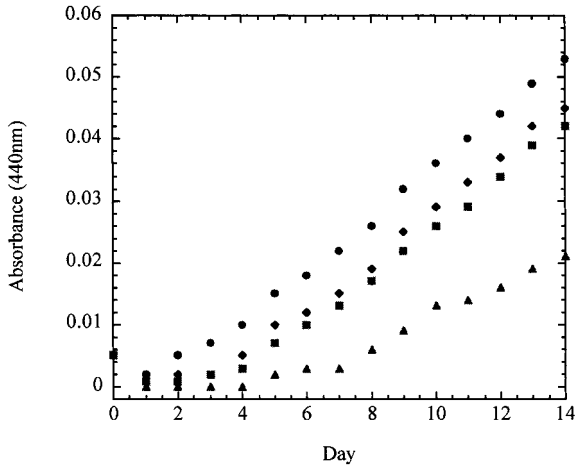

Figure 3. Influence of headspace volume on browning of catechin: ▲, 1 mL; ■, 3 mL; ◆, 5 mL; ●, 250 mL.

Table 1. Influence of Headspace Volume on the Lag Period, Maximum Absorbance, and Rate of Browning

headspace (mL)	lag period (days)	A_{440} at day 14	rate of browning[a]
1	7	0.021	0.00253
3	4	0.042	0.00396
5	3	0.044	0.00401
250	1	0.053	0.00405

[a] Rate of browning is calculated as the increase in absorbance versus time from the end of the lag period to day 14.

5, and 250 mL headspace volumes are shown in Figure 3. Table 1 compares the lag period, the absorbance after 14 days, and the rate of increase in browning (as the slope of the absorbance versus time plot) from the end of the lag period to day 14. Increasing the headspace volume shortens the lag period, with a consequent higher absorbance at day 14 (Table 1). The rate of browning, calculated as the change in absorbance as a function of time after the lag period, is similar for the 3-, 5-, and 250-mL headspace volume experiments. The rate for the 1-mL headspace is only about 60% of that for the 250-mL headspace.

The critical role of molecular oxygen in the initiation of the browning process is confirmed in Figure 4. Two solutions containing equal concentrations of catechin (100 mg/L) and ascorbic acid (200 mg/L) were prepared in the model wine base. One solution was aerated daily. However, the other was aerated daily, only until day 7,

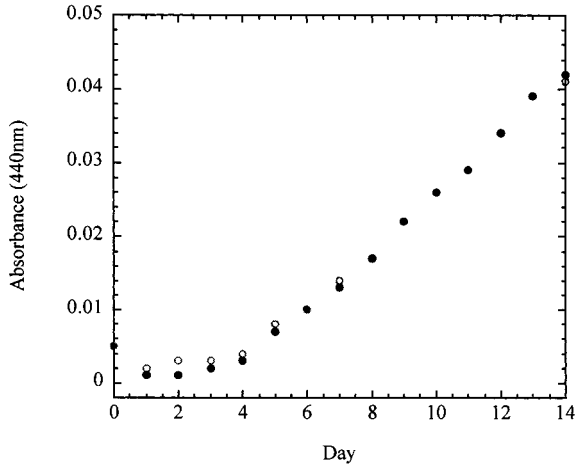

Figure 4. Influence of oxygen exclusion after the lag period on the catechin browning rate: ●, with daily aeration; ○, no aeration after day 7.

and then it was sealed until day 14. Obviously, only one further browning measurement could be taken for this latter sample. The two plots in Figure 4 are very similar, with the gradients of the absorbance versus time curves from day 7 to day 14 being 0.0040 (daily aeration) and 0.0037 (no aeration post day 7).

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. The decay of ascorbic acid in the model wine base over a 72-h period is evident from the decrease in absorbance as shown in Figure 5. Intriguingly, the increase in absorbance at 300 nm is greater than expected for the equimolar conversion of ascorbic acid to dehydroascorbic acid. The initial concentration of ascorbic acid in this experiment was 200 mg/L (1.1×10^{-3} M). Given that the molar absorptivity of dehydroascorbic acid is 720 cm^{-1} M^{-1} (*17*), the expected maximum absorbance at 300 nm using a 2-mm cell is 0.16 for complete conversion of ascorbic acid to dehydroascorbic acid. The absorbance at 300 nm after 72 h is 0.405, and this higher-than-expected value suggests that the dehydroascorbic acid is further reacting in the model wine base. The absence of an isosbestic point in Figure 5 is further evidence for a more complex reaction sequence than the production of an equimolar amount of dehydroascorbic acid from ascorbic acid.

Hydrogen peroxide is also produced in the aerobic oxidation of ascorbic acid. Addition of hydrogen peroxide (3×10^{-4}%; 9.7×10^{-5} M) to a catechin/model wine base solution did increase the extent of browning to some extent, but the increase was significantly less than that produced by ascorbic acid itself. Figure 6 shows that after 14 days, ascorbic acid increased the absorbance of the catechin/wine base solution to 0.056, whereas hydrogen peroxide brought about an increase to 0.024, compared to an increase to 0.015 for catechin alone.

Further evidence that ascorbic acid produces a reaction sequence different from that of hydrogen peroxide is obtained from the liquid chromatograms of the various reaction systems after a 14-day reaction period. Figure 7 presents the day 14 liquid chromatogram for catechin in wine base and this is compared with that for catechin plus hydrogen peroxide and catechin plus ascorbic acid. The day 14 chromatogram for ascorbic acid in wine base is also shown. It is obvious from these

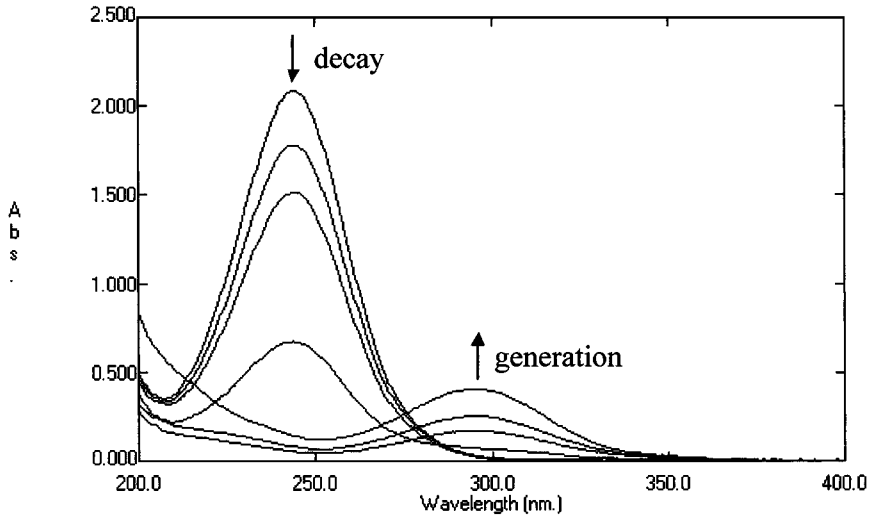

Figure 5. UV spectrum of ascorbic acid in model wine base over a 72-h period showing the decrease in ascorbic acid absorbance at 245 nm and the consequent increase in absorbance at 300 nm.

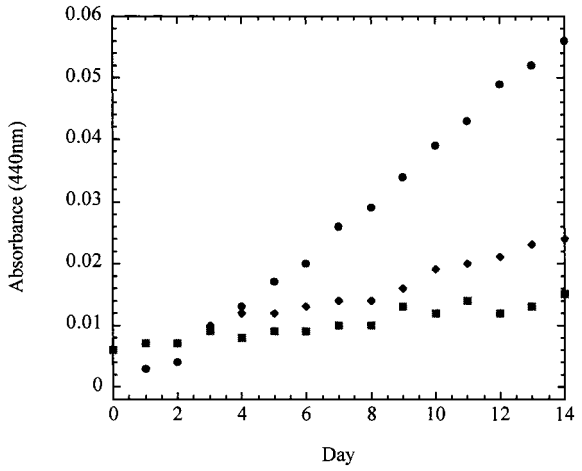

Figure 6. Hydrogen peroxide effect on the catechin browning rate in model wine base: ■, catechin; ◆, catechin plus hydrogen peroxide; ●, catechin plus ascorbic acid.

chromatograms that ascorbic acid is producing products different from those generated by hydrogen peroxide. The chromatograms for catechin and catechin plus hydrogen peroxide are very similar except that the absorbance values for the solution containing hydrogen peroxide are higher, as expected (see Figure 6). The chromatogram for the catechin plus ascorbic acid solution has an enhanced background absorbance, as well as three clearly defined peaks with retention times of 56, 81.5, and 88 min. Better definition of some of the other peaks is also apparent in the solution of ascorbic acid/catechin, presumably because of the higher overall absorbance.

DISCUSSION

The aerobic oxidation of ascorbic acid (AH_2) can be represented by the equation

$$AH_2 + O_2 \rightarrow A + H_2O_2$$

where A represents dehydroascorbic acid. At the pH of wine and in the model used in this study (pH 3.2), some 92% of the ascorbic acid exists as the neutral acid (*17*).

For the above reaction to occur, it is essential that the oxygen molecule is first converted from its triplet ground state to a singlet state. Presumably, this occurs at the elevated temperature of 45 °C used in this study. The presence of trace concentrations of metal ion catalysts cannot be discounted (*17*) even though all flasks were thoroughly cleaned prior to use. Irrespective, it is clear from Figure 3 that a sufficient quantity of molecular oxygen is required to initiate the browning reaction. At the low headspace volume of 1 mL, a long period was required before noticeable browning occurred, and the extent of browning after 14 days was minimal.

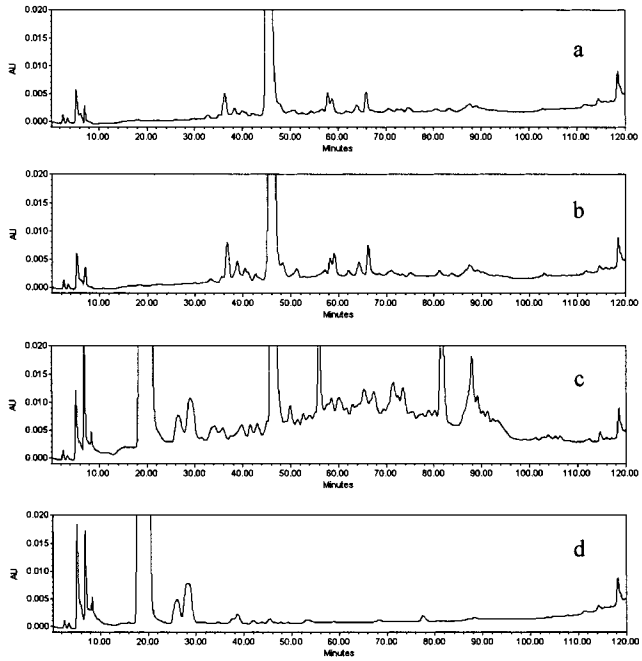

Figure 7. Liquid chromatograms (Day 14) for catechin in model wine base (detection at 278 nm): a, catechin (100 mg/L); b, catechin + hydrogen peroxide (9.7×10^{-5} M); c, catechin + ascorbic acid (200 mg/L); d, ascorbic acid (200 mg/L).

The lag period prior to the onset of browning decreases as the headspace volume was increased. Further, the rate of browning was essentially the same in the time interval from the end of the lag period to day 14 as shown by the slopes listed in Table 1. This observation suggests that molecular oxygen is required only to initiate the reaction. Confirmation is obtained

from the data in Figure 4 where the browning rate with oxygen exclusion parallels that for a daily aerated sample.

Use of a pre-oxidized acid solution (Figure 2) did not generate a lag period as was observed in the browning of a catechin in the presence of freshly dissolved ascorbic acid (Figures 1 and 2). As the length of this lag period is dependent on the amount of molecular oxygen initially present (Figure 3), it would appear that an oxidation product of ascorbic acid is intimately involved in the catechin browning process.

Hydrogen peroxide is frequently regarded as the critical component in any pro-oxidative reaction based on ascorbic acid. However, when added to a catechin solution in a model wine base, only a small increase in the rate of browning compared to catechin itself was observed (Figure 6). The extent of hydrogen-peroxide-induced browning is much less than that produced by ascorbic acid.

In a separate study using square wave voltammetry (*29*), we have shown that a solution containing 200 mg/L (1.1×10^{-3}M) ascorbic acid in the model wine base generated a maximum hydrogen peroxide concentration of 9.4×10^{-5} M. The maximum concentration was achieved after a 12-h reaction period. This amount is approximately 20% of the concentration expected for its 1:1 production from ascorbic acid.

The maximum observed concentration of hydrogen peroxide was used to generate the data in Figure 6. Although no lag period was observed (cf. Figure 1), the inability of the added hydrogen peroxide to generate the same level of browning as ascorbic acid questions the direct involvement of hydrogen peroxide in the browning of catechin. Rather, an alternative pathway would appear to be responsible for the ascorbic acid induced browning.

Dehydroascorbic acid is the other initial product in the aerobic oxidation of ascorbic acid, and it is now recognized as being capable of further interconversion processes (*24* and *25*). Niemela (*21*) has demonstrated that the aerobic oxidation of ascorbic acid, in alkaline conditions, can lead to the formation of up to 7 major products, with more than 50 compounds being observed in total.

Little is known about the reactivity of ascorbic acid at the pH of wine and in the presence of wine components such as ethanol and tartaric acid. The UV spectral study shown in Figure 5 demonstrates that the oxidation of ascorbic acid leads to the production of more than dehydroascorbic acid. The continuing increase in absorbance at 300 nm, significantly higher than that expected for a 1:1 conversion of ascorbic acid to dehydroascorbic acid, suggests the further reaction of dehydroascorbic acid to other products. Oxalic acid and threonic acid have been proposed as the major end products from the breakdown of dehydroascorbic acid (*6*, *20*, *21*). However, it is difficult to see how these two compounds can be directly involved in the browning reaction of catechin.

Further evidence for the different reactions induced by hydrogen peroxide and ascorbic acid can be seen in the liquid chromatograms shown in Figure 7. The chromatogram of the hydrogen peroxide-containing solution (Figure 7b) is similar to that of the solution containing only catechin (Figure 7a). The unique peaks at 56, 81.5, and 88 min in the chromatogram of the ascorbic acid/catechin solution (Figure 7c) clearly highlight the differences between the browning processes involving hydrogen peroxide and those involving ascorbic acid.

Our studies are continuing in an attempt to determine the identity of the products formed in both reaction systems. Recently, the formation of glyoxylic acid-bridged catechins and subsequent generations of xanthylium skeletons have been described (*30*−*32*), with iron(III) being used as the mediator in the oxidation of tartaric acid to produce the reactive glyoxylic acid. Whether a similar process occurs with ascorbic acid remains to be determined.

This work has demonstrated that ascorbic acid has a clear role in inducing 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. Whether the products of browning are due to the oxidation of catechin or its polymerization remains to be resolved. This work also questions the role of ascorbic acid in winemaking. Any ingress of molecular oxygen during the process, particularly at bottling, could induce the types of browning processes observed in this study.

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