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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.

Keywords: ascorbic acid; (+)-catechin; xanthylium pigments; model white wine; oxidation; browning; liquid chromatography; diode array detector; mass spectrometry.

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-visible 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 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 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 500mg 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 two minute stirring on a daily basis to replenish the molecular oxygen content in the samples.

2.2.4. 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 (250×4 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 minutes, down to 20 % in 5 minutes and to 0 % in 10 minutes; after 10 minutes at 0 %, up to 95 % in 5 minutes followed by 15 minutes at 95 %. The flow rate was 0.8 mL min^{-1}. Chromatograms and UV-visible 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 minutes, down to 50 % in 15 minutes, then to 20 % in 5 minutes and to 0 % in 15 minutes; after 30 minutes at 0 %,

up to 95 % in 10 minutes followed by 10 minutes at 95 %. Flow from the column was split so that it decreased from 0.8 mL min^{-1} 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-visible spectrum of each sample was recorded every second day for the first 12 days of the experiment and every four days for the 12 remaining days. UV-visible 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 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 four 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-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-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-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-visible 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-visible spectra indicated strong similarities between peaks 1-4 (Fig. 5A-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-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-B) because of the high intensity of peak 6 in the reference solution (*i.e.* 100 times more intense than peaks 6a and 6b).

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-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 min and 75.50 min respectively. Peaks 8-10 (Fig. 6A-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-visible spectra and MS data to the glyoxylic acid-derived xanthylium cations (data not shown). The final 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.

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Legends

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

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

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

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 9 measurements (3 measurements for each of the 3 replicated samples). Error bars represent the 95 % confidence limit (P = 0.05) calculated from the 9 values measured for each model system.

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**).

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.

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 3 replicates. Error bars represent the 95 % confidence limit (P = 0.05) calculated from the 3 values measured for each model system.