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Reaction of Acetaldehyde with Wine Flavonoids in the Presence of Sulfur Dioxide

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ABSTRACT: Acetaldehyde is responsible for many of the beneficial changes that occur in red wine as a result of oxidation. Ethylidene bridges are formed between flavonoids upon their reaction with acetaldehyde, which can contribute to improvements in color stability and SO_2-resistant pigments. In the present study, the reactions between acetaldehyde and various flavonoids (catechin, tannins from grape seed extract, and malvidin-3-glucoside) were examined in a model wine system. Lower pH conditions were seen to significantly increase the rate of reaction with acetaldehyde, whereas dissolved oxygen did not affect the rate. In systems containing SO_2, the rate of reaction of acetaldehyde with catechin was slowed but was not prevented until SO_2 was in great excess. Significant improvements in color stability were also observed after treatment with acetaldehyde, despite the presence of equimolar SO_2. These results demonstrate that acetaldehyde is reactive in its sulfonate form, which is contrary to widely held assumptions. In addition, the products of the reaction of flavonoids with acetaldehyde were characterized using MALDI-TOF MS in this study. Ethyl-bridged catechin nonamers were observed, as well as anthocyanin and pyranoanthocyanin derivatives of catechin and tannin oligomers. The results of this work illustrate the significance of acetaldehyde reactions in forming stable pigments in wine and the reactivity of acetaldehyde from its sulfonate form.

KEYWORDS: *wine oxidation, flavonoid, acetaldehyde, sulfur dioxide, anthocyanin, tannin*

INTRODUCTION

Flavonoids in red wine are directly related to many important indicators of wine quality. Changes in these compounds during wine production are also critical to improving quality parameters including mouthfeel and color stability.[1] Condensed tannins and anthocyanins have been shown to take part in reactions that lead to decreases in astringency and the formation of stable, polymeric pigments.[1,2] Many of the flavonoid modifications that occur in red wine are due to oxidation, wherein condensation reactions bind molecules together. Acetaldehyde, itself a major product of wine oxidation, drives some of the beneficial reactions of flavonoids by forming ethyl-bridged condensation products.[3] Oxygen is reduced by several metal-catalyzed steps leading to the formation of reactive oxygen species (e.g., hydroxyl radicals) that oxidize ethanol to acetaldehyde;[3−5] however, this process yields reactive intermediates, such as *o*-quinones and 1-hydroxyethyl radicals, that can lead to deleterious effects with respect to wine aroma and color.[5−7] Yeast metabolism also produces acetaldehyde as a side product of the alcoholic fermentation.[8]

Recent studies in our group and elsewhere have shown that exogenous acetaldehyde can be used to improve red wine color stability and astringency.[9−12] The use of exogenous acetaldehyde provides benefits for the wine without the risks of reactive intermediates formed due to oxygen exposure. Adducts formed by acetaldehyde are found to be bridged by an ethylidene moiety (Figure 1),[13,14] which can occur via C6 or C8 on the A-ring of the flavonoid molecule.[15−17] Ethylidene-bridging reactions can involve tannins and anthocyanins to form modified tannins as well as polymeric pigments.[13,17] Bridged products from reaction with acetaldehyde have been found in red wine and in model wine systems,[14,18−21] and evidence for ethylidene-bridged products of native tannins has been observed indirectly by phloroglucinolysis;[22] however, these compounds are difficult to observe without fragmentation during ionization for MS.

Another important fate of acetaldehyde under wine conditions is its reaction with bisulfite ions (Figure 1). Sulfur dioxide (SO_2) is added to wine to prevent faults due to chemical and microbial instability. Bisulfite, the predominant form of SO_2 at wine pH, ensures the chemical stability of wine by inhibiting oxidation reactions, specifically by reacting with hydrogen peroxide and quinones.[23−25] A major role that bisulfite plays in preserving wine quality is its binding of aldehydes, especially acetaldehyde, as it is typically the most abundant carbonyl.[26] This reaction is known to be fast (98% bound in 90 min at pH 3.3), and the resulting adduct is strongly bound ($K_d = 2.06 \times 10^{-6}$ at pH 3.5).[27−29] Due to the preference for the formation of the acetaldehyde−bisulfite adduct, 1-hydroxyethanesulfonate, previous studies have largely discounted the possibility that acetaldehyde remains reactive in the presence of SO_2.[23,30,31] However, there has been evidence for the antioxidant activity of this sulfonate in beer, as ethanol oxidation was inhibited by the sulfonate was observed to possess activity similar to SO_2 itself.[32]

The present work aims to characterize the reactions of acetaldehyde with flavonoids in a model wine system using wine-relevant concentrations of acetaldehyde, model flavanols,

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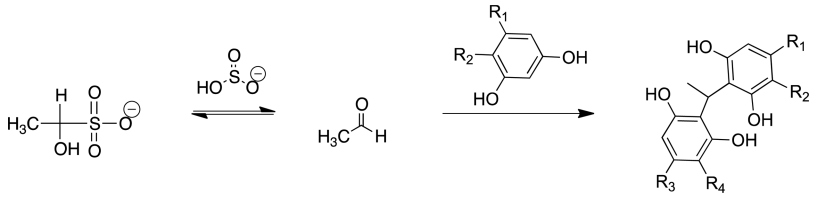

Figure 1. Reaction of acetaldehyde with bisulfite and with representative flavonoids to form an ethylidene-bridged adduct.

and anthocyanins. For the first time, the activity of acetaldehyde in bridging flavonoids was assessed in the presence of equimolar levels of bisulfite and under conditions in which acetaldehyde had been assumed to be present in its inert, bound sulfonate.

MATERIALS AND METHODS

Materials. (+)-Catechin hydrate and sodium metabisulfite were purchased from Sigma-Aldrich (St. Louis, MO, USA). Malvidin-3-glucoside (M3G) was purchased from Extraynthese (Genay, France). Grape seed extract (GSE; total phenolic content >85% gallic acid equivalents) was kindly donated from San Joaquin Valley Concentrates (Fresno, CA, USA). Potassium metabisulfite, acetaldehyde, and tartaric acid were purchased from Alfa Aesar (Ward Hill, MA, USA). Sodium iodide and LC-MS grade formic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetone, sodium hydroxide (10.00 N), 200 proof ethanol, 2,5-dihydroxybenzoic acid (DHB), and HPLC grade methanol were purchased from VWR International (Radnor, PA, USA). Glacial acetic acid was purchased from J. T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid and phosphoric acid were purchased from EMD Chemicals (Gibbstown, NJ, USA). Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA, USA) purification train.

Reaction Mixture Preparation. Model wine was composed of 5% w/v acetic acid and 12% ethanol v/v, and the pH was adjusted using 10 N NaOH. Preliminary experiments showed that there was no effect of organic acid (tartaric acid vs acetic acid) on the reaction of acetaldehyde with catechin (data not shown). Acetic acid was therefore used for all experiments described here to simplify the preparation of samples for MALDI-TOF MS, as it is volatile and easily removed.

Model solutions were prepared with 20 mg/L acetaldehyde (0.45 mM) added from a stock solution prepared in chilled model wine, 1000 mg/L (+)-catechin or GSE, and 500 mg/L M3G when needed. M3G was used as a model pigment as it is the dominant anthocyanin in red wine.[17,33] SO_2 was added from a freshly prepared stock solution of sodium metabisulfite. Concentrations of SO_2 in each sample were confirmed using an enzymatic assay for total SO_2 (Megazyme, Chicago, IL, USA). All experiments were prepared and analyzed in triplicate. Glassware was soaked in 5% nitric acid overnight and rinsed to minimize trace metals before use. Each reaction mixture was separated into 1 mL aliquots in vials with minimal headspace and stored at room temperature. Samples at each time point were normalized to controls for each mixture prepared without the addition of acetaldehyde. Samples representing individual experimental time points were stored at −80 °C and thawed immediately prior to analysis.

All samples, except those specified as aerobic, were stored in the absence of oxygen in an anaerobic chamber (Anaerobe Systems, Morgan Hill, CA, USA) with 95% argon/5% hydrogen gas and a palladium catalyst to remove residual oxygen. The anaerobic status of the chamber was confirmed using a PreSens oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany). All solutions were deoxygenated using argon gas before being introduced into the chamber, and all materials were left in the anaerobic chamber overnight prior to starting experiments.

Flavonoid Analysis. Flavonoid concentrations were determined by HPLC using a Shimadzu system with 10ADvp pumps and a SIL-20AC HT temperature-controlled autosampler (4 °C). Separation was achieved on a reverse phase Supelcosil LC-18 column (4.6 mm × 150 mm, 5 μm; Supelco, Inc., Bellefonte, PA, USA). Samples were filtered through 0.45 μm PTFE syringe filters prior to analysis. The injection volume was 10 μL, the column temperature was 30 °C, and the flow rate was held at 1 mL/min for all samples. Concentrations were calculated on the basis ofexternal calibration curves for the analytes, (+)-catechin and M3G.

For catechin analysis, the mobile phase consisted of 1% phosphoric acid in water (A) and methanol (B). Catechin was eluted using an isocratic method of 20% B and detected at 280 nm.

For M3G analysis, the mobile phase consisted of 5% formic acid in water (A) and acetonitrile (B). M3G was eluted according to the following binary gradient: 0 min, 10% B; 14 min, 65% B; 14−16 min, 65% B, followed by reequilibration at 10% B prior to the next injection. In samples containing GSE and M3G, the gradient was modified: 0 min, 10% B; 14 min, 65% B; 16 min, 90% B; 16−18 min, 90% B, followed by reequilibration at 10% B prior to the next injection. M3G was detected at 520 nm.

Pigment Analysis. Samples containing M3G were analyzed using a modified Somers assay to characterize pigment changes. The modified Somers assay was performed using a high-throughput procedure,[34] wherein solutions were incubated in 1.5 mL capacity microcentrifuge tubes before being transferred to 96-well plates (Greiner Bio One UV-Star, Monroe, NC, USA) for absorbance readings at 280, 420, and 520 nm using a Multiskan GO microplate reader (Thermo-Scientific, Waltham, MA, USA). All Somers color parameters were calculated as previously reported. Model wine samples were analyzed in duplicate.

Reaction Product Characterization. Samples for MALDI-TOF were prepared in acetic acid model wine with 200 mg/L acetaldehyde. After 4 weeks, aliquots of samples were dried under nitrogen and redissolved in methanol to achieve 10 mg/mL catechin or GSE. Samples were spotted from a 2,5-dihydroxybenzoic acid (DHB) matrix solution. DHB was recrystallized prior to sample preparation. Sample (1 μL, 10 mg/mL in methanol), sodium iodide (1 μL, 10 mg/mL in water), and DHB (10 μL, 200 mg/mL in acetone) were mixed, and 0.5 μL was spotted on a polished stainless steel plate (Bruker Corp, Billerica, MA, USA) for analysis.[35] MALDI-TOF mass spectra were collected in positive reflectron mode using a Bruker ultrafleXtreme instrument. Laser intensity was adjusted to optimize the resolution and signal for each spectrum. MALDI spectra were externally calibrated and acquired as an average of 1000 shots collected in random walk mode from m/z 500 to 4000.

Statistical Analysis. Two-way ANOVA analysis was paired with Tukey's test for determining significance between samples. For all samples, the amount of catechin consumed at the final time point was used for the comparison. Differences of $p < 0.05$ were considered significant. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Reaction of Acetaldehyde with Flavanols. Catechin concentrations were monitored over time to examine the effect of several matrix parameters on its reaction with acetaldehyde. As has been shown by other researchers,[36] there is a significant effect of pH on the rate of consumption of catechin by acetaldehyde (Figure 2). The greatest degree of monomeric catechin consumption after 14 days was observed at pH 2.0, whereas little to no catechin reacted at pH 3.5 during this time;

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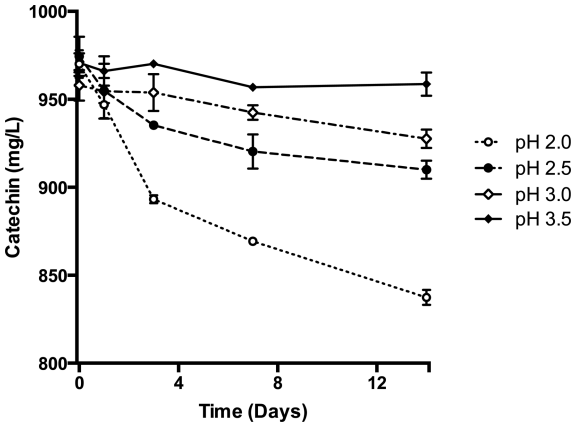

Figure 2. Catechin concentrations in model wine after treatment with 20 mg/L acetaldehyde at pH 2.0, 2.5, 3.0, and 3.5 as determined by HPLC-DAD.

however, it is likely, on the basis of previous studies, that catechin would have reacted by the same mechanism at pH 3.5, albeit at a slower rate, given sufficient time. As acetaldehyde protonation is the first step in this reaction, the overall reaction rate has been shown to increase as the pH decreases.[14] Our results, along with others, show that lower pH leads to an increased rate of reaction of acetaldehyde with flavonoids in real and model wine systems. Therefore, in the interest of expediency, the following experiments described were performed at pH 2.5 so that an acceptable rate of catechin consumption could be achieved within a reasonable time frame.

To confirm that catechin consumption was due to its reaction with exogenous acetaldehyde and not by other mechanisms (e.g., oxidation), catechin reactions were monitored under aerobic and anaerobic (<20 μg/L O_2) conditions. As such, no significant difference between catechin consumption rates was observed under aerobic versus anaerobic conditions (Figure 3), which confirms that the mechanism of

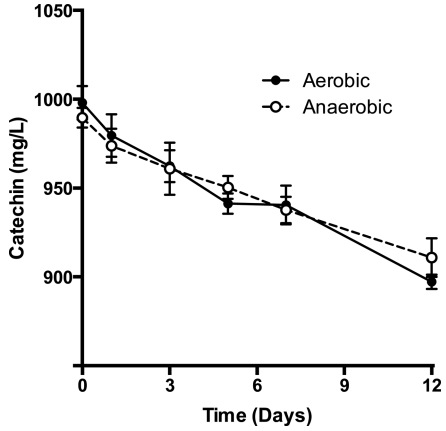

Figure 3. Catechin concentrations in model wine after treatment with 20 mg/L acetaldehyde with and without oxygen present at pH 2.5.

reaction with acetaldehyde does not involve oxygen as a reactant. These results also confirm that the consumption of catechin observed is not due to the formation of endogenous acetaldehyde or direct oxidation of catechin, as these would require oxygen as a reactant.

SO_2 was examined for its effect on the reaction of acetaldehyde with flavonoids due to its important role in the formation and fate of acetaldehyde. SO_2 is known to inhibit the metal-catalyzed oxidation of ethanol to acetaldehyde.[3,23] Bisulfite reacts swiftly with hydrogen peroxide, a reactive oxygen species intermediate formed during wine oxidation under wine conditions, as well as *o*-quinones that are formed during phenolic oxidation.[37−39] Due to the fact that bisulfite is highly reactive toward these species (hydrogen peroxide in particular), acetaldehyde is not expected to be generated in wine if SO_2 is present in excess. Bisulfite is also highly reactive toward acetaldehyde, forming a strongly bound sulfonate adduct (1-hydroxyethanesulfonate); this reaction strongly favors the formation of the sulfonate under wine conditions due to its low dissociation constant.[27] Previous studies have examined the effect of SO_2 on acetaldehyde formation and products of endogenous acetaldehyde in wine.[26,30,40,41] However, the reactivity of acetaldehyde when it is assumed to be bound as a sulfonate and inert (i.e., in the presence of SO_2) has not been directly assessed.

Catechin consumption was measured under aerobic and anaerobic conditions under low (40 mg/L, 0.62 mM) and high (80 mg/L, 1.25 mM) total SO_2 (TSO_2) concentrations. As was seen in the absence of SO_2, there was no significant difference between samples stored under aerobic or anaerobic conditions with respect to catechin consumption; however, there was a significant effect of SO_2 concentration on this reaction. At the higher test concentration of SO_2, when approximately double the concentration of bisulfite was present compared to acetaldehyde, catechin-consuming reactions ceased. This is due to the fact that the acetaldehyde is preferentially bound to the excess of bisulfite and, therefore, unable to react with catechin (Figure 4).

When SO_2 was present at a lower concentration (i.e., a slight molar excess of bisulfite compared to acetaldehyde), the consumption rate of catechin decreased but was not completely prevented. The concentration of catechin under these conditions was significantly lower than the control and high-SO_2 treatment samples, and the concentration of catechin was also significantly higher after 14 days than in the sample containing acetaldehyde alone. To our knowledge, this is the first clear evidence for the reactivity of acetaldehyde when it is assumed to be bound to bisulfite under wine conditions and suggests that the small amount of free acetaldehyde in equilibrium with its sulfonate is able to react with catechin.

Reaction of Acetaldehyde with M3G and Flavanols. After these effects in model systems containing catechin only were confirmed, M3G (500 mg/L) was added to the system. Catechin concentrations were measured to determine if M3G had an impact on the overall consumption of catechin (Figure 5). A small but significant difference in the consumption of catechin between samples with and without M3G was observed, which could be due to competition between M3G and catechin for acetaldehyde. This experiment was then repeated in the presence of SO_2 (40 mg/L TSO_2, 0.62 mM), under which conditions SO_2 was previously observed to retard the consumption of catechin compared to SO_2-free samples. However, there was significant consumption of catechin by acetaldehyde in the presence of SO_2 compared to a control. There was no significant difference in catechin consumption between SO_2-containing samples with or without M3G.

The consumption of M3G was followed to determine the effect of SO_2 on reactions of acetaldehyde with M3G as well as the effect of the type of flavanol present (i.e., monomeric catechin or tannin from grape seed extract) (Figure 6). No

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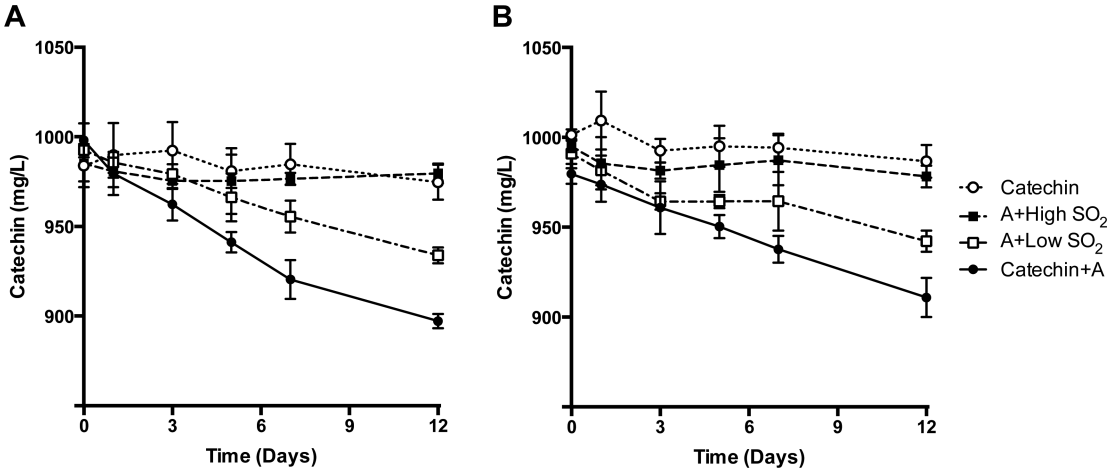

Figure 4. Catechin concentrations in model wine (pH 2.5) after treatment with acetaldehyde and SO_2 additions under aerobic (A) and anaerobic (B) conditions. Low SO_2 samples (A+Low SO_2) contained 40 mg/L TSO_2 and high SO_2 samples (A+High SO_2) contained 80 mg/L TSO_2.

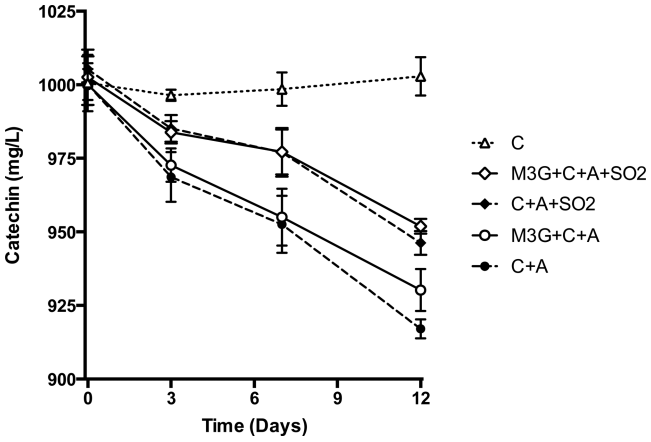

Figure 5. Catechin concentrations in all samples containing catechin over 12 days: catechin only (C), catechin with acetaldehyde (C+A), catechin with acetaldehyde and 40 mg/L SO_2 (C+A+SO2), M3G and catechin with acetaldehyde (M3G+C+A), and M3G and catechin with acetaldehyde and 40 mg/L SO_2 (M3G+C+A+SO2).

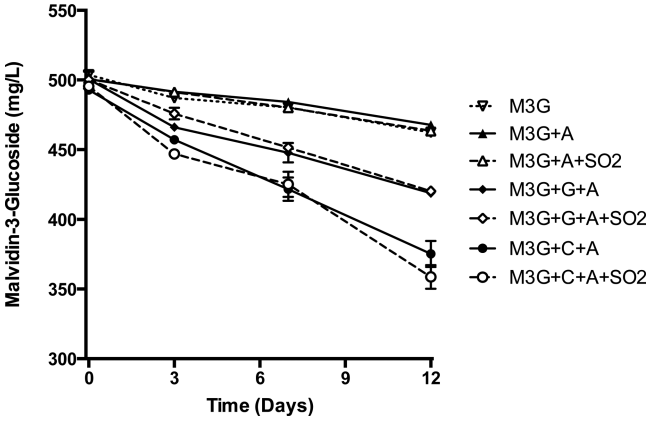

Figure 6. Malvidin 3-glucoside (M3G) concentrations in all samples containing M3G over 12 days: M3G only (M3G), M3G with acetaldehyde (M3G+A), M3G with acetaldehyde and 40 mg/L SO_2 (M3G+A+SO2), M3G and catechin with acetaldehyde (M3G+C+A), M3G and catechin with acetaldehyde and 40 mg/L SO_2 (M3G+C+A +SO2), M3G and GSE with acetaldehyde (M3G+G+A), and M3G and GSE with acetaldehyde and 40 mg/L SO_2 (M3G+G+A+SO2).

significant consumption of M3G in the presence of acetaldehyde was observed compared to a control without acetaldehyde. The reaction of M3G with acetaldehyde is slower than for catechin and may not be occurring at a rate that is sufficient for the effect to be seen in the time frame of the experiment. The presence of SO_2 did not have a significant effect on M3G consumption in any samples. Because the effect of SO_2 was seen in the consumption of catechin, it is possible the consumption rate of M3G was sufficiently slow such that no effect of SO_2 could be observed.

The presence of flavanols in the system did significantly affect M3G consumption. Samples containing catechin showed greater M3G consumption than those containing GSE and those with M3G alone. These results indicate that the catechin monomer, in conjunction with acetaldehyde, is more reactive toward M3G than the oligomeric tannins found in the GSE. The slow and nonsignificant reaction of M3G with acetaldehyde and itself illustrates that the rate of that reaction is significantly slower than that involving flavanols.

The reaction of M3G toward catechin compared to GSE illustrates the increased reactivity of monomers over oligomers. Monomeric catechin has two functional sites (C6 and C8) that are able to participate in ethylidene bridging, whereas oligomeric tannins should have only one functional site on each terminal subunit. Assuming most of the tannins in GSE are larger oligomers, the sites for ethylidene bridging will be lower in equivalent masses as compared to catechin.

Samples containing M3G were also characterized by a modified Somers assay to assess changes in color and color stability due to acetaldehyde treatment (Figure 7; Table 1). Control samples that did not contain acetaldehyde were compared to samples after 12 days of acetaldehyde treatment or acetaldehyde and SO_2 treatment, as described above. Statiscally significant differences were observed in several color stability parameters based on experimental conditions and treatment time. In general, acetaldehyde treatment significantly improved color stability, and acetaldehyde treatment with SO_2 present improved color stability, but to a lesser extent than acetaldehyde alone. The effects of both treatments (acetaldehyde or the combination of acetaldehyde and SO_2) were greater when M3G was treated with flavanols (catechin or GSE) than with M3G treated alone. A statistically significant decrease in the concentration of monomeric anthocyanins was observed in

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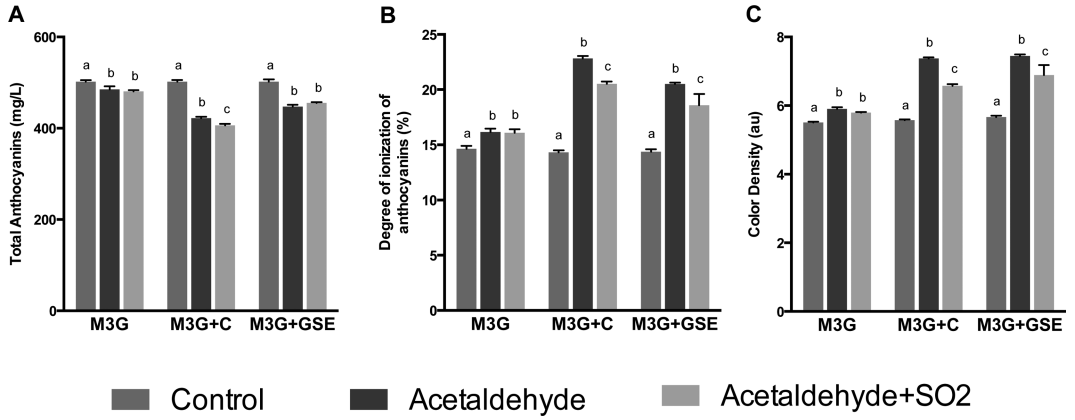

Figure 7. Select color parameters from the modified Somers assay for control and treatment groups (acetaldehyde and acetaldehyde+SO2) at 12 days: (A) total anthocyanins; (B) degree of ionization of anthocyanins; (C) color density. Values represent the average of three experimental replicates ± standard deviation. Columns in the same group with different letters indicate significant differences ($p < 0.05$).

Table 1. Color Parameters from the Modified Somers Assay for Control and Treatment Groups (Acetaldehyde or Acetaldehyde and SO_2) at 12 Days[a]

	chemical age 1 (au)	chemical age 2 (au)	color density SO_2-corrected (au)	hue	SO_2-resistant pigments (au)	total phenolics (au)
M3G samples						
control	0.098 ± 0.004 a	0.0146 ± 0.0002	5.29 ± 0.03 a	0.3614 ± 0.0004 a	3.84 ± 0.03 a	11.81 ± 0.06 a
acetaldehyde (12 days)	0.113 ± 0.003 b	0.0164 ± 0.0002	4.63 ± 0.43 b	0.3638 ± 0.0006 b	3.61 ± 0.08 b	11.54 ± 0.18 ab
acetaldehyde and SO_2 (12 days)	0.107 ± 0.021 ab	0.0170 ± 0.0035	5.19 ± 0.04 a	0.3689 ± 0.0009 c	3.73 ± 0.04 c	11.32 ± 0.04 b
M3G + C samples						
control	0.104 ± 0.001 a	0.0162 ± 0.0001 a	5.59 ± 0.08 a	0.3748 ± 0.0004 a	4.13 ± 0.03 a	20.89 ± 0.12 a[a]
acetaldehyde (12 days)	0.124 ± 0.001 b	0.0264 ± 0.0002 b	6.32 ± 0.25 b	0.4075 ± 0.0002 b	4.69 ± 0.02 b	19.12 ± 0.10 b
acetaldehyde and SO_2 (12 days)	0.127 ± 0.002 b	0.0258 ± 0.0003 b	6.16 ± 0.04 b	0.4144 ± 0.0010 c	4.38 ± 0.02 c	18.63 ± 0.22 c
M3G + GSE samples						
control	0.121 ± 0.001 a	0.0204 ± 0.0002 a	5.80 ± 0.06 a	0.4257 ± 0.0006 a	4.22 ± 0.02 a	23.47 ± 0.20 a
acetaldehyde (12 days)	0.178 ± 0.002 b	0.0350 ± 0.0003 b	5.91 ± 0.41 a	0.4413 ± 0.0007 b	4.46 ± 0.07 b	22.75 ± 0.31 b
acetaldehyde and SO_2 (12 days)	0.165 ± 0.003 b	0.0328 ± 0.0003 b	6.46 ± 0.07 b	0.4489 ± 0.0006 c	4.58 ± 0.03 c	22.90 ± 0.13 b

[a]Values represent the average of three experimental replicates ± standard deviation. Values in the same experimental group column with different letters indicate significant differences ($p < 0.05$).

all treated samples. With respect to the degree of ionization of anthocyanins, there was a significant increase in all acetaldehyde-treated samples. In samples containing M3G alone, the increase was modest, yet significant, in both the presence and absence of SO_2, although there was no significant difference in degree of anthocyanin ionization between the two acetaldehyde treatments. This is likely due to the relatively small amount of M3G that participates in these reactions, which was confirmed by measuring M3G concentrations. In samples containing M3G and catechin, there was a significant and much larger increase in degree of ionization with acetaldehyde treatment. Acetaldehyde treatment with SO_2 resulted in a significant increase in anthocyanin ionization compared to the control; however, this value was significantly lower than that for the corresponding treatment with acetaldehyde alone. Samples containing M3G with GSE showed a similar trend with respect to change in the degree of ionization of anthocyanins compared to M3G with catechin samples. These trends were also observed with respect to increases in color density associated with acetaldehyde treatment (Figure 7).

Taken together, these results suggest that acetaldehyde contributes to a decrease in monomeric anthocyanins while increasing the degree of ionization of anthocyanins and color density. This is consistent with previous results that were performed under similar conditions.[13,42,43] Furthermore, increases in SO_2-resistant pigments were seen in acetaldehyde-treated samples of M3G with catechin and GSE (Table 1). There was a slight but significant decrease in total phenolics for most samples, which could indicate precipitation of higher molecular weight oxidation products formed by ethylidene bridging.

In the model studies, pH was shown to have a significant effect on the rate of reaction of catechin with acetaldehyde with higher rates of consumption at lower pH, which is consistent with previous studies.[36] This is due to the important mechanistic step of protonation of the acetaldehyde. However, oxygen did not have a significant effect on the rate of reaction of acetaldehyde with catechin, which confirms that this mechanism does not involve the participation of oxygen and

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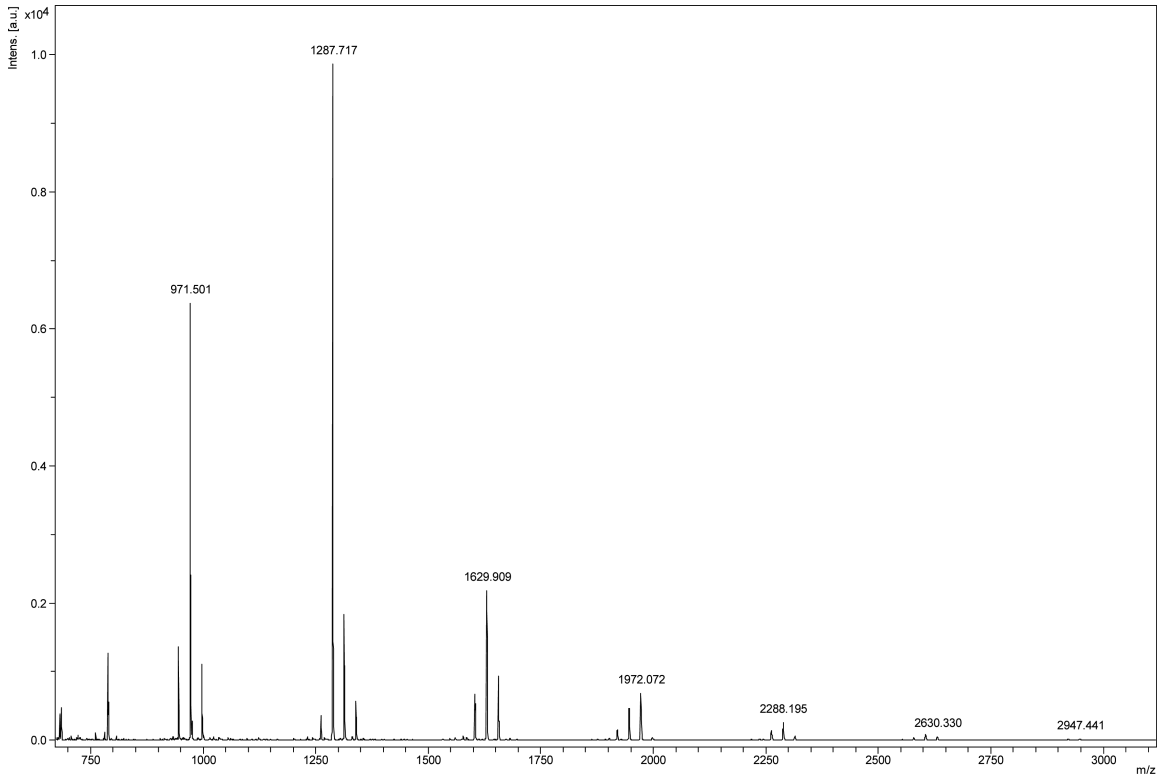

Figure 8. MALDI-TOF mass spectrum recorded in positive reflectron mode of catechin treated with acetaldehyde.

also that the loss of catechin observed is not due to other oxidative mechanisms.

Finally, acetaldehyde was found to react with catechin and M3G in the presence of an equimolar concentration of bisulfite. The rate of reaction was relatively slower compared to the SO_2-free system, as measured by following the loss of monomeric catechin; however, SO_2 did not affect the concentration of free M3G. Most importantly, there were statistically significant improvements in several color stability parameters in the presence of SO_2 for systems containing M3G with catechin or GSE. Whereas SO_2 may slow the formation of ethylidene-bridged catechin oligomers, bisulfite had little or no effect on polymeric pigment formation when it was present as equimolar concentrations, as was evident by measuring M3G concentrations and color stability indices.

Characterization of Products with MALDI-TOF MS. Samples were characterized by MALDI-TOF MS to directly observe the high molecular weight phenolic compounds formed by the acetaldehyde-bridging reactions described above. MALDI was selected here as it allows for a gentler ionization of compounds, which prevents fragmentation of larger molecules (e.g., tannins). Tannins and anthocyanin-derived pigments from grapes and wine have been previously observed by MALDI-TOF MS.[35,44,45] Analysis of the samples described here required minimal sample preparation because MALDI-TOF can be used without separation of analytes. By using a completely volatile mixture (with the exception of the analytes), samples were dried and immediately mixed with the matrix components for analysis.

The experimental conditions employed here were slightly different from those used to characterize the consumption of catechin and M3G. Briefly, higher concentrations of acetaldehyde (200 mg/L) and longer times (1 month) were used to prepare these samples so that concentrations of the resulting products were sufficiently abundant to be seen by MALDI-TOF without any separation steps. All products discussed below were found in treated samples after 1 month and were not seen in control MALDI-TOF spectra of the mixtures at the beginning of the experiment.

In samples containing catechin alone with acetaldehyde, oligomers up to the nonamer were observed by MALDI-TOF MS (Figure 8; Table 2). Oligomers are formed by the addition of a catechin subunit along with an ethylidene bridge due to reaction with acetaldehyde (i.e., the addition of 316 mass units). This sequence of increasing oligomer size is clearly seen from the dimer to the nonamer. These results are consistent with those previously seen using MALDI-TOF MS in systems containing catechin and formaldehyde[46] and by ESI and LSI-MS methods in systems contaning catechins and acetaldehyde.[15,36] Another sequence of mass additions was also observed corresponding to additional acetaldehyde products. These are believed to represent vinyl additions to catechin subunits, corresponding to an increase in mass of 26 units. Up to four addional vinyl catechin moieties were observed in the larger oligomers.

Oligomers of catechin can undergo depolymerization in wine and model wine systems. Acid-catalyzed cleavage of ethylidene-bridged catechins have been shown to result in the formation of vinyl catechins.[20] These vinyl moieties can be found on carbon-6 and carbon-8 of catechins, including those in oligomers (Figure 9). Vinyl-catechin, bivinyl-catechin, and vinyl oligomers have been observed by LSI-MS.[15] These reactive vinyl species are likely to be involved in further modification reactions.[17] On the basis of vinyl catechin products previously seen, we would expect only up to two vinyl moieties to be found in oligomers, those on terminal catechin subunits. The presence of sequential mass increases of 26 over the assumed two possible sites may indicate further functional sites for acetaldehyde reaction on

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Table 2. Predicted and Observed m/z Values As Recorded in Positive Reflectron Mode MALDI-TOF MS of Catechin Treated with Acetaldehyde

DP	vinyl additions	predicted	observed
2	0	629	629.188
	1	655	655.223
	2	681	681.286
3	0	945	945.469
	1	971	971.501
	2	997	997.536
4	0	1261	1261.691
	1	1287	1287.717
	2	1313	1313.744
	3	1339	1339.775
5	0	1577	1577.875
	1	1603	1603.886
	2	1629	1629.909
	3	1655	1655.940
6	1	1919	1920.026
	2	1945	1946.048
	3	1971	1972.072
	4	1997	1998.102
7	2	2261	2262.172
	3	2287	2288.195
	4	2313	2314.218
8	3	2603	2604.309
	4	2629	2630.330
9	3	2919	2922.430
	4	2945	2947.441

catechin beyond the A-ring or potentially another reaction product.

The masses predicted on the basis of ethyl-bridged catechin subunits and vinyl moieties corresponded closely with the observed spectrum (Table 2). The observation of multiple vinyl moieties is evidence that acetaldehyde may be responsible for greater changes in the flavonoid composition of wine than previously assumed. On the basis of the observed sequence of masses corresponding to vinyl catechin subunits, there may be another available functional site on catechin under certain conditions. These results also illustrate that determining the fate of acetaldehyde under wine conditions is complicated because of the complexities of ethylidene-bridged oligomers and vinyl catechins. There are likely higher molecular weight oligomers formed that should be explored in future research.

Samples containing M3G alone with acetaldehyde showed evidence for the formation of M3G dimers bridged by an ethylidene moiety. The dimer observed had m/z 1011.595 and corresponds to a previously described ethyl-linked M3G dimer with one neutral quinoidal base and one flavylium cation.[47] Previous studies have argued for the existence of anthocyanin trimers in grape pomace;[45] however, these compounds were not observed in the present study. Unlike catechin, which forms long oligomeric chains, M3G was not observed to polymerize beyond its dimer. This corroborates previous work demonstrating that anthocyanins may act as terminal subunits, thus preventing further increases in tannin size.

The combination of catechin and M3G treated with acetaldehyde yielded a relatively more complicated mixture of products than either flavonoid alone. In this mixture, catechin oligomers up to the tetramer were observed as well as catechin oligomers up to the trimer that also contained a M3G subunit. Products containing M3G also have the potential to rearrange to pyranoanthocyanins, resulting in a net decrease of 4 mass units (Table 3). Pyranoanthocyanins are cyclized forms of anthocyanin adducts formed by the reaction of vinyl catechins with an anthocyanin. The pyranoanthocyanin formed from an ethylidene-bridged adduct can be considered a rearrangement of the adduct; acid-catalyzed cleavage of the ethylidene bridge produces a vinyl catechin and releases the anthocyanin, M3G in this case. Reaction of the vinyl catechin with M3G forms an intermediate that recyclizes into a pyranoanthocyanin−catechin adduct (Figure 10).[17,48,49] Pyranoanthocyanin adducts have been observed in model wine and in red wine and are known to be more stable than their anthocyanin counterparts.[50−54]

It should be noted that the peak at m/z 809.409, corresponding to a catechin−ethyl−M3G adduct, was by far the most abundant in the spectrum for this mixture. Again, vinyl catechin moieties were observed in this reaction mixture of M3G and catechin. Although we did not observe adducts larger than the tetramer, they are likely present in the reaction mixture. The detector may have been saturated due to the presence of smaller m/z compounds, which would include M3G monomers as well as any doubly charged adducts formed that would contain multiple M3G subunits. It is conceivable that prior chromatographic separation of the components in

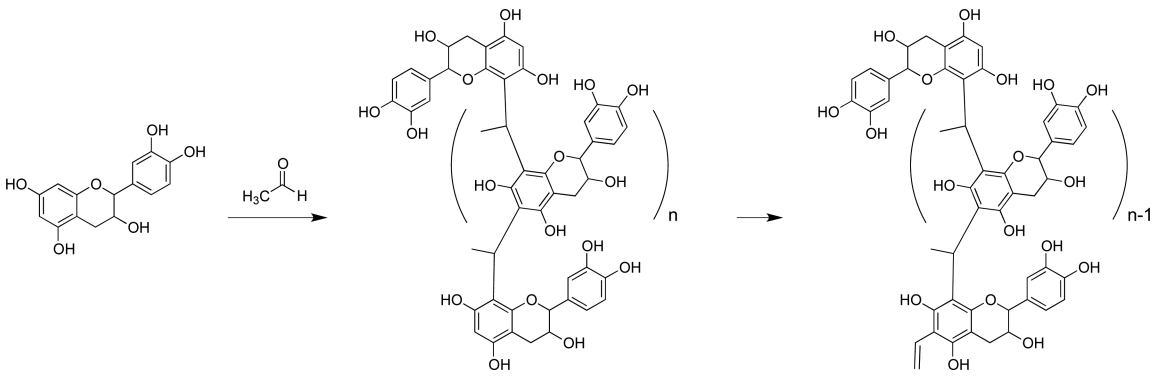

Figure 9. Reaction of catechin with acetaldehyde to form ethylidene-bridged oligomers followed by cleavage to form vinyl catechin moieties.

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Table 3. Predicted and Observed *m/z* Values As Recorded in Positive Reflectron Mode MALDI-TOF MS of Catechin and M3G Treated with Acetaldehyde

DP of catechin	M3G additions	vinyl additions	predicted adduct/*rearranged*	observed	
1	1	0	809	809.409	
		1	835/*831*	835.446	*831.407*
2	1	0	1125	1125.657	
		1	1151/*1147*	1151.691	
		2	1177/*1173*		*1173.694*
3	0	0	945	945.519	
		1	971/*967*	971.556	
	1	0	1441	1441.847	
		1	1467/*1463*	1467.881	*1463.857*
		2	1493/*1489*		*1489.889*
4	0	1	1287	1287.774	

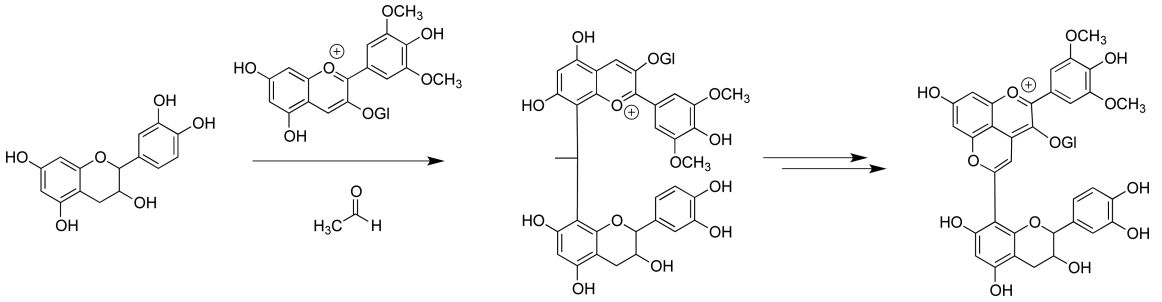

Figure 10. Reaction of catechin with M3G and acetaldehyde to form an ethylidene-bridged polymeric pigment and subsequent rearrangement to pyranoanthocyanins.

advance of MALDI-TOF MS analysis would allow for improved characterization of the mixture.

Samples containing tannins from GSE were treated with acetaldehyde to observe the effect on native tannins. In this mixture of products, tannins up to the pentamer were observed in their native and modified states. These predicted masses and the corresponding observed *m/z* values are summarized (Table 4). Procyanidins and their gallate equivalents were all found to be modified by vinyl and/or catechin additions. It is likely that larger modified tannins would form given longer treatment times, resulting in concentrations that could be more easily detected.

The combination of GSE and M3G treated with acetaldehyde resulted in complicated product mixtures, as was seen in catechin and M3G samples. These samples contained native tannins as well as those that have been modified to include M3G subunits. The MALDI-TOF spectrum showed evidence for native tannins up to the tetramer modified by an additional M3G subunit. These predicted and observed mass values are summarized (Table 5). Additionally, the rearranged pyranoanthocyanin version of adducts containing M3G were observed in these oligomers. As was seen in samples of GSE alone with acetaldehyde, there would likely be larger, modified tannins formed that could be observed by MALDI-TOF with longer reaction time.

MALDI-TOF characterization confirmed the formation of many products resulting from the reaction of grape flavonoids with acetaldehyde. Acetaldehyde was shown to form polymeric pigments by adding M3G subunits to native tannins from GSE and to catechin oligomers formed by ethylidene bridges. These polymeric pigments existed in their ethyl-linked forms and as rearranged pyranoanthocyanins. M3G was also observed to form dimers bridged by ethylidene moieties in the presence of acetaldehyde. The ability of acetaldehyde to formed bridged catechin oligomers was confirmed by the observation of nonamers in samples containing only catechin and acetaldehyde. These results further demonstrate the advantages that MALDI confer for the analysis of complex wine flavonoids,

Table 4. Predicted and Observed *m/z* Values As Recorded in Positive Reflectron Mode MALDI-TOF MS of GSE Treated with Acetaldehyde

DP	gallate groups	vinyl additions	catechin additions	predicted	observed
2	0	0	0	601	601.145
		1	1	943	943.485
3	0	0	0	889	889.39
		1	1	1231	1231.668
	1	0	0	1041	1041.481
		1	1	1383	1383.731
4	0	0	0	1177	1177.587
		1	1	1519	1519.801
	1	0	0	1329	1329.651
		1	1	1671	1671.863
5	0	0	0	1465	1465.74
		1	0	1491	1491.783
	1	0	0	1617	1617.785
		1	0	1643	1643.82

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Table 5. Predicted and Observed *m/z* Values As Recorded in Positive Reflectron Mode MALDI-TOF MS of GSE and M3G Treated with Acetaldehyde

DP of tannin	gallate groups	M3G additions	vinyl additions	predicted adduct/*rearranged*	observed	
1	0	0	0	313		
		1	0	809/*805*	809.392	*805.356*
		1	1	835/*831*	835.438	*831.393*
	1	0	0	465		
		1	0	961/*957*	961.491	*957.463*
2	0	0	0	601	601.145	
		1	0	1097/*1093*	1097.613	*1093.564*
		1	1	1123/*1119*		*1119.597*
	1	0	0	753	753.259	
		1	0	1249/*1245*	1249.675	*1245.64*
3	0	0	0	889	889.39	
		1	0	1385/*1381*		*1381.731*
		1	1	1411/*1407*		*1407.77*
	1	0	0	1041	1041.481	
		1	0	1537/*1533*		*1533.797*
4	0	0	0	1177	1177.587	
		1	0	1673/*1669*		*1669.83*

especially given the minimal sample preparation required compared to other MS techniques.

In summary, these results illustrate the important role that acetaldehyde plays, even in the presence of relatively low bisulfite concentrations. Although past research has focused on free acetaldehyde, the bound form should not be considered a true end point. Instead, future work should examine the best ways to take advantage of the activity of acetaldehyde in wine that is protected by SO_2. Additionally, MALDI-TOF MS characterization of several reaction products confirms the complexity of adducts formed by reaction with acetaldehyde—including the polymeric pigments formed by the addition of anthocyanins to monomeric and oligomeric condensed tannins.

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The authors declare no competing financial interest.

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