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Stability of Apigeninidin and Its Methoxylated Derivatives in the Presence of Sulfites

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3-Deoxyanthocyanin pigments are more stable than anthocyanins and show promising bioactive properties. However, little is known about their stability in the presence of food additives such as sulfites. This work investigates the stability of apigeninidin and its derivatives in the presence of sulfites. Pigment (apigeninidin, 5-mono-, and 5,7-dimethoxyapigeninidin) stability at pH 1.8, 3.0, and 5.0, in the presence of sodium metabisulfite (molar ratio ~ 1:40, pigment/SO_2) was monitored over 21 days at room temperature. The structure of sulfite complexation products was monitored using HPLC-MS and NMR spectroscopy. All pigments were significantly bleached within 30 min in the presence of sulfites; the bleaching effect was more severe at pH 5.0 and 3.0 compared to pH 1.8. Apigeninidin was more resistant to bleaching than its methoxylated derivatives. However, all pigments regained some or all of the bleached color within 14−21 days at pH 3.0 and 1.8 in the presence of sulfites, indicating equilibrium favored flavylium cation at these pH values. Formation of colorless sulfonates via bisulfite ion addition at C-4 was responsible for the bleaching effect. Both structure and pH significantly affected stability of 3-deoxyanthocyanidins in the presence of sulfites. The pigments may have potential applications in low pH systems containing sulfites.

KEYWORDS: 3-Deoxyanthocyanin; anthocyanin; apigeninidin; sulfur dioxide; natural colorant

INTRODUCTION

Anthocyanins have been widely investigated for their potential as natural food colorants. However, color stability of anthocyanin pigments is generally poor, especially at elevated pH and in the presence of some food additives utilized for bacteriostatic, antioxidant, or nutritional purposes, for example, sulfur dioxide and vitamin C. Thus, even though the natural colorants are increasingly used by the food industry, their use remains relatively low compared to artificial colorants. 3-Deoxyanthocyanins, found in large quantities in sorghum, are a class of anthocyanins unsubstituted at position 3 (**Figure 1**). The 3-deoxyanthocyanins have generated considerable interest due to their better stability in various food processing conditions and with food additives relative to the common anthocyanins (*1*−*4*). Additionally, recent findings suggest that the 3-deoxyanthocyanins have a higher potency against cancer cell proliferation than their anthocyanin analogues (*5*, *6*). Thus, the 3-deoxyanthocyanin pigments present an opportunity as biologically important natural colorants.

Sulfites are commonly used to preserve many processed foods and beverages. However, interaction of sulfite with anthocyanins often leads to bleaching, a phenomenon that has been documented for a long time (*7*, *8*). The bleaching reaction is generally reversible depending on structure of pigments involved (*9*), pH, and availability of oxygen in solution (*10*). However, the reaction mechanisms involved are still somewhat ambiguous (*8*, *9*, *11*, *12*), especially with respect to the effect of solution pH and molecular structure of anthocyanins involved. Additionally, limited information exists on sulfite reaction with 3-deoxyanthocyanins.

Sulfites dissociates almost instantaneously in solution into three species depending on the thermodynamic constant and the pH of the solution. These molecular species are sulfur dioxide (SO_2), sulfite ion (SO_3^{2-}), and bisulfite ion (HSO_3^-). The bleaching effect of sulfites on anthocyanins in solution results from the nucleophilic addition of these molecular species to the C-ring of the flavylium cation leading to formation of colorless sulfonates (*8*, *11*, *12*). Berké et al. (*12*) showed that this reaction occurred at C-4 of flavylium cation using malvidin-3-*O*-glucoside as a model. In agreement with this observation, Sarni-Manchado et al (*13*) demonstrated that cycloaddition products of wine anthocyanins and 4-vinylphenol were relatively resistant to sulfite bleaching and hypothesized that this was due to nonavailability of C-4 for nucleophilic attack by sulfite.

3-Deoxyanthocyanins on the other hand often show different reactive patterns when compared to anthocyanins. Jurd (*8*) proposed that sulfite addition to the 3-deoxyanthocyanidins flavylium cation occurred at C-2 and not C-4 as reported for anthocyanins; however, no evidence was presented to support this. Timberlake and Bridle (*9*) demonstrated that sulfites formed a relatively weak complex with 3-deoxyanthocyanidins, apigeninidin, and luteolinidin, compared to their anthocyanin analogues pelargonidin and cyanidin at pH 1.05. However, the authors did not investigate the structure of these weak complexes. Understanding precisely how sulfites reacts with the 3-deoxyanthocyanin pigments is important since it can be useful in designing

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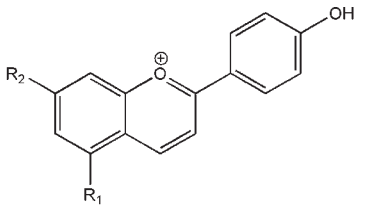

Apigeninidin: $R_1 = R_2 = OH$
5-Methoxyapigeninidin: $R1 = OCH_3$, $R_2 = OH$
5,7-Dimethoxyapigeninidin: $R_1 = R_2 = OCH_3$

Figure 1. Chemical structure of apigeninidin and its methoxylated derivatives used in this study.

strategies to protect the pigments from the bleaching effect of bisulfite. Additionally, recent data indicate that methoxylation of the 3-deoxyanthocyanins on the A-ring influences the mechanism of their reaction (*4*). Thus, it is important to determine how methoxyl substitution affects the reaction of these compounds with sulfites. The aim of this work was to establish how solution pH and structure of 3-deoxyanthocyanidins affect their stability in the presence of sulfites, and reaction mechanisms of the pigments with the sulfites.

MATERIALS AND METHODS

3-Deoxyanthocyanidin Samples and Buffer Solutions. Apigeninidin, 5-methoxyapigeninidin, and 5,7-dimethoxyapigeninidin (**Figure 1**) were purchased from Alsachim (Strasburg, France). Purity of the compounds was checked by HPLC with a diode array detector; all compounds were ≥97% pure. For pH 1.8, HCl/KCl buffer solution was prepared by mixing 10.2 mL of 0.2 M HCl with 39.8 mL of 0.2 M KCl, and adjusting to 100 mL with deionized water. For pH 3.0 and 5.0, citric acid/sodium citrate buffers were used. The pH 3.0 buffer was prepared by mixing 46.5 mL of 0.1 M citric acid with 3.5 mL 0.1 M sodium citrate and adjusting to 100 mL with deionized water. For pH 5.0 buffer, 20.5 mL of 0.1 M citric acid was mixed with 29.5 mL of 0.1 M sodium citrate and the solution was adjusted to 100 mL with deionized water.

Sample Stability in the Presence of Sulfites. Each sample was accurately weighed and dissolved in 50% aqueous methanol adjusted to pH 1.0 with HCl to produce a concentrated stock solution (the methanol was used to ensure rapid and complete solubilization of the pigments). From the concentrated solutions, 0.1 mL aliquot was adjusted to 10 mL with appropriate buffer solutions to produce the working control samples. Blank solutions (no pigment) were initially used to check the pH of the final mixture; pH of each working solution was confirmed. Working sample molar concentrations were apigeninidin, 6.87×10^{-5}; 5-methoxyapigeninidin, 6.56×10^{-5}; 5,7-dimethoxyapigeninidin, 6.7×10^{-5}. The absorbance spectra of these samples were recorded immediately after mixing with buffer (fresh solution) and then after 2 h (after equilibration), 24 h, 7, 14, and 21 days, by scanning from 200−700 nm using a Shimadzu UV2450 UV−vis spectrophotometer (Shimadzu Corp., Tokyo, Japan).

For the sulfite stability test, concentrated sodium metabisulfite solution was prepared using deionized water and aliquots were added to the buffer solutions (5% v/v) to achieve a final SO_2 concentration of 2.52×10^{-3} M. The pH of buffer + metabisulfite solutions were checked and adjusted as appropriate with HCl or NaOH. These solutions (9.9 mL) were then mixed with equilibrated 3-deoxyanthocyanidin solutions (0.1 mL) to achieve pigment concentrations similar to control samples above. The samples were then vortexed for 30 s and spectra were immediately recorded (as 1 min). Additional spectra were recorded after 5 min, 30 min, 24 h, 7, 14, and 21 days. The initial SO_2/pigment molar ratios ranged from 37:1 to 42:1. Samples were kept at room temperature throughout analyses. All analyses were repeated two times.

LC-MS Analysis. HPLC separation was achieved using an Agilent 1100 system, equipped with a diode array detector (DAD), quaternary pump, and an automatic injector. A reversed phase 150 × 2.00 mm i.d., 5 μm Luna C-18(2) column (Phenomenex, Torrance, CA) thermostatted at 35 °C was used. Sample injection volume was 5.00 μL; spectra were recorded from 200−720 nm and the monitoring wavelength was 480 nm. The mobile phase consisted of (A) 1% formic acid in water, and (B) 1% formic acid, 50% acetonitrile in water. The elution gradient was as follows: 0−2 min, 10% isocratic; 2−30 min, 10−70% B; 30−32 min, 70−100% B; 32−36 min, 100% isocratic; 36−37 min, 100−10% B; 37−43 min, 10% isocratic; followed by 2 min of re-equilibration of the column before the next run at a flow rate of 0.250 mL/min.

MS analysis was performed using a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer equipped with an API2 source, Performance Pack (with wider orifice in the skimmer and an extra turbo pump on the source) and an electrospray ionization (ESI) interface (ThermoFinnigan, San Jose, CA). The mass spectrometer was connected to an integrated Thermo-Finnigan LC system consisting of a P4000 quaternary LC pump and SCM1000 vacuum degasser, an AS3000 autosampler, and a UV6000LP diode-array detector. The electrospray needle voltage was 4.5 kV and the heated inlet capillary was equilibrated at 250 °C. All voltages were optimized to maximize ion transmission and minimize unwanted fragmentation. Spectra were recorded in positive ion mode between m/z 150 and 1000.

NMR Spectroscopy. NMR spectra were obtained on a Varian Inova 400 spectrometer housed in the Chemistry Department of Texas A&M University. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature in dimethyl sulfoxide (DMSO-d_6)/trifluoroacetic acid (TFA-d_1)/sulfite (as SO_2) (20:1:5), at 400 MHz for ^{1}H-measurements. For ^{13}C measurements, an auto switchable 5 mm broad-band probe tuned to observe ^{13}C (at 100 MHz) and decouple ^{1}H (at 400 MHz) was used. With appropriate power settings, typical values of 5.6 and 10.1 μs for proton pulse width and ^{13}C pulse width, respectively, were used. While proton spectra were obtained with 128 scans, ^{13}C data were collected overnight; data was processed on VNMRJ 2.2D application software.

Table 1. Effect of Sulfites on Stability (Absorbance at λ_{max}) of Apigeninidin at pH 1.8 and 3.0[a]

	pH 1.8			pH 3.0		
time[b]	control	with sulfites	% change[c]	control	with sulfites	% change[c]
1 min	2.89	2.58	−10.7	2.16	0.76	−64.5
5 min	2.89	2.26	−21.8	2.16	0.67	−69.0
30 min	2.87	2.13	−25.8	2.14	0.32	−85.0
Day 1	2.85	0.97	−66.0	2.16	0.15	−93.1
Day 7	2.81	2.61	−7.1	2.09	0.18	−91.4
Day 14	2.79	2.78	−0.4	1.99	0.73	−63.3
Day 21	2.74	2.77	+1.1	1.84	1.36	−26.1
% CV	2.3	6.2		2.3	6.2	

[a] Samples were kept at room temperature; initial SO_2/apigenindin molar concentration was 37:1. λ_{max} in pH 1.8 = pH 3.0 buffer = 468 nm. [b] All measurements were recorded after control had been equilibrated for 2 h in buffer solution. [c] Percent changes are relative to corresponding controls; CV values are based on two separate experiments.

RESULTS AND DISCUSSION

Effect of pH on Color Stability of 3-Deoxyanthocyanidins in the Presence of Sulfites. *Control Solutions.* Control samples (without sulfites) were generally stable at pH ≤ 3.0 over 21 days under the conditions used in this study, with average absorbance of the flavylium species (λ_{max} 464−468 nm) dropping by less than 10% (**Tables 1**−**3**). At these pH values, only the flavylium cation absorbance peaks (464−468 nm) were apparent (not shown), which agrees with the pH jump study by Brouillard et al (*14*) that showed that the 3-deoxyanthocyanidins are present in solution almost exclusively as flavylium cations at pH values below 3.0. This is partly explained by the relatively slow hydration of these compounds due to their larger acidity constants relative to hydration constants. By contrast, anthocyanidins hydrate much more readily at pH above 1.0, owing to their larger hydration constants, which leads to rapid color loss even at pH 2.0 (*3*, *14*).

However, at pH 5.0, the pigments generally lost absorbance in the visible region over time (**Figure 2**). Initial loss of absorbance of

Table 2. Effect of Sulfites on Stability (Absorbance at λ_{max}) of 5-Methoxyapigeninidin at pH 1.8 and 3.0[a]

	pH 1.8			pH 3.0		
time[b]	control	with sulfites	% change[c]	control	with sulfites	% change[c]
1 min	2.71	0.87	−67.9	2.71	0.64	−76.4
5 min	2.71	0.54	−80.1	2.71	0.34	−87.5
30 min	2.67	0.21	−92.1	2.65	0.12	−95.5
Day 1	2.76	0.46	−83.3	2.69	0.10	−96.3
Day 7	2.67	2.48	−7.1	2.66	0.70	−73.7
Day 14	2.73	2.80	+2.6	2.60	1.89	−27.3
Day 21	2.68	2.77	+3.4	2.54	2.12	−16.5
% CV	2.3	6.2		2.3	6.2	

[a] Samples were kept at room temperature; initial SO_2/pigment molecule molar concentration was 42:1. λ_{max} in pH 1.8 = pH 3.0 buffer = 464 nm. [b] All measurements were recorded after control had been equilibrated for 2 h in buffer solution. [c] Percent changes are relative to corresponding controls; CV values are based on two separate experiments.

Table 3. Effect of Sulfites on Stability (Absorbance at λ_{max}) of 5,7-Dimethoxyapigeninidin at pH 1.8 and 3.0[a]

	pH 1.8			pH 3.0		
time[b]	control	with sulfites	% change[c]	control	with sulfites	% change[c]
1 min	1.69	0.79	−53.3	3.12	0.43	−86.2
5 min	1.69	0.20	−88.2	3.12	0.39	−87.5
30 min	1.65	0.10	−93.9	3.09	0.06	−98.1
Day 1	1.69	0.11	−93.5	2.99	0.03	−99.0
Day 7	1.62	1.31	−19.1	3.01	0.03	−99.0
Day 14	1.65	2.20	+33.3	2.99	0.09	−97.0
Day 21	1.61	2.38	+47.8	2.97	0.66	−77.8
% CV	2.3	6.2		2.3	6.2	

[a] Samples were kept at room temperature; initial SO_2/pigment molecule molar concentration was 39:1. λ_{max} in pH 1.8 = pH 3.0 buffer = 468 nm. [b] All measurements were recorded after control had been equilibrated for 2 h in buffer solution. [c] Percent changes are relative to corresponding controls; CV values are based on two separate experiments.

the flavylium cation species at pH 5.0 during equilibration corresponded to an increase in chalcone species (λ_{max} 375−400 nm). Since the chalcone species are pale yellow, whereas the apigeninidin flavylium species are yellow, overall loss of absorbance of flavylium species did not accurately represent (overestimated) overall color loss of solution. Furthermore, significant absorbance observed in the spectra beyond 530 nm at this pH (spectra were generally back to baseline at 520−530 nm in pH 1.8 and 3.0 buffers) indicated the presence of the quinoidal species, which are also colored. However, during the 21 days, significant loss of all colored species was observed; apigeninidin and 5-methoxyapigeninidin lost 71.7 and 29.4%, respectively, of initial absorbance of flavylium cation species (λ_{max} 474−477 nm), and 49.4 and 25.0%, respectively, of initial absorbance of the chalcone species (λ_{max} 389−398 nm) at equilibrium. Thus, the 5-methoxyl apigeninidin derivative was significantly more stable at this pH than apigeninidin, which agrees with previous findings (*15*). The chalcone species were also generally more stable than the flavylium cation at this pH, which indicates the equilibrium favored the chalcones. This by itself indicates unusual behavior of 3-deoxyanthocyanidins, since Chen and Hrazdina (*16*) demonstrated that anthocyanins do not form any chalcone species at pH below 6.0, but instead hydrate to colorless carbinol species.

The 5,7-dimethoxyl substituted apigeninidin had a somewhat different behavior at pH 5.0; immediately after mixing in buffer solution, the pigment's absorbance spectrum (measured after 1 min) was virtually identical to its spectra at pH 1.8 and 3.0, except for a slight hump at 525−560 nm, indicative of some quinoidal anhydrobase formation. Thus, immediately upon mixing in pH 5.0 buffer, the compound did not produce any chalcone peak, whereas its flavylium cation absorbance remained strong (**Figure 2c**). However, after 2 h at room temperature (which was the standard equilibration time), the dimethoxyapigeninidin flavylium species absorbance (λ_{max} 468 nm) dropped from 2.80 to 0.86 with the chalcone species (λ_{max} 378 nm) becoming dominant (absorbance 1.25). In fact, even after 24 h, conversion of flavylium cation to chalcone was still evident for this compound, indicated by a drop in flavylium cation absorbance from 0.86 to 0.55, and an increase in chalcone absorbance from 1.25 to 1.49. By contrast, apigeninidin and its 5-methoxylated derivative immediately produced major chalcone peaks upon adjustment to pH 5.0, with a correspondingly reduced flavylium cation absorbance compared to pH 3.0 (**Figure 2a**,**b**). Slower hydration of the dimethoxylated apigeninidin due to its more hydrophobic nature relative to the other two compounds may partly explain this observation.

Effect of Sulfites. In the presence of sulfites (~40:1 molar ratio, as SO_2), all pigments were significantly bleached within 30 min at all pH levels (**Table 1**−**3**; **Figure 2**). The initial bleaching effect of sulfites was generally more rapid and pronounced at pH 5.0 and 3.0 compared to pH 1.8. For example, at pH 1.8, apigeninidin lost 10.7% of absorbance within the first minute of mixing with sulfites, and 25.8% after 30 min; by contrast, at pH 3.0, this compound lost 64.5% of absorbance within 1 min, and 85.0% within 30 min of mixing with sulfites (**Table 1**). This observation is likely because at the lower pH (higher hydrogen ion concentration), the equilibrium more strongly favors the flavylium cation over the colorless bisulfite adduct (*8*) as represented by the equation below:

$$HSO_3^- + H^+ \leftrightarrows H_aSO_3$$
$$HSO_3^- + AH^+ \leftrightarrows A^+HSO_3^-$$
where $AH^+$is the flavylium cation

Over time, all the compounds regained the flavylium cation absorbance at pH 1.8 and 3.0 (**Table 1**−**3**). The color restoration was more rapid at pH 1.8 than pH 3.0, with all compounds regaining lost absorbance within 14 days in solutions containing sulfites at pH 1.8. However, at pH 3.0, absorbance values for all the compounds, although much higher than initial absorbance in the presence of sulfites, were still significantly below that of their respective controls after 21 days. At pH 5.0, apigeninidin and 5-methoxyapigeninidin regained some absorbance after 21 days, but these were generally minimal (**Figure 2**). HPLC chromatograms (not shown) generally confirmed these spectrophotometric observations; the loss of flavylium cation absorbance at pH 1.8 and 3.0 corresponded to a reduction in parent 3-deoxyanthocyanidin peaks and a concomitant increase in the new sulfonate adduct peaks (λ_{max} = 276 nm). The reverse observation was true when the color was restored over time; sulfonate adduct peaks declined, while the parent 3-deoxynathocyanidin peaks increased. On the other hand, at pH 5.0, HPLC chromatograms showed that the sulfonate adduct peaks continued to increase slightly over the 21 days; the parent 3-deoxyanthocyanidin peaks also remained relatively strong likely due to reconversion of the various species to flavylium cation in the low pH mobile phase.

Anthocyanins have been shown to form relatively stable complexes with the sulfites at elevated pH (*9*), where equilibrium disfavors the highly reactive flavylium cation (AH^+). On the other hand, at lower pH, the sulfonate adducts formed are less stable. Thus, the sulfonate adducts will dissociate faster at low pH and more slowly at high pH. Hence, a likely explanation for the rapid color restoration at pH 1.8 is that as the SO_3H^- reacted with the abundantly available AH^+, concentration of bisulfite in

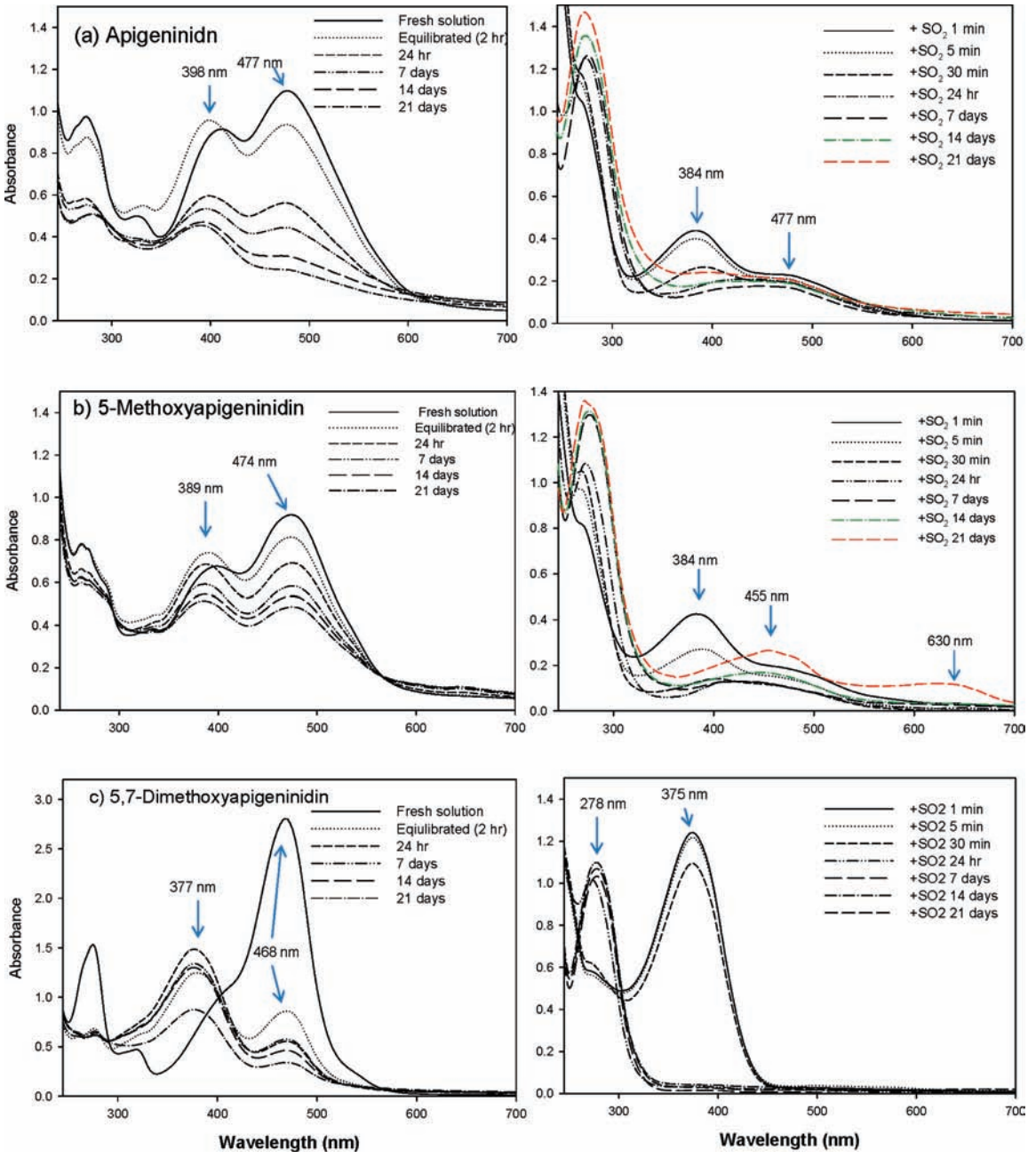

Figure 2. Spectral characteristics of (a) apigeninidin and (b, c) its methoxylated derivatives at pH 5.0 in the presence and absence of sulfites. Samples were incubated at room temperature over 21 days; initial pigment/SO_2 molar ratio was ~1:40.

solution decreased to the point where the reverse reaction (dissociation) was favored, especially since the formed sulfonates complexes are weak. However, this cannot fully explain why initial color was completely restored at pH 1.8 (i.e., AH^+ absorbance was similar to control samples) after 21 days in the presence of sulfites, and even significantly higher than control for 5,7-dimethoxyapigeninidin (**Table 3**). A possible explanation is that over time, loss of SO_2 from solution (expected to be higher at lower pH due to more rapid dissociation) reduced effective sulfite concentration and shifted equilibrium, which led to faster dissociation of the sulfonate adducts. However, on the basis of literature evidence, loss of SO_2 under conditions used in this study (expected to be < 25%) (*17*) cannot adequately explain the complete and rapid color restoration, especially given the large excess of initial SO_2 concentration relative to pigment concentration. It is possible that the 3-deoxyanthocyanin compounds, due to their hydrophobic pyrilium ring, will tend to more readily self-associate over time (compared to anthocyanins) in a way that protects the AH^+ from further nucleophilic attack after it dissociates from bisulfite ion. Additionally, some of the sulfonate complexes could possibly copigment with the AH^+ to not only stabilize color, but also increase color intensity, as was observed for the dimethoxyapigeninidin (**Table 3**). This phenomenon should be further investigated.

Substitution on A-ring had a significant effect on the stability of the pigments in the presence of sulfites. Compared to the methoxylated derivatives, apigeninidin was relatively resistant to bleaching in the presence of sulfite species (**Table 2**−**4**). Despite the large of excess of sulfites in solution relative to the 3-deoxyanthocyanidin molecules, apigeninidin lost only 25.8% of its absorbance at λ_{max} within 30 min of reaction with sulfites, and 66% after 24 h, at pH 1.8. By contrast, 5-methoxyapigeninidin and 5,7-dimethoxyapigeninidin lost > 90% of their absorbance at λ_{max} within 30 min at this pH. Similar trends were observed at pH

Table 4. HPLC/MS Data Indicating the Molecular Weights of the 3-Deoxyanthocyanidin Pigments and Their Retention Times, in Relation to Their Sulfonate Adducts

	parent compound		sulfonate adduct	
compound	*m/z*	retention time (min)	*m/z* $(M + H)^+$	retention time (min)
apigeninidin	255	7.8	337	5.8
5-methoxyapigeninidin	269	8.2	351	6.6
5,7-dimethoxyapigeninidin	283	8.7	365	7.8

3.0. Hydroxylation of anthocyanins at positions 5 and 7 reduces the acidity of the heterocyclic ring and makes it less susceptible to nucleophilic attack (*9*). Thus, methoxyl substitution at these positions is likely to increase heterocyclic ring acidity and increase susceptibility of the molecule to attack by a strongly nucleophilic molecule such as SO_2. Additionally, the bisulfite complexes formed would be more stable than in compounds with hydroxyl groups at positions 5 and 7. This likely explains why apigeninidin was the most resistant to sulfite bleaching, while the 5,7-dimethoxylated derivative was the most susceptible. For example, dimethoxyapigeninidin was completely bleached at pH 5.0, whereas apigeninidin and 5-methoxyapigeninidin were not.

Another interesting observation was that the formation of chalcones at pH 5.0 by the 3-deoxyanthocyanidins allowed for a clear demonstration of the fact that the flavylium cation is the form that reacts with sulfite species as has been previously reported (*8*). Addition of bisulfite led to rapid depletion of the flavylium peaks, but a much slower reduction in chalcone peaks (**Figure 2**). This implies that as the flavylium cation reacted directly with the sulfite species to form colorless adducts, equilibrium shifted to favor conversion of the now dominant chalcones to flavylium cations which are further depleted by reaction with the bisulfite ions. **Figure 2c** clearly demonstrates this; flavylium peak disappeared immediately upon adding the dimethoxylated compound to the sulfite-containing buffer solution, whereas the chalcone peak remained unchanged (compared to control) after 5 min in sulfite solution. Even after 30 min, more than 90% of the chalcone peak was still present.

Mechanisms of Reaction of Bisulfite with the 3-Deoxyanthocyanidins. Anthocyanins are known to form colorless sulfonate adducts via reaction at C-4. However, how the 3-deoxyanthocyanins react with sulfites is yet unknown, even though Jurd (*8*) proposed that it may be via C-2. The mechanism of reaction is important since the knowledge can be used to devise strategies to protect the pigments from nucleophilic attack by sulfites, for example, via cyclic condensation reations that protect the vulnerable C-4. Furthermore, recent evidence indicates that the methoxyl substitution on the A-ring of the 3-deoxyanthocyanin molecules affects how they react with other molecules (*4*). Thus, it is important to establish whether the structure affects the mechanism reaction of the 3-deoxyanthocyanidins with sulfites.

LC-MS analysis revealed that all the 3-deoxyanthocyanidin samples formed colorless (λ_{max} 276−278 nm) sulfonic acid adducts ($A^+ HSO_3^-$) ($m/z + 81$) (**Table 4**) which were primarily responsible for the observed bleaching effects. Additionally, isolation of the sulfonate adducts via HPLC revealed similarities in terms of spectra and elution times relative to their flavylium cation (AH^+) parents, which suggested they had similar substitutions relative to their parent compounds. Thus, it is likely that the type of adducts formed from bisulfite reaction with 3-deoxyanthocyanidins was not influenced by methoxyl substitution on the A-ring.

Of particular interest was whether the bisulfite addition was at position 2 or position 4 on the C-ring. Since LC-MS data indicated the sulfonate adduct was likely similarly substituted in the 3-deoxyanthocyanidins, we used the one compound (5,7-dimethoxyapigeninidin) to establish the structure of the adducts. Comparison of ^{1}H NMR of control dimethoxyapigeninidin and that of the sulfonate adduct revealed that the most dramatic upfield shift occurred for the H-4 ($\Delta\delta$ = 4.72 ppm) and H-3 ($\Delta\delta$ = 2.75 ppm) in the sample with sulfites (**Table 5**). This indicates a loss of conjugation of the flavylium ion (*12*). The large upfield shift of the H-4 resonance relative to H-3 strongly suggest that the sulfonate adduct formed at position 4 (C-4) and not C-2 as previously suggested by Jurd (*8*).

Table 5. ^{1}H and ^{13}C NMR Spectra of 5,7-Dimethoxyapigeninidin in the Presence and Absence of Sulfites[a]

	flavylium cation		flavylium-sulfonate adduct	
carbon	^{1}H NMR δ (ppm) *J* (Hz)	^{13}C NMR δ (ppm)	^{1}H NMR δ (ppm) *J* (Hz)	^{13}C NMR δ (ppm)
2		172.5		131.3
3	8.37, *d* (8.8)	112.4	5.62, *d* (6.0)	121.2
4	9.16, *d* (8.8)	149.4	4.44, *d* (6.4)	55.0
4a		115.1		102.2
5		160.2		158.7
6	6.98, *d* (2.0)	100.9	6.25, *d* (2.4)	112.4
7		173.8		159.0
8	7.53, *d* (2.0)	94.5	6.27, *d* (2.4)	95.0
8a		158.8		154.2
1′		121.1		118.3
2′, 6′	8.45, *d* (9.2)	133.9	7.50, *d* (8.8)	130.6
3′, 5′	7.11, *d* (9.2)	118.6	6.78, *d* (8.8)	115.3
4′		168.1		160.0
-OCH_3 (C-5)	4.08	58.1	3.74	56.1
-OCH_3 (C-7)	4.07	57.9	3.70	55.8

[a] Control and sulfonated samples were dissolved in DMSO-d_6/TFA-d_1 (19: 1.0) and DMSO-d_6/TFA-d_1/SO_2 (46.5:2.5:1.0) solutions, respectively, at ambient temperature. Chemical shifts are relative to TMS.

Additional evidence from the ^{13}C spectra indicated the strongest upfield shift occurred in C-4 resonance in the dimethxyapigeninidin-sulfonate adduct relative to the dimethoxyapigeninidin flavylium cation ($\Delta\delta$ = 94.4 ppm). The C-4 carbon resonance at δ = 55.0 ppm is indicative of a heteroatom benzylic carbon (*12*). Additionally, the relatively downfield resonance of C-2 (δ = 131.3 ppm) and C-3 (δ = 121.2 ppm) even after loss of conjugation in the C-ring are indicative of a double bond between C-2 and C-3 (*12*, *18*). This further rules out sulfonate addition at C-2. Thus, it is apparent that the bleaching effect of bisulfite on the 3-deoxyanthocyanins is via a mechanism similar to that reported for anthocyanins.

Methoxyl substitution on the A-ring of the apigeninidin molecule has a major effect on its stability in the presence of sulfites. It is apparent that the loss of a hydroxyl group at position 5 or 7 significantly reduces stability of the molecule to the bleaching effect of sulfites, likely due to increased heterocyclic ring acidity which makes it more susceptible to nucleophilic attack. Even though the pigments were rapidly bleached at all pH values, most of the color was restored within 7 days for all compounds at pH 1.8, indicating an equilibrium reaction that strongly favors the flavylium cation despite the large excess of sulfite species in solution. This suggests the 3-deoxyanthocyanins may be applicable in low pH food systems containing sulfites. However, given that a large excess of SO_2 was used in this study to produce measurable bleaching effects, it is likely that the pigments would be more stable at the lower SO_2 concentrations typically encountered in food processing. The fact that colorless sulfonate adduct formation occurs via bisulfite ion addition at C-4 implies that protecting the C-4 via cyclic condensation, as occurs in the

presence of pyruvic acid, would further improve the pigment stability. Further investigation of these pigments in complex systems that closely mimic food and beverage processing and handling conditions is warranted.

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