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# Glyceraldehyde Bridging between Flavanols and Malvidin-3-glucoside in Model Solutions

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Hydroxyl radicals (•OH) seem to have an important role in the oxidation of wine constituents and the production of important electrophilic aldehydes and ketones. In this experiment, glyceraldehyde, a •OH oxidation product of glycerol, recently described in wine, reacts with (+)-catechin, (−)-epicatechin, and malvidin-3-glucoside (Mv3gl), in model solutions, yielding new condensed phenolic compounds. The adduct compounds formed were separated by means of reversed phase liquid chromatography and detected and characterized using UV−vis and electrospray ionization mass spectrometry. Flavanol−flavanol and anthocyanin−flavanol adducts linked with glyceraldehyde yielded compounds with *m*/*z* ratios for their main ions, in positive ion mode, of 653.2 for the (+)-catechin dimer or the (-)-epicatechin dimer and 855.5 for Mv3gl/(+)-catechin or Mv3gl/(−)-epicatechin dimers. The possible occurrence of these compounds in wine is suggested, and the potential role of these and related reactions in wine aging is discussed.

**KEYWORDS: Oxidation; glycerol; glyceraldehyde; flavanol; malvidin-3-glucoside; wine**

## INTRODUCTION

Oxidation reactions involving phenolic compounds, including coupling reactions with aldehydes, have been the focus of significant study (*1*−*3*). In wine, such reactions might have a foremost role in color, mouthfeel, and flavor (*1*, *4*), yet their mechanistic chemistry is only partially understood (*3*, *5*).

It has long been thought that in the presence of oxygen, part of the wine phenolic pool would "autoxidize" to quinones, also forming hydrogen peroxide (*6*). Quinones are highly reactive structures that can be substituted with a number of substances including phenolics, whereas hydrogen peroxide is the alleged oxidant responsible, mainly, for the chemical conversion of ethanol to acetaldehyde (*1*, *6*). Instead, current hypotheses based on the early observations of Fenton, Fenton and Jackson, and Haber and Weiss (*7*−*9*) indicate that these types of oxidation could be caused by hydroxyl radicals (•OH), as the product of hydrogen peroxide reacting with iron or copper salts (*3*, *5*). Given the high reactivity/low selectivity of •OH toward organic constituents, it is now hypothesized that this radical could also react with other important wine substances, thus generating numerous reactive aldehydes and ketones (*5*).

The latter hypothesis was supported in an experiment in which Fenton-generated •OH yielded glyceraldehyde and dihydroxyacetone as the main oxidation products of glycerol (**Figure 1**), even in the presence of high ethanol concentrations (12%) (*10*). In that study, it was suggested that, as with other aldehydes, these electrophilic compounds could eventually condense with flavanols and anthocyanins, thus potentially improving wine color.

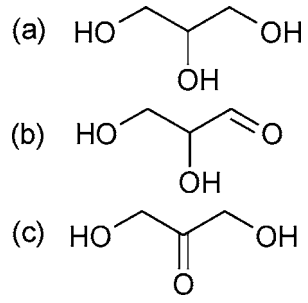


**Figure 1.** Chemical structures of (**a**) glycerol, (**b**) glyceraldehyde, and (**c**) dihydroxyacetone.

Monomeric anthocyanins, the main compounds responsible for the color of young red wine, exhibit chromatic properties that change as they copigment, self-associate, and polymerize (*11*, *12*). As the wine ages, the fraction of monomeric anthocyanins seems to decrease mainly due to direct tannin addition (*13*), yet other forms of polymerization have also been described.

In 1976, Timberlake and Bridle (*14*) suggested one type of pigmentation reaction where acetaldehyde, the main oxidation product of ethanol, served as mediator in the condensation of anthocyanins and flavanols. Since then, the formation of these types of ethyl-linked adducts has been extensively studied (*15*−*20*). Another example of an aldehyde oxidation product mediating this type of polymerization reaction is glyoxylic acid. This compound, resulting from the chemical oxidation of tartaric acid, was observed in a study of the iron-catalyzed oxidation of (+)-

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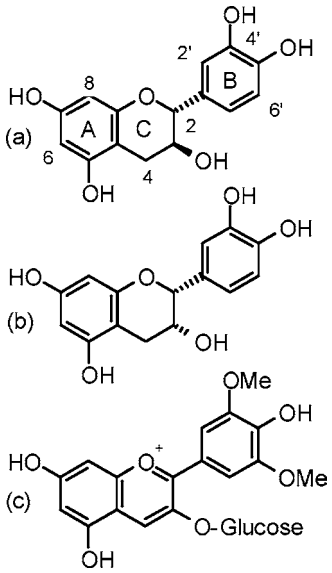


**Figure 2.** (**a**) (+)-Catechin, (**b**) (-)-epicatechin, and (**c**) malvidin-3-glucoside chemical structures.

catechin (*21*) and was subsequently described as an alternative to acetaldehyde in the bridging of flavonoids in wine (*22*). Later observations demonstrated that the resulting products from the reactions between flavanols and glyoxylic acid can further rearrange to xanthylium pigments (*23*).

Considering that wine's glycerol concentration, 5−20 g $L^{-1}$, is comparable to or higher than that of tartaric acid, 2−8 g $L^{-1}$ (approximately 54−217 mM for glycerol and 13−53 mM for tartaric acid) (*24*, *25*), and in view of the scarcity of information on this compound in wine, we chose to study glyceraldehyde as a new possible mediator for flavonoid polymerization. The aim of this work was to evaluate the chemical interaction between (+)-catechin, (−)-epicatechin, and malvidin-3-glucoside (**Figure 2**) in the presence of glyceraldehyde in acidic wine-like model solutions and to characterize the UV−vis and mass spectra of the newly formed products resulting thereof.

## MATERIALS AND METHODS

**Reagents.** Solutions and dilutions were prepared using deionized water purified through a Milli-Q system (Millipore, Bedford, MA). (+)-Catechin, (−)-epicatechin, and DL-glyceraldehyde (95%) were purchased from Sigma-Aldrich (Milwaukee, WI), whereas malvidin-3-glucoside (Mv3gl) was kindly provided by Professor Peter Winterhalter (Technische Universitat Braunschweig, Germany). Methanol, HPLC grade, and acetic acid glacial, approximately 17.4 N, were obtained from Fisher (Fair Lawn, NJ), whereas ethanol (100%) was purchased from Gold Shield Chemical Co. (Hayward, CA).

**Model Solution and Reactions (Flavanol−Flavanol and Anthocyanin−Flavanol).** An acidic wine-like model solution, containing 12% ethanol and 4% acetic acid, giving a pH value of 2.5, was used as the solvent for all reactions. It was difficult to attain this pH with other more typical fruit acids. Solutions of (a) (+)-catechin and DL-glyceraldehyde; (b) (−)-epicatechin and DL-glyceraldehyde; (c) (+)-catechin, (−)-epicatechin, and DL-glyceraldehyde; (d) Mv3gl, (+)-catechin, and DL-glyceraldehde; and (e) Mv3gl, (−)-epicatechin, and DL-glyceraldehyde were prepared as follows: Solutions a and b were separately prepared in 2.0 mL reaction vials by dissolving 1.2 mg of each flavanol and an excess of 18.0 mg of DL-glyceraldehyde per vial, to give a molar concentration of approximately 2 mM for the flavonoids and 100 mM for the aldehyde. Similarly, solutions containing mixtures of (+)-catechin and (−)-epicatechin (solution c), Mv3gl and (+)-catechin (solution d), and Mv3gl and (−)-epicatechin (solution e) were prepared at equimolar concentrations (1 mM each) and were also treated with 100 mM DL-glyceraldehyde. All solutions, in triplicate, were kept in the dark at 36 °C, and the progress of these reactions was monitored at hourly or daily intervals (depending on time since the reaction had been started) by high-performance liquid chromatography with diode array (UV−vis) and electrospray mass spectrometry (HPLC/DAD-ESI/MS) detections.

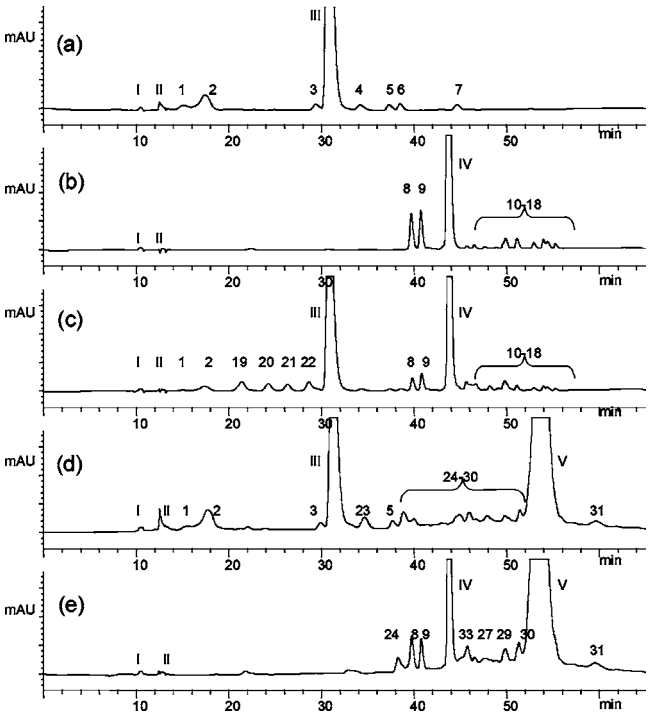


**Figure 3.** Chromatographic profiles (280 nm traces) of the reactions between (**a**) (+)-catechin (peak III) and DL-glyceraldehyde; (**b**) (–)-epicatechin (peak IV) and DL-glyceraldehyde; (**c**) (+)-catechin, (–)-epicatechin, and DL-glyceraldehyde; (**d**) malvidin-3-glucoside (Mv3gl) (peak V), (+)-catechin, and DL-glyceraldehde; and (**e**) Mv3gl, (–)-epicatechin, and DL-glyceraldehyde in an acidic wine-like model solution.

**HPLC/DAD-ESI/MS Analyses.** The chromatographic separations were performed on a Hewlett-Packard, reversed phase HPLC, 1100 series, with photodiode array UV−vis and electrospray ionization mass spectrometry detectors (HP 1100 MSD) coupled to Agilent Chemstation software (A 09.03) (Palo Alto, CA). UV−vis spectra were recorded from 240 to 700 nm with automatic detection traces at 280, 316, 420, and 520 nm, whereas the MS detector was set to scan in positive ion mode, from *m*/*z* 100 to 1500. The MS parameters used were a capillary voltage of 3500 and fragmentor at 50 V. The drying gas flow was set at 12 L $min^{-1}$, the nebulizer pressure at 241.32 kPa, and the drying gas temperature at 350 °C. The chromatographic separation was achieved using a C18 LiChrospher column (4 mm × 250 mm, 5 $\mu$m particle size) (Merck, Whitehouse Station, NJ) protected with a guard column of the same material. The mobile phase consisted of a binary gradient of (A) aqueous 1% acetic acid solution and (B) 0.5% acetic acid in methanol as follows: 0 min, 20% B; 20 min, 25% B; 40 min, 50% B; 50 min, 80% B; 60 min, 20% B; and 65 min, 20% B, with 10 min of equilibration time between runs. The sample injection volume was 20 $\mu$L and the flow rate 0.2 mL $min^{-1}$.

**HPLC/ESI Tandem Mass Spectrometry (MS/MS) Analyses.** Analytes were separated using a reversed phase HPLC system (Shimadzu Scientific, Columbia, MD) equipped with an SIL-10A autoinjector and binary LC 10 AD pumps, operated under the same chromatographic conditions previously described. The column eluent was directed into a Quattro LC triple-quadrupole mass spectrometer (Micromass, Altrincham, U.K.) equipped with a dual orthogonal (ZSPRAY) ion source. The peaks with *m*/*z* of interest were analyzed in selected ion monitoring (SIM) mode using ESI-MS/MS. The MS was operated in positive ion mode using a capillary voltage of 3 kV. The cone and extractor voltages were set to 50 and 2 V, respectively. The source and desolvation temperatures were 140 and 350 °C respectively, and the nebulizer and desolvation flows were set at 67

**Table 1.** Spectral Information on the Observed Peaks after Reactions between (+)-Catechin and DL-Glyceraldehyde (Peaks I–III and 1–7); between (–)-Epicatechin and DL-Glyceraldehyde (Peaks I, II, and IV and 8–18); between (+)-Catechin, (–)-Epicatechin, and DL-Glyceraldehyde (Peaks I–IV and 1, 2, 8–22); between Mv3gl, (+)-Catechin, and DL-Glyceraldehyde (Peaks I–III and V and 1–3, 5, and 23–31); and between Mv3gl, (–)-Epicatechin, and DL-Glyceraldehyde (Peaks I, II, IV, and V and 8, 9, 24, and 27–32)

| peak | RT (min) | $\lambda_{UV-vis\ max}$ (nm) | molecular ions (*m*/*z*) |
|---|---|---|---|
| I | 10.45 | nd | 203.3 |
| II | 12.65 | 280 | 159.2 |
| III (catechin) | 30.83 | 280 | 291.3 |
| IV (epicatechin) | 43.78 | 280 | 291.3 |
| V (Mv3gl) | 53.54 | 532 | 493.3 |
| 1 | 15.11 | 280 | 159.3 |
| 2 | 17.41 | 280 | 653.5 |
| 3 | 29.37 | 280 | 653.4 |
| 4 | 34.19 | 280 | 653.5 |
| 5 | 37.31 | 280 | 653.5 |
| 6 | 38.49 | 280 | 653.5 |
| 7 | 44.68 | 280 | 653.4 |
| 8 | 39.69 | 280 | 653.5 |
| 9 | 40.72 | 280 | 653.5 |
| 10 | 45.69 | 280 | 291.3 |
| 11 | 46.48 | 280 | 635.4 |
| 12 | 47.62 | 280 | 635.3 |
| 13 | 49.88 | 278 | 275.3 |
| 14 | 51.10 | 280 | 653.4 |
| 15 | 52.93 | 280 | 653.4 |
| 16 | 53.96 | 280 | 653.4 |
| 17 | 54.40 | 280 | 653.5 |
| 18 | 55.26 | 280 | 363.4 |
| 19 | 21.40 | 280 | 653.4 |
| 20 | 24.25 | 280 | 653.4 |
| 21 | 26.34 | 280 | 653.4 |
| 22 | 28.64 | 280 | 653.4 |
| 23 | 34.78 | 545 | 855.5 |
| 24 | 38.88 | 544 | 855.5 |
| 25 | 40.05 | 280 | 493.4 |
| 26 | 44.97 | 280 | 207.4 |
| 27 | 46.03 | 274 | 493.3 |
| 28 | 47.98 | 318 | 493.3 |
| 29 | 49.82 | 324 | 325.3 |
| 30 | 51.43 | 348 | 349.3 |
| 31 | 59.58 | 538 | 521.3 |
| 32 | 45.74 | 540 | 855.5 |

and 467 L $h^{-1}$, respectively. Fragment ions were generated using data-dependent scanning techniques. The data were collected and processed using MassLynx software (v 3.5) (Micromass, Beverley, MA).

## RESULTS AND DISCUSSION

**HPLC/DAD-ESI/MS and MS/MS Analyses.** After the appropriate chromatographic conditions were determined, the reactions' progress was monitored with HPLC-DAD/ESI-MS over successive sample injections. The general trend of all reactions showed a decrease in the peak areas of the flavonoid reactants used and the appearance of several new peaks with distinctive retention times and spectral characteristics, indicating, as suggested (*10*), the formation of new phenolic compounds. **Figure 3** provides examples of typical HPLC chromatograms, with traces collected at 280 nm, for the reactions of (a) (+)-catechin and DL-glyceraldehyde; (b) (−)-epicatechin and DL-glyceraldehyde; (c) (+)-catechin, (−)-epicatechin, and DL-glyceraldehyde; (d) Mv3gl, (+)-catechin, and DL-glyceraldehde; and (e) Mv3gl, (−)-epicatechin, and DL-glyceraldehyde. All chromatographic samples analyzed showed two early eluting peaks, labeled I and II, with *m*/*z* ratios for their main ions at 203.3 and 159.2, which were not further identified (**Figure 3**).

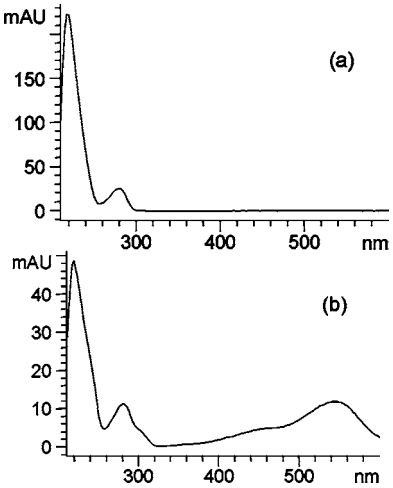


**Figure 4.** Example of UV–vis spectra of compounds formed during the reaction of (**a**) flavanols [(+)-catechin and (–)-epicatechin] and DL-glyceraldehyde and (**b**) malvidin-3-glucoside, flavanols, and DL-glyceraldehyde.

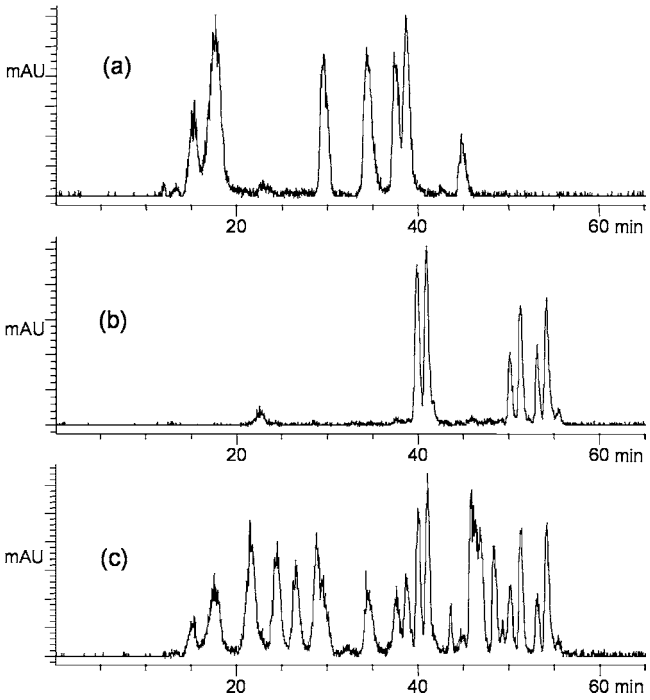


**Figure 5.** Single ion monitoring (SIM) chromatograms for ion *m*/*z* 653.4, corresponding to flavanol dimers bridge by glyceraldehyde: reactions between (**a**) (+)-catechin and DL-glyceraldehyde; (**b**) (–)-epicatechin and DL-glyceraldehyde; and (**c**) (+)-catechin, (–)-epicatechin, and DL-glyceraldehyde.

The results of flavanol−flavanol and flavanol−anthocyanin reactions were as follows.

*Flavanol−Flavanol Reactions.* After approximately 12 h, the chromatographic trace at 280 nm of the reaction of (+)-catechin (peak III, **Figure 3a**) and DL-glyceraldehyde (reaction a) showed seven significant peaks, labeled 1−7 (**Figure 3a**). The relative peak areas of the newly formed peaks were comparable, except for that eluting at 17.4 min (peak 2), thus suggesting that the reaction was favored toward the formation of this particular compound. In the case of reaction b, (−)-epicatechin (peak IV, **Figure 3b**) and DL-glyceraldehyde, 11 new peaks were observed, 2 of which had higher peak areas (peaks 8 and 9, with retention times at 39.7 and 40.7 min, respectively) than the other analytes detected (**Figure 3b**). Moreover, reaction c, (+)-catechin, (−)-

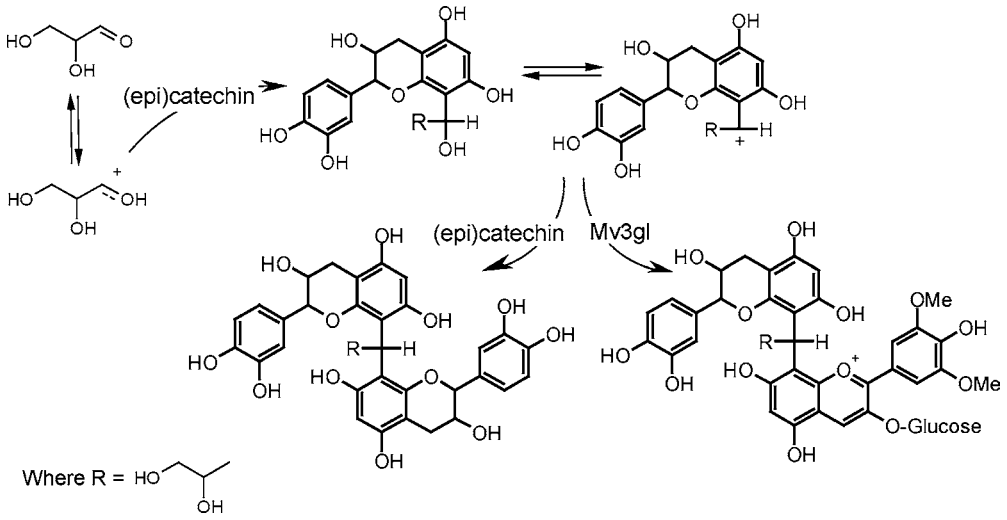


**Figure 6.** Reaction of glyceraldehyde with (+)-catechin, (–)-epicatechin, and/or malvidin-3-glucoside in acidic media.

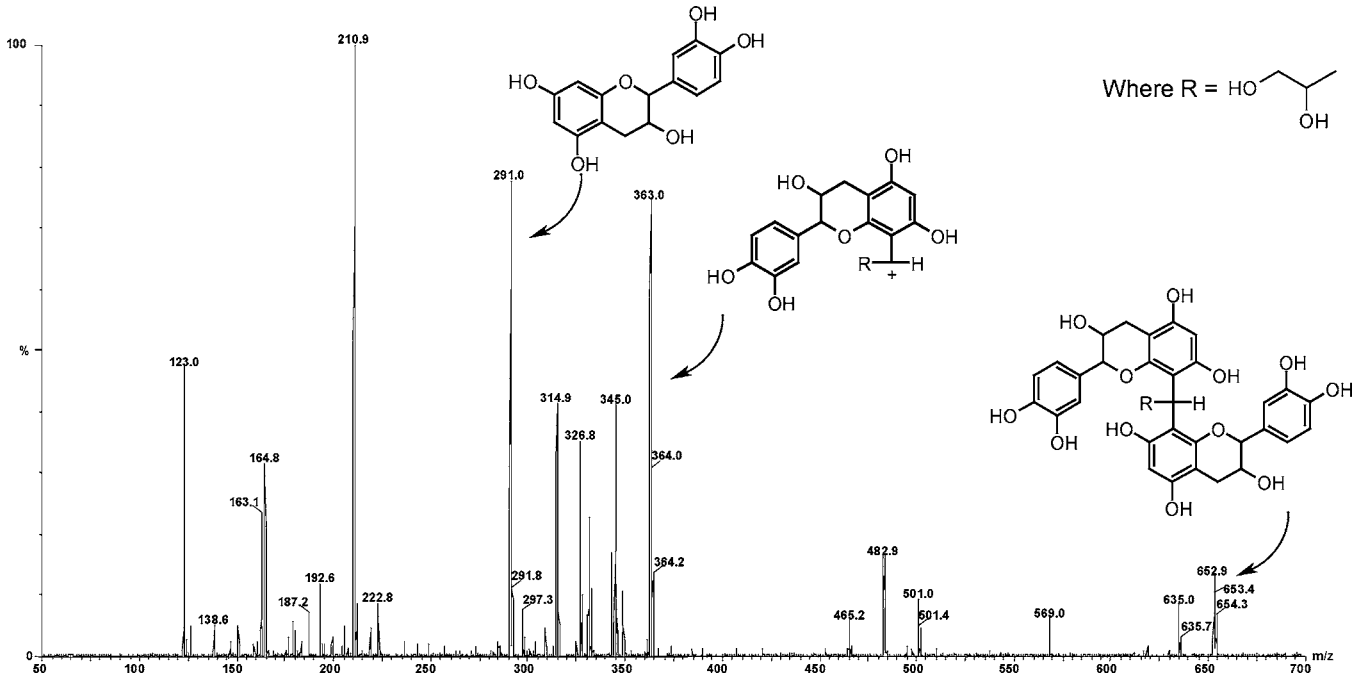


**Figure 7.** Chemical structures and MS/MS example for (+)-catechin and DL-glyceraldehyde reaction products.

epicatechin (peaks III and IV, **Figure 3c**), and DL-glyceraldehyde, showed evidence of more than 16 peaks, several of which had retention times and spectral properties matching those of peaks observed for reactions a and b (**Figure 3c** and **Table 1**).

The UV−vis spectra of the products formed were similar to those of the pure reactants used, with $\lambda_{max}$ of 280 nm (**Figure 4a**), hence indicating that these reaction products probably retain their original flavanol structures (*26*). Nevertheless, in the case of some of the later eluting products, besides the main absorption peak at 280 nm, a small absorption peak at approximately 245 nm was also noted (data not shown). At this point, it is not clear whether this variation is due to specific chromatic characteristics of these compounds or just owed to coelution with minor intermediates.

Total ion current chromatogram (TIC) analyses of reactions a−c showed that, for each reaction, several of the peaks observed, at different retention times, had the same *m*/*z* ratios for their main ions at 653.4 (**Table 1**), therefore indicating that the structures formed might have the same constitutive units, but linkages at different positions. In **Figure 5**, SIM analysis at *m*/*z* 653 shows seven peaks for reactions a and b and ca. 17 peaks for reaction c. This *m*/*z* ratio observed was consistent with the calculated molecular weight of a dimeric (+)-catechin and/or (−)-epicatechin structure bridged by glyceraldehyde (MW 652.6) (**Figure 6**). Moreover, the *m*/*z* ratios of other ions observed in these chromatograms include 363.4, quotient matching the theoretical value for the intermediate catechin−glyceraldehye adduct (**Figure 6**), 291.3 corresponding to the flavanols (+)-catechin or (−)-epicatechin, and the unknown 275.3.

The presence of several ion peaks detected under the same *m*/*z* ratio suggest the occurrence of isomeric structures for each C-6 or C-8 position of the A ring of the flavonoids used, and the *R* or *S* configuration of the interflavonoid carbon (*22*). In working with these epimers, that is, (+)-catechin and (−)-epicatechin, Fulcrand cited isomerization at C-2 to yield the enantiomer of the other as a likely cause for the difference in the number of peaks expected and observed (*16*).

Peaks showing *m*/*z* values matching possible glyceraldehyde addition products were further investigated using tandem mass

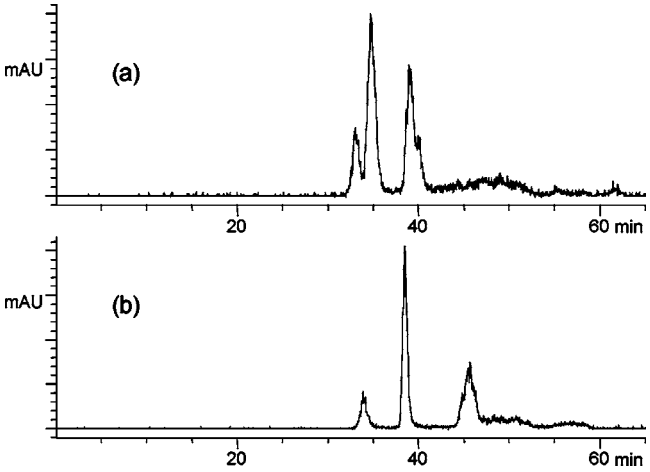


**Figure 8.** Selected ion monitoring (SIM) chromatograms for ion *m/z* 855.4: reactions between (**a**) malvidin-3-glucoside, (+)-catechin, and DL-glyceraldehyde and (**b**) malvidin-3-glucoside, (–)-epicatechin, and DL-glyceraldehyde.

spectrometry. The information on ion fragmentation obtained showed the existence of three main product ions at 210.9, 291.0, and 363.0. The ion corresponding to *m/z* 210.9 might have resulted from the retro-Diels–Alder (RDA) decomposition of the flavanol unit ($[M - 152]^+$) in the (+)-catechin [or (−)-epicatechin]–glyceraldehyde adduct (*27*), *m/z* 291.0 corresponds to monomeric (+)-catechin [or (−)-epicatechin], and *m/z* 363.0 corresponds to the (+)-catechin [or (−)-epicatechin]–glyceraldehyde adduct, respectively (**Figures 6** and **7**). Other fragments observed were the RDA decomposition of the glyceraldehyde dimer adduct (*m/z* 501) and its water loss $[M - 18]^+$ to give *m/z* 482.9. The ion at *m/z* 345 $[M - 18]^+$ resulted from the water loss of the ion *m/z* 363.0.

*Anthocyanin–Flavanol Reactions.* Reactions d and e, Mv3gl/(+)-catechin and Mv3gl/(−)-epicatechin, respectively, exhibited the formation of several new peaks with various maximum absorptions of visible light [**Figure 3d**,**e**, where peak III is (+)-catechin, peak IV is (-)-epicatechin, and peak V is Mv3gl]. As expected, some of these newly formed compounds have the same retention times and UV–vis spectra ($\lambda_{max}$ 280 nm) as the peaks observed in the reactions of flavanols alone (reactions a–c), thus indicating also the occurrence of the flavanol–flavanol condensation products.

It has to be noted that although the maximum absorption of Mv3gl is close to 530 nm, five of the newly formed products here exhibited a shift in their maximum absorption to approximately 538–546 nm (**Table 1** and **Figure 4b**). Such a change would alter the appearance of a Mv3gl solution, changing the appearance of the solution's hue from red to purplish red. In our previous study, excess additions of DL-glyceraldehyde or dihydroxyacetone to wine were shown to change the color density of a young red wine by increasing light absorption at 420 and 520 nm (*10*). On the basis of the observations here, it is possible that these color changes might have occurred via a flavonoid condensation reaction mechanism similar to those previously reported for acetaldehyde, glyoxylic acid, and furfural and its derivatives (*14*, *15*, *22*, *28*).

TIC and SIM mass spectral analyses of reactions d and e showed that, for each reaction, three of the peaks observed had the same *m/z* ratios for their main ions at 855.5 (**Table 1** and **Figure 8**). This value is in agreement with the expected molecular weight of the calculated product (854.6) between Mv3gl and either of the flavanols used linked by glyceraldehyde. The *m/z* ratios of other ions observed in these chromatograms were 493.4, like the *m/z* of monomeric Mv3gl, and the unknowns 207.4, 325.3, 349.3, and 521.3 (**Table 1**). Note that peaks 29 and 30 (*m/z* 325.3 and 349.3) were the only compounds with odd $\lambda_{max}$ at 324 and 348, respectively. Such $\lambda_{max}$ might indicate that these peaks are derived from the Mv3gl chromophore, but no further identification was attempted. In all cases, TIC traces were collected by scanning ions over a mass range of *m/z* 100–1500, encompassing the masses of the monomeric flavanols and the theoretical addition products up to four phenolic units, which were not observed.

In addition, **Figure 9** shows the fragmented mass spectra of ion *m/z* 855.5, with three product ions at 693.3 [malvidin aglycone–glyceraldehye–(+)-catechin], 565.1 (Mv3gl–

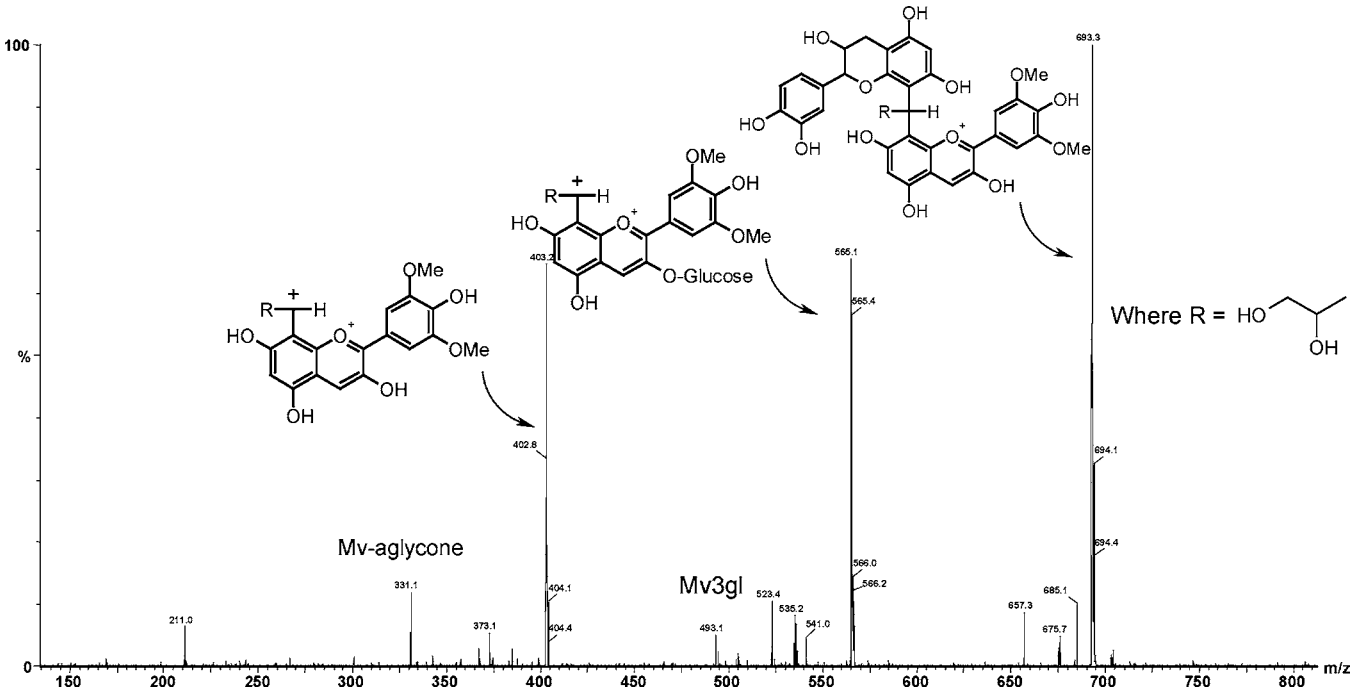


**Figure 9.** Chemical structures and MS/MS example for malvidin-3-glucoside, (+)-catechin, and DL-glyceraldehyde reaction products.

glyceraldehyde−adduct), and 403.2 (malvidin aglycone−glyceraldehyde adduct). Also, the fragments corresponding to the loss of one or two water molecules from *m*/*z* 693 to give *m*/*z* 675 and 657, respectively, and the fragments corresponding to Mv3gl (*m*/*z* 493) and its aglycone at *m*/*z* 331 were observed. Once again, the masses and the fragments obtained here are in agreement with the theoretical masses and formation mechanism of these types of compounds (*14*), hence indicating a polymerization mechanism similar to the one proposed for acetaldehyde and glyoxylic acid. In brief, according to this reaction mechanism, a protonated aldehyde can undergo a nucleophilic attack by a flavonoid at positions C-6 or C-8 of the A ring (**Figure 2**), forming a flavonoid adduct. If this adduct loses a water molecule to generate a carbocation, it can be further attacked by other flavonoids, forming a dimer (**Figure 6**). If the process is repeated at additional positions, larger oligomers could be produced, but steric interactions between the 6- and 8-position linkages might make such polymerization slow (*14*, *22*, *28*).

Even though a much smaller molar fraction of aldehyde to phenolics is expected under wine conditions, the long times available under wine aging conditions suggest these compounds may still make a contribution to the color and other sensory properties of red wine, an important goal of future investigations.

To conclude, the spectrometric analyses used for chemical elucidation of the new pigments formed were consistent with calculated data suggesting that polymerization of flavonoids with glyceraldehyde occurs via the same mechanism described for acetaldehyde-bridged polymerization (*14*). This type of polymerization has been well documented for other types of aldehydes, and it would appear that additional aldehydes or ketones could be observed from other alcohols, including fruit acids and sugars. However, the significance of these products in color enhancement compared to that of direct anthocyanin−tannin addition, as well as their significance in flavor and mouthfeel properties, requires further study. Their concentrations compared with that of acetaldehyde as well as their relative reactivities will affect their relative contributions to products, but their importance in changing color or flavor is more difficult to assess. Finally, further NMR characterization of these compounds could help to clarify the specific configuration that determines the preference in synthesis of one isomer over another.

## ABBREVIATIONS USED

OH, hydroxyl radicals; Mv3gl, malvidin-3-glucoside; HPLC/DAD/MSD, high-performance liquid chromatography, diode array, and mass spectrometry detection; MS, mass spectrometry, MS/MS, tandem mass spectrometry; UV−vis, ultraviolet−visible; $\lambda_{max}$, wavelength of absorption maximum; TIC, total ion current chromatogram; SIM, single ion monitoring; NMR, nuclear magnetic resonance spectroscopy.

## ACKNOWLEDGMENT

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