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(+)-Catechin−Aldehyde Condensations: Competition between Acetaldehyde and Glyoxylic Acid

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(+)-Catechin reaction with two aldehydes (acetaldehyde and glyoxylic acid) was studied in winelike model solution. The two aldehydes were reacted either individually or together with (+)-catechin and in molar excess. The reactions were followed by HLPC-UV and HPLC-ESI/MS to monitor (+)-catechin disappearance as well as dimer and polymer appearance. In all reactions a reaction order of close to 1 for (+)-catechin disappearance was observed. (+)-Catechin disappearance was slower in the presence of acetaldehyde ($t_{1/2} = 6.7 \pm 0.2$ h) compared to glyoxylic acid ($t_{1/2} = 2.3 \pm 0.2$ h). When the two aldehydes were reacted together, (+)-catechin disappearance was faster ($t_{1/2} = 2.2 \pm 0.5$ h). When aldehydes were reacted separately, the dimer appearance was independent of the type of aldehyde used but the ethyl-bridged dimer disappearance was slower with acetaldehyde. When aldehydes were reacted together, the dimer appearance changed. Ethyl-bridged dimers appeared before carboxymethine-bridged dimers, and their disappearance occurred earlier. Copolymers containing both ethyl and carboxymethine bridges were also observed.

KEYWORDS: Phenolic compounds; flavanols; (+)-catechin; glyoxylic acid; acetaldehyde; condensation; LC-UV; LC-ESI/MS

INTRODUCTION

Phenolic compounds play an important role in wine quality because of their color and taste properties. Condensed tannins (proanthocyanidins) influence bitterness and astringency (*1*, *2*) and are involved in wine colloidal (*3*) and color stability (*4*−*6*). In the grape, they consist of polymers of flavan-3-ol units [(+)-catechin, (−)-epicatechin (−)-epigallocatechin, and (−)-epicatechin-3-*O*-gallate] with C4−C6 or C4−C8 linkages (*7*).

During winemaking and aging, tannins undergo enzymatic or chemical modifications. An important chemical fate is the acid-catalyzed cleavage of the interflavan bond and subsequent condensation reactions (*4*, *8*, *9*). Among these reactions, nucleophilic substitutions have been demonstrated in model wine involving the C6 or C8 of the A-ring of monomeric flavanols (*10*). Electrophilic molecules identified in these bridging reactions include acetaldehyde and glyoxylic acid (*10*−*14*). Acetaldehyde may be produced in two ways: production by yeasts (*Saccharomyces cerevisiae*) (*16*) and oxidation of ethanol (*17*). Glyoxylic acid is a product of tartaric acid oxidation (*14*, *18*−*20*).

In acidic media and for flavanol monomers, the first step of the condensation reaction with glyoxylic acid or acetaldehyde is the formation of colorless dimers, linked by a carboxymethine or ethyl bridge, respectively (**Figure 1**). It has been postulated (*9*, *11*−*14*) that the overall reaction pathways are similar.

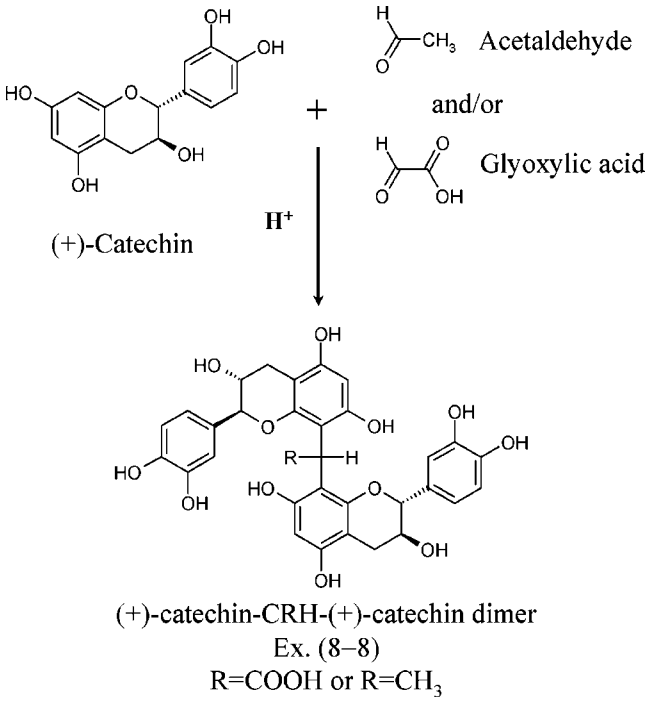

Figure 1. Reaction pathway of (+)-catechin–aldehyde condensation leading to bridged dimers.

Overall, the dimers are the first intermediates before further polymerization (*3*, *11*), and these products may also be

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Table 1. Reaction Media Realized

condensation type	[(+)-catechin]	[glyoxylic acid]	[acetaldehyde]
(+)-catechin–glyoxylic acid	200 mg·L^{-1} (0.7 mmol·L^{-1}) 600 mg·L^{-1} (2.1 mmol·L^{-1}) 1000 mg·L^{-1} (3.5 mmol·L^{-1})	10 g·L^{-1} (135 mmol·L^{-1})	
(+)-catechin–acetaldehyde	200 mg·L^{-1} (0.7 mmol·L^{-1}) 600 mg·L^{-1} (2.1 mmol·L^{-1}) 1000 mg·L^{-1} (3.5 mmol·L^{-1})		9.8 g·L^{-1} (222 mmol·L^{-1})
(+)-catechin–glyoxylic acid–acetaldehyde	200 mg·L^{-1} (0.7 mmol·L^{-1}) 600 mg·L^{-1} (2.1 mmol·L^{-1}) 1000 mg·L^{-1} (3.5 mmol·L^{-1})	10 g·L^{-1} (135 mmol·L^{-1})	5.9 g·L^{-1} (133 mmol·L^{-1})

precursors to other reactions. One possible secondary reaction is the oxidation of dimers leading to the formation of xanthylium salts (*11*, *19*−*23*).

To further investigate the reaction between flavanols and glyoxylic acid/acetaldehyde, (+)-catechin was allowed to react in a model solution with these compounds. Because these reactions involve only the A-ring of flavanols, (+)-catechin serves a good model compound for equivalent proanthocyanidin reactions.

The purpose of this study was to study the reaction kinetics of (+)-catechin with acetaldehyde and glyoxylic acid. The aldehydes were incubated either alone or together but in molar excess compared to (+)-catechin.

MATERIALS AND METHODS

Materials. The water used was deionized water purified with a Milli-Q water system (Millipore, Bedford, MA). Acetonitrile (HPLC grade) was obtained from Fischer Chemicals (Elancourt, France), ethyl alcohol (HPLC grade) from Carlo Erba (Val de Reuil, France), and acetaldehyde (RP) from Riedel-De Haën (Val de Reuil, France); methanol (HPLC grade), acetic acid (RP), and l-tartaric acid were obtained from Prolabo-VWR (Fontenay s/Bois, France). (+)-Catechin and glyoxylic acid were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Reactions. A model wine solution was prepared with 12% (v/v) ethanol and 5 g·L^{-1} tartaric acid and adjusted to pH 3.2 with 1 N sodium hydroxide. The reagents used were (+)-catechin, glyoxylic acid, and acetaldehyde. The reaction media used are presented in **Table 1**. Each reaction was replicated three times, with averages and standard deviations calculated for each reaction. Each mixture was separated into sealed vials (1.5 mL) and incubated at 40 °C. The reactions were monitored by high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) and a mass spectrometer (MS).

Analytical HPLC-UV Analysis. HPLC-UV analyses were performed by means of a Beckman System Gold (Beckman, Roissy Charles-de-Gaulle, France), which included a manual injector, a 126 pump module, and a 168 diode array detector and with all systems operated using 32Karat 5.0 software. UV−vis absorption spectra were recorded from 200 to 900 nm. The column was a reversed-phase Interchrom UP3 ODB-10QS (3 μm packing, 100 × 4.6 mm i.d.) (Interchim, Montluçon, France). Elution conditions were as follows: flow rate, 1 mL·min^{-1}; room temperature (20 °C); 20 μL sample loop; solvent A, water/acetic acid (99:1, v/v); solvent B, acetonitrile/solvent A (80:20, v/v). The elution gradient for the reaction of (+)-catechin−glyoxylic acid was as follows: 0−34% B in 28 min, 34−100% B in 0.50 min, 100% B for 3 min, 100−5% B in 0.5 min, 0% B for 4 min. The elution gradient for the reaction of (+)-catechin−acetaldehyde was as follows: 0−20% B in 1 min, 20−30% B in 5.5 min, 30−50% B in 12.5 min, 50−100% B in 0.50 min, 100% B for 3 min, 100−5% B in 0.5 min, 0% B for 4 min. The elution gradient for the reaction of (+)-catechin−glyoxylic acid−acetaldehyde was as follows: 0−50% B in 48 min, 50−100% B in 0.50 min, 100% B for 3 min, 100−5% B in 0.5 min, 0% B for 4 min.

MS Apparatus and LC-MS Analysis. LC-MS analyses were performed on a Micromass Platform II simple quadruple mass spectrometer (Micromass-Beckman, Roissy Charles-de-Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in the negative-ion mode. Source temperatures were 120 and 45 °C, capillary voltage was set at ±3.5 kV, and cone voltages of −30 and −90 V were used. Mass spectra were recorded from 100 to 2000 amu. HPLC separations were performed on a Hewlett-Packard 1100 series (Agilent, Massy, France) including a pump module and a UV detector. Both systems were operated using Masslynx 3.4 software. Column and separation conditions were identical to those used for analytical HPLC-UV analysis. Flow rate was 1 mL·min^{-1} for the column and 0.1 mL·min^{-1} for the MS source, and the sample loop was 50 μL. The absorbance was recorded at 280 nm.

Kinetics Studies and Compound Identification and Quantification. Kinetics studies were monitored by HPLC-UV. Compound quantification was made by comparing peak area (280 nm) with that of a (+)-catechin standard. All results were expressed in milligrams per liter of (+)-catechin equivalents. Peak identity was determined by mass spectrometry and by comparison with previous studies (*3*, *11*, *12*).

(+)-Catechin Disappearance Kinetic Order and $t_{1/2}$ Calculation. (+)-Catechin disappearance rate was calculated with an integral method. Because aldehydes were in excess, the (+)-catechin disappearance rate (v) was defined as $v = -k'[(+)\text{-catechin}]^{\beta}$. k' corresponded to the reaction constant and β to the reaction order. These coefficients were calculated by fitting $\ln(v_0) = \ln(k') + \beta \ln([(+)\text{-catechin}]_{initial})$. To calculate the initial (+)-catechin disappearance rate (v_0), the experimental data points of kinetic curves were approximated by mathematical regressions for each (+)-catechin initial concentration. v_0 corresponded to the value at $t = 0$ for the differential mathematical regressions.

Kinetic Index Calculation of Dimer Kinetic Evolution. The kinetic curves were approximated by fitting the experimental data points by mathematical regressions. The theoretical curves obtained were used to calculate the maximal concentrations and the corresponding indices. t_{max} was defined as the time when the maximal dimer concentration (C_{max}) was reached, and t_{50app} corresponded to the half-appearance time of dimers.

Polymer Analysis. To stop the reaction and to purify polymers, a solid-phase extraction (SPE) step was used. Each sample was purified on a C_{18} cartridge (Supelco, St Quentin Fallavier, France) as follows: the column was conditioned, and the sample was applied. The column was washed with 15 mL of water to remove tartaric acid and excess aldehyde. The polymers were then eluted with 5 mL of methanol. The methanol fraction was injected directly onto the mass spectrometer. Direct injections were performed as described above but with the following conditions: no column was used, the flow rate was 0.1 mL·min^{-1} for the MS source, the sample loop was 20 μL, the source temperature was 45 °C, the cone voltage was −90 V, and the run time was 5 min: 100% solvent B was used for analytical HPLC-UV analysis. For both glyoxylic acid-mediated and acetaldehyde-mediated condensations, polymer analyses were performed on reaction media containing 1000 mg·L^{-1} (+)-catechin and after a 24 h reaction time. When the

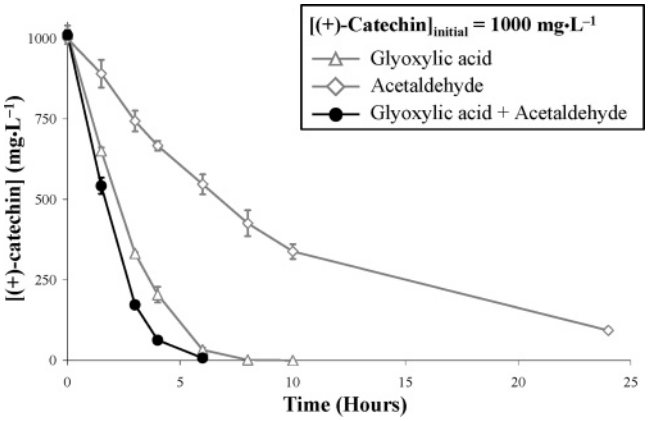

Figure 2. Comparison of (+)-catechin disappearance kinetics during (+)-catechin–aldehyde condensation. The aldehydes were incubated separately or together, and initial [(+)-catechin] was 1000 mg·L^{-1} (±SD, $N = 3$).

Table 2. β and $t_{1/2}$ Coefficients for (+)-Catechin Disappearance in the Presence of Acetaldehyde, Glyoxylic Acid, or Acetaldehyde + Glyoxylic Acid[a]

reactant	β	$t_{1/2}$ (h)
glyoxylic acid	1.3 ± 0.1	2.3 ± 0.2
acetaldehyde	0.9 ± 0.0	6.7 ± 0.2
glyoxylic acid + acetaldehyde	0.8 ± 0.1	2.2 ± 0.5

[a] Aldehydes were incubated separately or together. Reaction rate is defined as $r = -K'$ [(+)-cat]$^{\beta}$ (±SD, $N = 3$).

aldehydes were reacted together, the polymer analyses were carried out after a 4 h reaction time.

RESULTS AND DISCUSSION

(+)-Catechin disappearance was investigated in a winelike solution containing glyoxylic acid and acetaldehyde. The aldehydes were reacted separately or together with (+)-catechin and were present in excess. The solutions were maintained at 40 °C to accelerate reaction rates, and the disappearance of (+)-catechin and the appearance of new compounds were monitored as a function of time.

(+)-Catechin Disappearance. As previously described (*3*, *11*−*14*), (+)-catechin reacts with aldehydes in winelike model systems (**Figure 2**), and the rate of (+)-catechin disappearance depends on the type of aldehyde used. In this study, (+)-catechin was consumed in <7 h for glyoxylic acid, whereas >24 h was necessary with acetaldehyde. When the two aldehydes were reacted together, (+)-catechin was consumed in <6 h.

To calculate the (+)-catechin disappearance kinetic order and the half-reaction times ($t_{1/2}$), reactions at three (+)-catechin initial concentrations were monitored (**Table 2**). Consistent with previous work (*3*) and for acetaldehyde, the (+)-catechin disappearance followed first-order kinetics. With glyoxylic acid the order was close to 1, although higher (1.3 ± 0.1). When the two aldehydes were reacted together, the order was still close to 1, although lower (0.8 ± 0.1).

Consistent with first-order kinetics, the $t_{1/2}$ data were independent of (+)-catechin initial concentration (**Table 2**). In addition, (+)-catechin disappearance was fastest when the two aldehydes were reacted together. When the aldehydes were reacted separately, the reaction of (+)-catechin was 3 times faster with glyoxylic acid.

Evolution of Reaction Intermediates RI_1. The pathway proposed for the reaction of (+)-catechin with aldehyde is an acid-catalyzed nucleophilic substitution. This pathway can be divided into four steps (R1 → R4). With regard to R1 and R2, aldehydes and (+)-catechin react to form the first reaction intermediate (RI_1). Here, aldehyde protonation to form a C^+ carbocation (R1) is followed by nucleophilic attack (R2) by C-6 or C-8 of (+)-catechin to form the corresponding benzylic alcohol (RI_1, **Figure 3**).

The different RI_1 intermediates were monitored (**Figure 4**) after identification by LC-MS (*11*, *13*, *15*), and it was found that the kinetics did not follow the same trend. The intermediates formed with glyoxylic acid [RI_1(G)] were produced in larger quantity and were consumed more quickly than with acetaldehyde [RI_1(A)]. For the acetaldehyde reaction, after an initial increase, RI_1(A) plateaued and remained constant thereafter (i.e., the apparent rates of formation and disappearance were similar). When the two aldehydes were reacted together, the apparent appearance rate of the different RI_1 did not change, but the RI_1 disappearance rates did: RI_1(G) disappeared earlier, for RI_1-(A) a plateau did not occur and they disappeared after 3 h of reaction.

Differences in aldehyde structure could explain these differences. Specifically, glyoxylic acid has both an aldehyde and a carboxylic acid functional group and, therefore, has some conjugation associated with its structure, which leads to an increase in aldehyde polarizability. Acetaldehyde has aldehyde and methyl functional groups (i.e., no conjugation). The glyoxylic acid protonation (R1) to C^+(G) therefore would be easier than acetaldehyde protonation to C^+(A). It is predicted that these structural differences would lead to an enhanced reactivity (R1) for glyoxylic acid compared to acetaldehyde.

Changes in the amounts of RI_1 intermediates reflect not only the reactivity of acetaldehyde and glyoxylic acid toward (+)-catechin but also their further reaction leading to dimers and larger oligomers. Thus, the lower amounts of (+)-catechin−acetaldehyde adduct [$R1_1$(A)] observed may reflect not only a reduced reactivity of the acetaldehyde compared to glyoxylic acid but also the rapid progression of the (+)-catechin−acetaldehyde adduct to condensed dimers. This would also be consistent with higher amounts of (+)-catechin−ethyl-(+)-catechin dimers being formed compared to (+)-catechin−carboxymethine-(+)-catechin products (discussion below).

If R1 was slower than R2, it would be the rate-limiting step for the R1 + R2 reaction sequence. For R1 + R2 only R2 involves (+)-catechin. In our experiments the RI_1 appearance rates were similar when the aldehydes were incubated alone or together (**Figure 4**), suggesting that the protonation step (R1) is the rate-limiting step for the R1 + R2 reaction.

Dimer Evolution. The two last reaction steps, R3 + R4, of the (+)-catechin−aldehyde condensation (**Figure 5**) involve RI_1 protonation (R3) leading to a benzylic carbocation RI_2. RI_2 undergoes subsequent (+)-catechin nucleophilic attack leading to the formation of bridged dimers. Four possible bridged dimers would be possible: one (8−8) isomer, two stereoisomers (6−8), and one (6−6) isomer (*3*, *11*, *12*). During the reaction, the amount of each dimer (8−8, 6−8, and 6−6) was monitored (**Figure 6**). The condensation reaction was regioselective with regard to C-8 substitution; the 8−8-bridged dimers were always higher than 6−8- and 6−6-bridged dimers. Overall, 6−6-bridged dimers were formed in very small quantities. This is consistent with previous data (*11*) and can be explained by the sterically favored substitution at C-8 as opposed to substitution at C-6 (*24*).

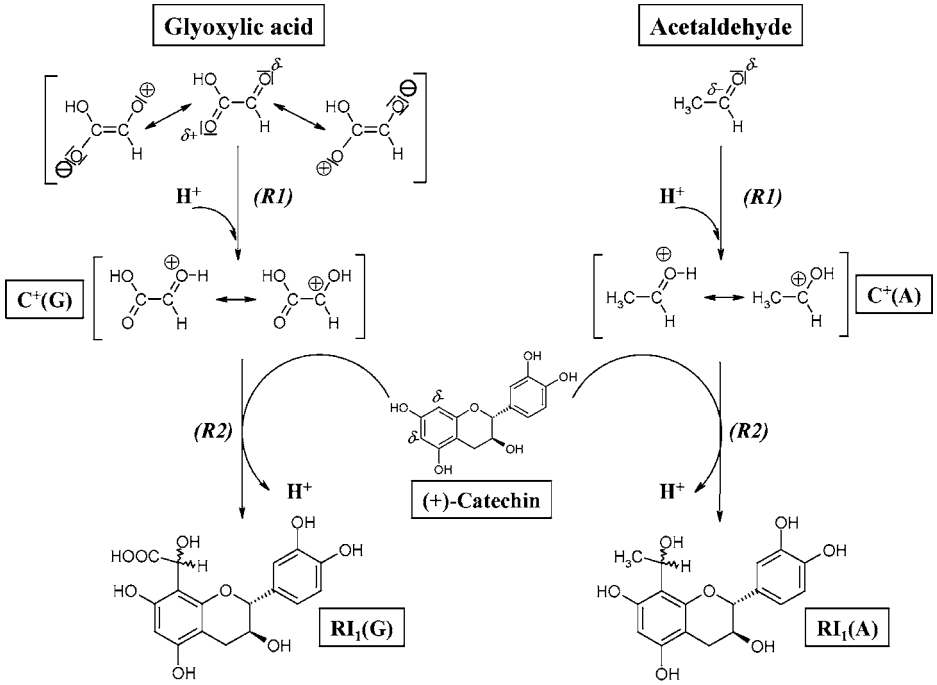

Figure 3. Formation of RI_1 intermediates during (+)-catechin–aldehyde condensation.

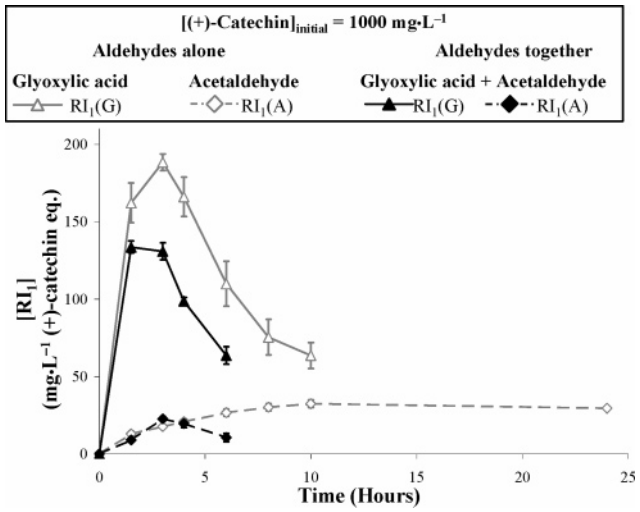

Figure 4. Aldehyde influence on the RI_1 appearance. The aldehydes were incubated separately or together, and initial [(+)-catechin] was 1000 mg·L^{-1} (±SD, $N = 3$).

The total dimer concentrations were monitored to compare the relative kinetic evolution of the different reactions (**Figure 7**). For comparison, the following kinetic indices were calculated: the half-appearance time for dimers (t_{50app}) and the time to maximal dimer concentration (t_{max}, **Table 3**).

When glyoxylic acid and acetaldehyde were reacted separately, the rates of appearance for the dimers were similar (i.e., independent of aldehyde used). The disappearance of dimer, however, was slower with acetaldehyde than with glyoxylic acid. That R1 + R2 was faster with glyoxylic acid suggests that the similar rates of appearance observed for bridged dimers could be due to the rates of R3 + R4, which either would be faster in the case of acetaldehyde (to compensate for the reduced rate of R1 + R2) or would be the rate-limiting steps for the entire condensation reaction.

The structural differences between the glyoxylic acid and acetaldehyde reaction intermediates RI_1 and RI_2 could explain these experimental results. In this case, the carboxyl functional group of RI_1(G) and RI_2(G) would form intramolecular hydrogen bonds and could therefore have higher steric hindrance in contrast to the methyl functional group of RI_1(A) and RI_2(A), which would not have available intramolecular hydrogen bonding. Specifically, and for RI_1(G), the intramolecular hydrogen bonds between the carboxyl functional group and the OH of the benzylic alcohol may not favor its subsequent protonation and dehydration (R3). For RI_2(G), intramolecular hydrogen bonding between the carboxyl functional group and the hydroxyl of carbon 7 of (+)-catechin as well as steric hindrance may make (+)-catechin nucleophilic attack (R4) less favorable.

When the two aldehydes were reacted together, the appearance rates were different (**Table 3**). The dimers bridged by acetaldehyde were formed more rapidly than those bridged by glyoxylic acid. When the aldehydes were reacted separately, dimers bridged by acetaldehyde seemed to be formed more quickly compared to when aldehydes were reacted together. However, the differences in t_{50app} and t_{max} could be explained by differences in rates of dimer disappearance. Because the dimers disappeared earlier and more rapidly, t_{max} and t_{50app} would decrease as result. Specifically, and for ethyl-bridged dimers, t_{max} was reached in one-third of the time when the aldehydes were reacted together. If dimer disappearance is earlier when the two aldehydes are reacted together, the reason for this could be that they react with additional aldehyde and become more polymerized.

If R4 is slower than R3, it would be the rate-limiting step for the R3 + R4 reaction sequence. Competition for (+)-catechin can occur in only R4. Our experiments showed that bridged dimer appearance and disappearance were modified when the two aldehydes were reacted simultaneously (**Table 3**). R4 could be the rate-limiting step of R3 + R4 and may be the rate-limiting step for the entire reaction sequence R1 → R4.

Polymers. Under our conditions, aldehydes were present in large excess (64−322 equiv). If all aldehydes were in a reactive

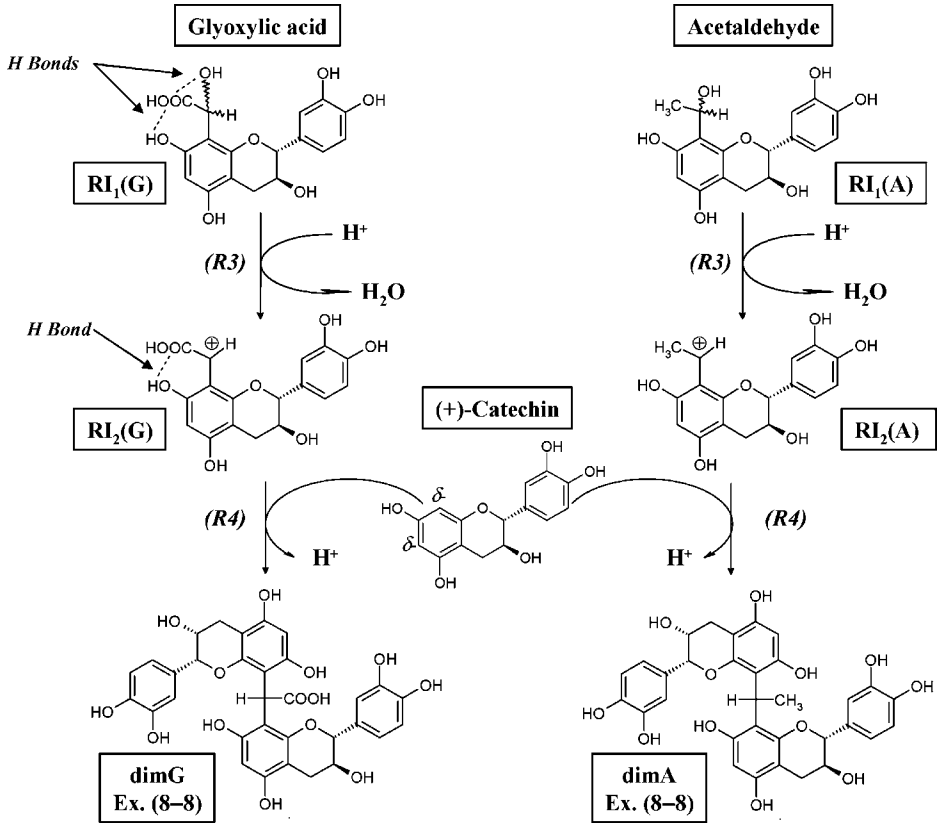

Figure 5. Formation of dimers from RI_1 intermediates during (+)-catechin–aldehyde condensation.

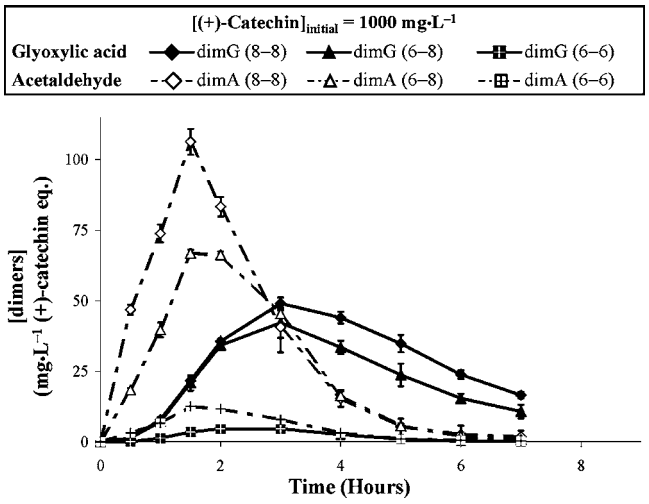

Figure 6. Comparison of different dimer kinetics during (+)-catechin–aldehyde condensation. The aldehydes were incubated together, and initial [(+)-catechin] was 1000 mg·L^{-1} (±SD, $N = 3$).

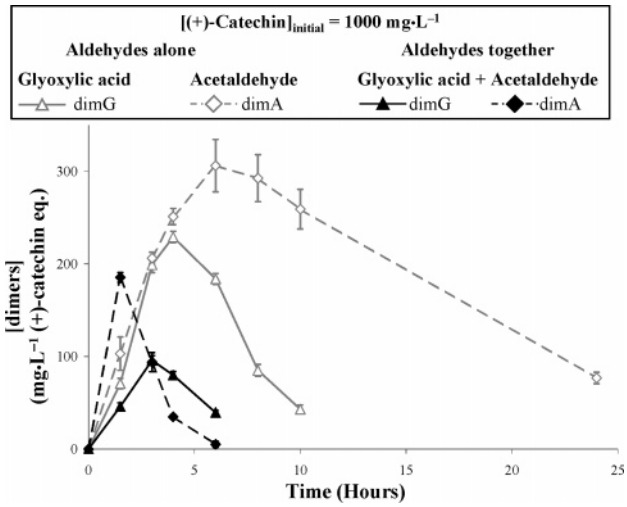

Figure 7. Comparison of dimer concentration during (+)-catechin–aldehyde condensation. The aldehydes were incubated separately or together, and initial [(+)-catechin] was 1000 mg·L^{-1} (±SD, $N = 3$).

form, it is reasonable to assume that only (+)-catechin substituted by aldehyde at C-6 and C-8 would be observed and, therefore, no polymerization would occur. Given the reaction pathway, however, only the protonated form of the aldehyde is reactive. Furthemore, under the aqueous conditions used in this study, aldehydes would be expected to be predominately hydrated (up to 99%) (*25*). Under our conditions, therefore, although aldehydes were used in large excess, the reactive form (i.e., the protonated aldehyde) would not be in molar excess, and therefore this would explain the polymerization observed.

Mass spectrometry experiments in the direct injection mode revealed molecular ion masses corresponding to polymers up to tetramers when the aldehydes were incubated alone or together (**Figure 8**), in agreement with a polymerization reaction. In this study the disappearance of dimers can be explained by the formation of higher molecular weight oligomers.

When the two aldehydes were reacted together, the presence of molecular ion masses corresponding to "mixed polymers" was also observed. These mixed polymers consisted of (+)-catechin units linked by both carboxymethine and ethyl bridges (**Figure 8**). This may explain why bridged dimers were consumed earlier and more quickly when the two aldehydes were reacted together. As expected, molecular ion masses analogous to the first reaction intermediates were observed [i.e.,

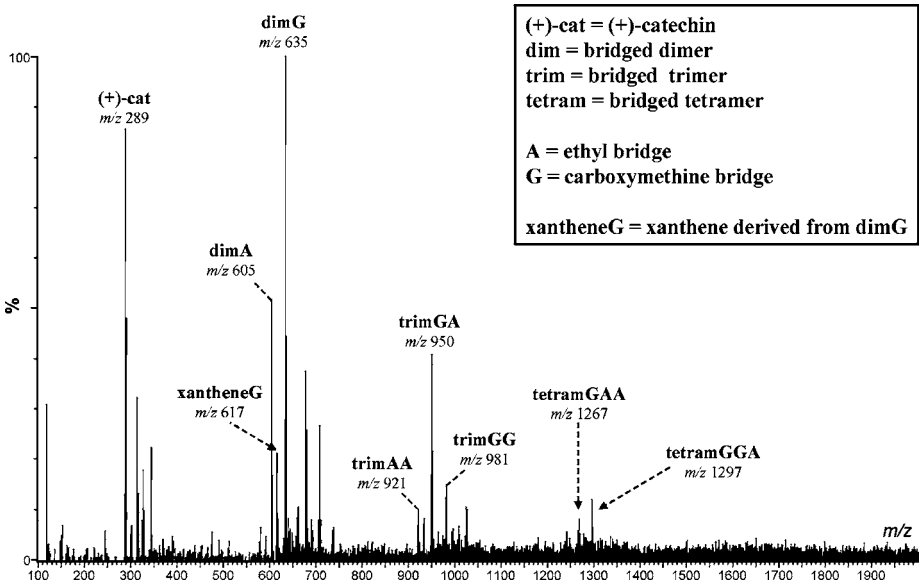

Figure 8. Mass spectrum of LC-MS analysis of (+)-catechin–glyoxylic acid–acetaldehyde condensation after 4 h of reaction. Initial [(+)-catechin] was 1000 mg·L^{-1}; tetram GGA corresponded to four (+)-catechin units linked by two carboxymethine bridges and one ethyl bridge.

Table 3. t_{50app} and t_{max} Indices of Dimers in the Presence of Acetaldehyde, Glyoxylic Acid, or Acetaldehyde + Glyoxylic Acid[a]

	dimG		dimA	
reactant	t_{50app} (h)	t_{max} (h)	t_{50app} (h)	t_{max} (h)
glyoxylic acid	2.0 ± 0.1	3.7 ± 0.1		
acetaldehyde			2.3 ± 0.4	6.2 ± 1.0
glyoxylic acid + acetaldehyde	1.7 ± 0.2	3.4 ± 0.4	0.9 ± 0.1	1.7 ± 0.2

[a] t_{max} is the necessary time to obtain maximal dimer quantity, and t_{50app} is the necessary time to obtain 50% of maximal dimer quantity (±SD, $N = 3$).

(+)-catechin, ethyl-bridged dimer−ethyl adduct (*m*/*z* 649), carboxymethine-bridged dimer−carboxymethine adduct (*m*/*z* 679), and bridged dimer−aldehyde adduct (*m*/*z* 709)]. An additional fate for dimers therefore appears to exist.

Xanthylium salts were not detected in our study, and this is not consistent with previous work (*11*, *20*, *22*). This may be due to the lack of a catalyst such as iron or copper or a limited time of reaction under our conditions. Molecular ion masses consistent with xanthene structures were observed, however, when (+)-catechin was reacted with glyoxylic acid. In the negative-ion mode, *m*/*z* 617 would correspond to xanthene derived from carboxymethine-bridged dimers (*11*) and *m*/*z* 963 to the xanthene linked to a (+)-catechin by a carboxymethine bridge.

Our results show a competition between glyoxylic acid and acetaldehyde condensation with (+)-catechin. A synergistic effect between glyoxylic acid and acetaldehyde may also occur to form new flavanol copolymers. This may have enological consequences. The oxidation of tartaric acid into glyoxylic acid and the presence of acetaldehyde may favor the flavanol condensation to copolymer formation in wines. These copolymers may influence the bitterness, astringency, and color of wine. Future work will focus on quantifying these products in wine and their evolution toward orange xanthylium forms.

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