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The Production and Isomeric Distribution of Xanthylium Cation Pigments and their Precursors in Wine-like Conditions: Impact of Cu(II), Fe(II), Fe(III), Mn(II), Zn(II) and Al(III)

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ABSTRACT

This study establishes the influence of Cu(II), Fe(II), Fe(III), Zn(II), Al(III) and Mn(II) on the oxidative production of xanthylium cations from (+)-catechin and either tartaric acid or glyoxylic acid in model wine systems. The reaction was studied at 25 °C using UHPLC and LC-HRMS for the analysis of phenolic products and their isomeric distribution. In addition to the expected products, a colorless product, tentatively assigned as a lactone, was detected for the first time. The results show the importance of Fe ions, and a synergistic influence of Mn(II), in degrading tartaric acid to glyoxylic acid, while the other metal ions had minimal activity in this mechanistic step. Fe(II) and Fe(III) were shown to mediate the (+)-catechin-glyoxylic acid addition reaction, a role only previously attributed to Cu(II). Importantly, the study demonstrates that C-8 addition products of (+)-catechin are promoted by Cu(II) while C-6 addition products promoted by Fe ions.

KEYWORDS

Xanthylium cations; metal ions; isomers; wine color, (+)-catechin, tartaric acid oxidation.

INTRODUCTION. The reaction of molecular oxygen in wine can lead to eventual changes in the color, flavor and turbidity of the wine.[1-3] Metal ions in wine, such as Cu, Fe and Mn, in their various redox forms, are known to mediate the reaction between molecular oxygen and phenolic compounds or ascorbic acid in the wine, and hence induce an accelerated rate of consumption of molecular oxygen.[4-6] Provided the concentration of sulfur dioxide is sufficiently high in wine, the reaction products of molecular oxygen and the wine components are mostly scavenged.[7] However, once the concentration of sulfur dioxide is sufficiently low, the changes in wine color, flavor and turbidity become particularly evident.[7]

Fe has a key role in the interaction of molecular oxygen with phenolic compounds, whereby it cycles through Fe(II) and Fe(III) redox states generating oxidation products such as *o*-quinone and hydrogen peroxide.[4] In the process a variety of intermediates are proposed including semiquinone radicals and an $[Fe(III)\text{-}O_2^{\cdot}]^{2+}$ intermediate.[8] A key outcome is that the hydrogen peroxide generated can react with Fe(II) and allow the conversion of ethanol in wine to acetaldehyde via Fenton chemistry reactions. However, trace amounts of glyoxylic acid can also be generated from g/L concentrations of tartaric acid via Fenton chemistry, even in the presence of 12% (v/v) aqueous ethanol.[9,10] Glyoxylic acid can also be formed by visible light and Fe(III) tartrate via a photo-Fenton mechanism.[11]

Cu(II) appears to have a synergistic role with Fe ions in terms of oxygen consumption in wine, as the influence of Cu(II) on oxygen consumption in a model wine system was only substantial provided some Fe ions were present.[3,8] However, incremental increases in Cu(II) concentration when Fe ions were present had a marked influence on the rate of oxygen consumption,[3,8] and this was similarly observed in white wines containing ascorbic acid.[6] Although the mechanism for this synergy between Cu(II) and Fe ions has not been

established, it was proposed[3] that Cu(II) aids the conversion of Fe(II) to Fe(III). Similar to Cu(II), Mn(II) has been shown to provide minimal oxygen consumption in a wine-like solution with phenolic compounds.[5] However, in the presence of sufficient Cu(II) and Fe ions, it demonstrated a synergistic effect that was attributed to Mn(II)/Mn(III) redox cycling, which enhanced conversion of Fe(II) to Fe(III).

The colored compounds formed from oxidative reactions in wine can stem from the glyoxylic acid, as this acid can react with (+)-catechin to generate xanthylium cation pigments.[9,10,12] These specific pigments were first observed during the oxidation of (+)-catechin in a model wine system containing tartaric acid in the presence of added Fe(II) (room temperature to 39 ºC),[10] and were subsequently detected in red and white wines.[13,14] The mechanism of their formation[15] involved the production of glyoxylic acid from tartaric acid (step 1) (Figure 1), and its subsequent reaction with (+)-catechin (step 2) to afford two (+)-catechin addition products, **1** and **2**, which react with a second (+)-catechin unit (step 3) to form four carboxymethine-linked (+)-catechin dimers, **3**-**6**. This initial stage of the reaction is akin to the well-established reaction between acetaldehyde and wine flavanols.[16,17] The carboxymethine-linked (+)-catechin dimers were initially postulated to be in equilibrium with lactone forms (step 4b) which in turn were presumed to be direct precursors to colored products.[18-20] However, it was shown that the dimers themselves underwent dehydration (step 4a) to form six xanthenes[15,21], **7**, rather than lactones. The xanthenes then underwent oxidation (step 5) to provide six xanthylium cation pigments (one represented by $\mathbf{X_2}^+$). Esterification of the acid functionality by ethanol was reported to involve the carboxymethine-linked (+)-catechin dimers and allow production of the ethyl ester of the xanthylium cation, $\mathbf{X_{est}}^+$.[21] The same mechanism was shown for Cu, albeit under conditions of either high temperature (45 ºC)[22] or at concentrations ten-fold higher than in wine (5 mg/L).[23] However, in conditions

more relevant to wine (20-25 ºC and 0.3 mg/L Cu), Cu(II) induced negligible molecular oxygen consumption by (+)-catechin[3] and consequently negligible (+)-catechin oxidation products. The main role of Cu(II) and Fe ions in the overall mechanism for xanthylium cation production was attributed degradation of tartaric acid by Fenton chemistry.[9,22,23] However, later it was shown that Cu(II) could accelerate the reaction between glyoxylic acid and (+)-catechin (combined steps 1 and 2) (Figure 1), but not the reaction between acetaldehyde and (+)-catechin, indicating that the acid moiety of glyoxylic acid was important to this catalytic effect of the metal.[24] The influence of Mn(II), or other metals (i.e., Al(III) and Zn(II)), on xanthylium cations production has not been reported.

At low concentrations the xanthylium cations will induce yellow coloration to solutions, but in the presence of an hydroxycinnamic acid, tend towards orange/brown coloration.[25] In white wine, this coloration is deemed negative in terms of consumer sensory appraisal. The ability of Cu(II) to accelerate the reaction between glyoxylic acid and (+)-catechin, to ultimately form the pigmented xanthylium cation, has been used as a colorimetric measure of Cu(II) activity in white wine and model wines with variable hydrogen sulfide concentrations.[26] The results showed that $CuS_{(s)}$ formed in the model wine system was less active in catalyzing the reaction between (+)-catechin and glyoxylic acid compared to $CuS_{(s)}$ formed in wine. For such a colorimetric measure to allow assessment of metal activity between different wines, an assessment of the ability of other metal ions to induce production of glyoxylic acid in wine and/or to catalyze its reaction with (+)-catechin is necessary.

This study was conducted in order to assess the ability of Cu(II), Fe(II), Fe(III), Mn(II), Al(III) and Zn(II) to influence specific steps of xanthylium cation production (steps 1-5) (Figure 1). This was conducted at a temperature (25 ºC) more relevant to wine storage than

100 many other past studies, and at metal concentrations typically encountered in wine. The
101 individual isomers of products were assessed along with the summed concentration of
102 isomeric products.
103
104 **MATERIALS AND METHODS. Materials.** Grade 1 water was used for solutions and
105 dilution preparation and obtained from Milli-Q purification system (Millipore, Billerica, MA).
106 All glassware was soaked for at least 16 h in 10% (v/v) nitric acid and then rinsed with
107 copious amounts of grade 1 water. Fe(III) and Cu(II) samples were prepared from 1000 mg/L
108 Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) standards, Mn(II),
109 Al(III) and Zn(II) as 1000 mg/L Flame Atomic Absorption Spectroscopy (FAAS) standards,
110 and all were obtained from Fluka (St Louis, MO). Fe(II) was added as ferrous sulfate
111 heptahydrate (≥ 99.0%, Biolab, Montrose, Australia). Sodium borohydride (≥ 96%), (+)-
112 catechin monohydrate (≥ 98%, HPLC grade), and glyoxylic acid (≥ 98%) were all purchased
113 from Sigma-Aldrich (St Louis, MO).
114
115 **Reactions.** A model wine solution containing 0.011 M potassium hydrogen L-(+)-tartrate,
116 0.007 M L-(+)-tartaric acid, 12% (v/v) aqueous ethanol was prepared, with adjustment to pH
117 3.25 using 1 M sodium hydroxide. By means of fresh stock solutions of 5.0 mM (+)-catechin
118 hydrate, and 2.5 mM mg/L glyoxylic acid monohydrate, both in model wine solution, final
119 concentrations of 0.50 mM (+)-catechin with/without 0.25 mM glyoxylic acid were prepared.
120 Metal ions were added from stock solutions of 1000 mg/L to obtain the concentrations and
121 combinations as shown in Table 1. Triplicate 25 mL portions of each reactive treatment were
122 placed in separate 50 mL plastic bottles with screw top lids (with a headspace of around 25
123 mL). All samples were incubated at 25 °C in darkness, and only opened on measurement days

for the collection of 2 mL which was filtered (0.2 μm) prior to analysis. All products were monitored by UHPLC during the experiment. Quantitation of products by UHPLC[16] was performed using an external (+)-catechin calibration graph at 280 nm and concentrations are expressed in mg/L (+)-catechin equivalents. The exception was for the xanthylium cation pigments whose peak area (440 nm) was used to reflect the relative concentration changes during the storage period.

Method for isolation of 1, 2 and 3, and determination of their specific products. For the isolation of the reactive products **1-3** (Figure 1), a 50 mL model wine solution containing (+)-catechin hydrate (3.24 mM) and glyoxylic acid monohydrate (1.62 mM) was placed in a 100 mL Scott bottle and held at 40 °C for 2 h in darkness. HPLC separation and analysis on a semi-preparative scale were performed by using the Empower Pro software (Waters, Milford, MA) with Waters chromatography equipment consisting of 2996 photodiode array detector, 600 pump, in-line degasser and temperature controller modules (Waters). Sample introduction was achieved by manual injection with a Rheodyne injection valve using a 2.0 mL sample loop. Detection was afforded by UV/Vis spectra recorded from 210 to 600 nm after splitting the flow from the column by 1:10. The column was a 250 × 10 mm i.d., 4 μm, Synergy Hydro-RP C18 polar end capped, with a 10 × 10 mm i.d. ODS Octadecyl C18 guard column (Phenomenex, Torrance, CA). Elution conditions were as follows: flow rate 2 mL/min; column temperature 35 °C; solvent A 0.01% (v/v) formic acid in water; solvent B 0.01% (v/v) formic acid in methanol; elution from 0-15% B in 10 min, from 15-25% B in 20 min, from 25-50% B in 5 min, from 50-100% B in 3 min, 100% B for 4 min, and from 100-0% B in 3 min, and 0% B for 10 min. The compounds corresponding to peaks **1-3** were collected within the aqueous methanol mobile phase. The purity of the collected products were determined by UHPLC (> 90% of total peak area) and the identity by LC-HRMS analysis. Once the identity

of the collected compounds was confirmed, methanol was removed by sparging gently with nitrogen at room temperature. Subsequently, for compounds **1** and **2** (but not **3**) the remaining aqueous solutions were diluted 2-fold with 1.0 mM (+)-catechin solution to provide a final (+)-catechin concentration of 0.5 mM. Afterwards the collected compounds were incubated at 40 °C for 4 days in darkness.

Reduction of xanthylium cations. The extraction of xanthylium cations from catechin and glyoxylic acid solutions was conducted as described by George et al.[25] The extracts were dried with a centrifugal evaporator, and then dissolved in methanol (3 mL). Immediately, sodium borohydride (50 mg) was added over 3 min to reduce the xanthylium cations to their respective xanthenes,[15,27] and water (3.0 mL) was then added.

UHPLC with high-resolution mass spectrometry (HRMS). HRMS measurements were performed with an 6530 Quadrupole-Time of Flight LC-MS instrument with a Dual Jet Stream Technology Electrospray Ionization source and an 1290 Infinity LC system, run by MassHunter Workstation software B.05.01 (Agilent Technologies, Mulgrave, Australia). The mobile phase solvents were 0.04% (v/v) formic acid in water (solvent A) and 0.04% (v/v) formic acid in methanol (solvent B). The column, gradient and chromatographic settings were as for UHPLC[16] but the the injection volume was 3 μL. The HRMS was operated in Extended Dynamic Range (2 GHz) mode and in negative ion mode, with the following settings: range *m/z* 100 – 1500, scan rate 1 spectrum/s, reference ions for internal mass correction were TFA (*m/z* 112.9855) and the formic acid adduct of HP-0921 (*m/z* 966.0007), drying gas temperature 350 °C, drying gas flow 9 L/min, nebulizer pressure 35 psi, sheath gas temperature 350 °C, sheath gas flow 11 L/min, capillary voltage 4000 V, nozzle voltage 1000

V, fragmentor voltage 80 V, skimmer 1 voltage 65 V and octopole RF peak voltage 750 V. Targeted MS/MS experiments were conducted using the following settings: isolation width ~ 1.3 Da and collision energy 16 eV.

RESULTS AND DISCUSSION. Assignment of chromatographic peaks to phenolic products. The 280 nm and 440 nm chromatograms from an incubated sample are shown in Figure 2, and the peaks attributed to products **1-6**, xanthylium cations ($\mathbf{X_{1\text{-}4}}^+$) and xanthylium cation esters ($\mathbf{X_{est}}^+$) are indicated. The peak assignment was based on matching retention time, UV/Vis spectra and high-resolution mass data to that described previously.[13,15,21,23,24] As described below, confirmation of precursors was achieved by isolation of individual compounds, then their incubation at 40 °C to induce product formation, and consequent identification of products.

The isomeric products **3**, **6,** and **4** or **5** (the latter two diastereoisomers were not distinguished from each other) were assigned based on their elution order and relative peak intensity, a strategy that is well established in reverse phase chromatography systems.[13,15,24,28] It has been shown, in terms of elution time and concentration, that **3** (the 8-8 connected isomer) is the earliest and highest, respectively, followed by **4** and **5** (the 6-8 and 8-6 connected isomers) and finally **6** (the 6-6 connected isomer).[13,15,24,28] A similar elution order and pattern of relative peak intensities have been observed for the acetaldehyde-bridged (+)-catechin dimers[29] and can be attributed to the C-8 of (+)-catechin being less sterically hindered and more electron rich, and hence favoring nucleophilic attack compared to C-6.[13,28]

196 Using UHPLC and LC-HRMS, the isomeric products **1** and **2** were confirmed to be
197 precursors to **3-6**, after their semipreparative collection and separate incubation with (+)-
198 catechin. More specifically, incubation of product **1** with (+)-catechin provided **3** as the major
199 product, with smaller amounts of **4** and **5** and negligible **6** being evident (data not shown).
200 This confirmed **1** as having the glyoxylic acid-moiety attached to C-8 of (+)-catechin.
201 Incubation of product **2** with (+)-catechin led to production of **6** with smaller amounts of **4**
202 and **5** and trace amounts of **3**. This confirmed the identity of **2** as having the glyoxylic acid
203 moiety attached to C-6 of (+)-catechin. The minor amount of **3** generated from **2** suggested
204 some acid-catalyzed conversion of **2** to **1** during the incubation period which has been
205 reported previously for related products.[21] The relative intensity and elution order of **1** and **2**
206 were consistent with other (+)-catechin/aldehyde addition products.[13,30]
207
208 To confirm the location of the xanthene peaks in the UHPLC chromatogram, the xanthylium
209 cations ($\mathbf{X_1}^+$-$\mathbf{X_4}^+$) (Figure 2b) were isolated and reduced with sodium borohydride.[15,27] The
210 resulting peaks (Figure 2c) were confirmed to have the required UV/Vis spectrum, accurate
211 high resolution mass, and also fragmentation patterns consistent with their assignment as the
212 xanthenes. In the samples, it was found that the peaks attributed to the xanthenes did not
213 accumulate to any significant extent and for this reason the xanthene compounds are not
214 described further in this study.
215
216 The unknown peaks $\mathbf{U_1}$-$\mathbf{U_3}$ had HRMS precursor ions and UV/Vis spectra (λ_{max} 278 nm) that
217 were consistent with both the lactone and xanthene structures. However, the xanthenes,
218 formed from reduction of the xanthylium cations (Figure 2c), were already shown to elute at
219 different retention times to $\mathbf{U_1}$-$\mathbf{U_3}$ (Figure 2a). Furthermore, the HRMS fragmentation data,

which was identical for all three $\mathbf{U_1}$-$\mathbf{U_3}$ peaks, were consistent with the lactone structure rather than the xanthene. The most intense fragment ion (*m/z* 327.0511) corresponded to a loss of (+)-catechin from the lactone form, but was not reconcilable with the xanthene form. Formation of such a lactone involves the acid group of the carboxymethine-linked (+)-catechin dimer undergoing an esterification reaction with a hydroxyl group on the neighboring phenolic ring (Figure 1). For **3**, the lactone would be formed by reaction of the acid group with the hydroxyl group at C-7 on one of the neighboring (+)-catechin units. This would lead to the formation of two diastereoisomers based on the chirality of the carbon bridging the (+)-catechin moieties. When **3** was collected and incubated alone (without (+)-catechin present), as expected, two of the major products were $\mathbf{U_1}$ and $\mathbf{U_2}$, as well as $\mathbf{X_2}^+$ and $\mathbf{X_{est}}^+$. This confirmed that $\mathbf{U_1}$ and $\mathbf{U_2}$ were both derived from an 8-8 connected isomer of the carboxymethine-linked (+)-catechin dimer and most likely the lactone diastereoisomers shown in Figure 1. The single acidic xanthylium cation isomer ($\mathbf{X_2}^+$) is expected from **3** based on Figure 1, and hence confirms that $\mathbf{X_2}^+$ is the 8-8 derivative. $\mathbf{U_3}$ was generated from the incubation of **2** with (+)-catechin, suggesting that it was at least one lactone stemming from **4**-**6**, but more likely multiple lactone isomers unresolved from each other. Based on preliminary MS data published in past studies,[18-20] the lactone was originally speculated to be the direct precursor to pigments formed from (+)-catechin and glyoxylic acid, but this postulation was discounted after characterization of the xanthylium cations and recognition that they instead were responsible for the ions observed in the preliminary MS data.[15,21] Consequently the lactone structure has not previously been identified in the (+)-catechin and glyoxylic acid reaction system. Therefore, although compelling evidence has been presented for assignment of $\mathbf{U_1}$-$\mathbf{U_3}$ as lactones (Figure 1), the assignment remains tentative without NMR characterization. The different isomers of the ethyl ester of the xanthylium cation ($\mathbf{X^+_{est}}$) were observed to co-elute and only exhibit a single peak.

245

246 **Products from (+)-catechin and tartaric acid: summed concentration of isomers.** For the
247 samples with added Fe(III) and Fe(II), it is appreciated that the redox forms of the iron will
248 gradually reach an equilibrium state between the two ions, regardless of the form initially
249 added, as the metals interact with molecular oxygen and phenolic compounds within the
250 model wine systems.[4] However, it was decided to assess if the presence of iron in an initial
251 state of Fe(II) or Fe(III) would favor any of the mechanistic steps of Figure 1.

252

253 After 28 days, in the samples incubated without glyoxylic acid present, the samples with
254 added Mn(II), Al(III), Zn and Cu(II) had less than a 2.0% (3 ± 1 mg/L) decrease in the initial
255 (+)-catechin concentration. Alternatively, for samples with an individual metal ion present,
256 Fe(III) had the highest loss of (+)-catechin with a 7.0% (11.3 ± 0.5 mg/L) decrease, followed
257 by Fe(II) with 6.4% (10.2 ± 0.7 mg/L). When Cu(II) was combined with Fe(II), no further
258 loss of (+)-catechin was induced, however, Mn(II) in combination with both Fe(II) and Cu(II)
259 led to the highest loss of (+)-catechin observed (14 ± 1 mg/L). These results suggested that Fe
260 ions were the most efficient metal at inducing loss of (+)-catechin in the model wine system,
261 and that Mn(II) demonstrated a synergistic effect in combination with Fe(II). Such a
262 synergistic effect of Mn(II) with Fe has been observed previously when following oxygen
263 consumption and free sulfur dioxide loss in wines.[5]

264

265 The initial products **1** and **2** (Figure 1) did not accumulate in any of the samples, suggesting
266 that they reacted rapidly after formation. The overall production of the carboxymethine-linked
267 (+)-catechin dimers **3-6** (Figure 3a), $\mathbf{U_1}$-$\mathbf{U_3}$ (Figure 3b), xanthylium cations (Figure 3c) and
268 esters of the xanthylium cations were followed throughout the storage period. It is evident that
269 Fe(III) leads to faster formation and higher concentrations of dimers, **3-6** (Figure 3A). Cu(II)

shows no significant influence on **3-6** production in combination with Fe(II), while Mn(II) combined with Cu(II) and Fe(II) shows a synergistic effect, which is consistent with the outcome for (+)-catechin decay. A similar outcome is observed for $\mathbf{U_1}$-$\mathbf{U_3}$ (Figure 3b), xanthylium cations (Figure 3c) and esters of the xanthylium cations, suggesting that the main influence of Fe ions and Mn(II) was occurring prior to the production of **3-6**. That is, either during production of glyoxylic acid from tartaric acid, or during the initial reaction of glyoxylic acid with (+)-catechin. Cu(II), without Fe(II) present, could not induce production of any of the phenolic products outlined in Figure 1 under the conditions adopted (25 ºC, 0.3-1.0 mg/L Cu(II)). This suggested that the xanthylium cations produced by Cu(II) in previous studies[22,23] were induced by either high temperatures (45 ºC) or high copper concentrations (≥ 5.0 mg/L). The lag period for the initial production of the xanthylium cations (Figure 3c) has been shown previously,[24,28] and is a consequence of the time required for the production and decay of its precursors, **1-6**. A similar lag period is evident for $\mathbf{U_{1\text{-}3}}$ (Figure 3b), and this is expected given their tentative assignment as lactones generated from **1-6**.

Products from (+)-catechin and tartaric acid: concentration of individual isomers. In terms of the individual isomers, Figures 4a-c presents the concentrations for each of the connected isomers: **3** (8-8 isomer), **4** and **5** (6-8 and 8-6 diastereoisomers), and **6** (6-6 isomer). For Fe(III), Fe(II) and Mn(II), the relative influence of the metals on the concentration of the separate isomers (Figures 4a-c) are similar to that of the overall data for **3-6** (Figure 3a), where Fe(III) shows a faster accumulation and higher concentration than Fe(II), and Mn(II) inducing synergistic effect with Fe(II). Alternatively, for **3** (Figure 4a), Cu(II) provides a synergistic impact with Fe(II) for its concentration, which was not observed in the summed data (Figure 3a). The **4** and **5** isomers show negligible synergistic impacts of Cu(II) with

294 Fe(II), while the **6** isomer shows that Cu(II) can lower its concentration when combined with
295 Fe(II). Therefore, although the overall data for **3**-**6** suggested minimal synergistic impacts by
296 Cu(II) in the presence of Fe(II), it is evident that the outcome for the individual isomers is
297 quite different, with **3** enhanced by Cu(II), **6** inhibited and **4**-**5** not impacted. It would appear
298 that while Fe(II) is required for the formation of **3**-**6**, Cu(II) can bias the isomers formed
299 towards **3** and away from **6** when in the presence of Fe ions. Additionally, in an outcome
300 independent of metal ions, **4**-**5** reach a plateau in concentration (Figure 4B) while **3** and **6**
301 continue to increase (Figure 4A and 4C) throughout the storage period. This implies that **4**-**5**
302 are degraded more rapidly than **3** and **6** to form their products or precursors (steps 3-4)
303 (Figure 1).
304

305 Table 1 presents the relative isomer distribution, based on peak area ratios, for $\mathbf{U_1}$ and $\mathbf{U_3}$
306 (with respect to $\mathbf{U_2}$) and xanthylium cations (with respect to $\mathbf{X_2}^+$) for the samples. While the
307 carbon 8-8 link between (+)-catechin units is the favored connected isomer for the
308 carboxymethine-linked dimer (Figure 4), the conversion of the 8-8 isomer **3** to xanthylium
309 cation $\mathbf{X_2}^+$ seems to be slower than for the 6-8 and 8-6 connected isomers **4** and **5**. This is
310 reflected in the concentration plateaus for the 8-6 and 6-8 forms of the carboxymethine-linked
311 dimers compared to the concentration increases for 8-8 and 6-6 (Figure 4B versus 4A and C),
312 and also because the ratio of $\mathbf{X_2}^+$ (8-8 connected isomer of the xanthylium cation) relative to
313 $\mathbf{X_1}^+$ (a non 8-8 isomer) is less than 1. This means that the 6-8 and 8-6 isomers favor
314 xanthylium cation formation (steps 4a and 5) (Figure 1) over either remaining as
315 carboxymethine-linked dimers, forming the lactone products $\mathbf{U_3}$ and/or reverting back to
316 glyoxylic acid-substituted (+)-catechin (step 3) (Figure 1). It also explains the larger
317 uncertainty for the ratio of the carboxymethine-linked (+)-catechin isomers (i.e., (**4**+**5**)/**3**)

(Table 1) given that the concentration of **4** and **5** were rapidly changing while **3** remained relatively constant throughout days 10-28.

The differences induced by the presence of Cu(II) in products **3-6** appear to hold in the subsequent production of $\mathbf{U_1}$-$\mathbf{U_3}$, whereby Cu(II) favors production of the products derived from the 8-8 connected isomers ($\mathbf{U_1}$ and $\mathbf{U_2}$ over $\mathbf{U_3}$) from the carboxymethine-linked (+)-catechin dimers (Table 1). There was a trend to higher ratios of the 8-8 derived xanthylium cations over other isomers for Cu(II) compared to Fe(II) ($\mathbf{X_2}^+/\ \mathbf{X_1}^+$) but the differences were not significant and most likely complicated by the low concentrations of xanthylium cations in these samples. However, this again supported Cu(II) inducing increased amounts of the 8-8 connected isomer of the xanthylium cation $\mathbf{X_2}^+$ compared to Fe ions.

Products from (+)-catechin and glyoxylic acid: summed concentration of isomers. Glyoxylic acid was added at a relatively large concentration (0.25 mM) compared to that expected to be generated in the samples.[31] However, to account for the glyoxylic acid generated *in situ*, the data presented (Figures 5 and 6) for samples with added glyoxylic acid is corrected by subtracting the concentration of products generated in the absence of added glyoxylic acid. For the samples containing (+)-catechin and glyoxylic acid with an individual metal ion present, the high Cu(II) concentration (1.14 mg/L) showed the largest loss of (+)-catechin (88.9 ± 0.5 mg/L) after 28 d, followed by Fe(III) and Fe(II), which had losses that were not significantly different from each other (78 ± 3 and 74 ± 1 mg/L, respectively). The lower wine-like concentration of Cu(II) (0.3 mg/L) provided a lower 53.7 ± 0.2 mg/L loss of (+)-catechin. The remaining metal ions produced losses of (+)-catechin that were either not significantly different (Mn(II) and Zn(II)), or only marginally different (Al(III); 26 ± 2 mg/L)

342 to the sample without added metal ions (20.4 ± 0.1 mg/L). The sample with combined Cu(II)
343 and Fe(II) led to an increase in (+)-catechin loss (78.5 ± 0.3 mg/L), compared to the samples
344 with Cu(II) or Fe(II) alone. The combination of Mn(II), Cu(II) and Fe(II) led to no increase in
345 (+)-catechin decay compared to Cu(II) and Fe(II), suggesting that Mn(II) had no impact in
346 these circumstances. This suggested significant impacts by both Cu(II) and Fe ions on the
347 reaction between (+)-catechin and glyoxylic acid.
348
349 Unlike the situation without added glyoxylic acid, products **1** and **2** were observed to
350 accumulate for the samples with added glyoxylic acid (Figure 5A). The results showed that
351 the maximum concentration for **1** and **2** was reached after 2-days, and that the metal ions
352 influenced the maximum concentration in a similar manner to the loss of (+)-catechin (Figure
353 5A). Similarly, the summed concentration of **3**-**6** (Figure 5B) showed an influence of the
354 metal ions that was similar to that observed for (+)-catechin loss and **1**-**2** production (Figure
355 5A), but with a more pronounced influence of 1.14 mg/L Cu(II) than the other samples.
356 Interestingly, it is evident that Al(III) has a slight but significant effect on the generation of
357 products **3**-**6** (Figure 5B) and this is consistent with a slight increase in (+)-catechin
358 consumption in the sample with Al(III) compared to the sample without added metal.
359 Consequently it would appear that Al (III) has some minor activity in mediating the reaction
360 between glyoxylic acid and (+)-catechin. The summed concentration of $\mathbf{U_1}$-$\mathbf{U_3}$ (Figure 5C)
361 shows a similar influence of metal ions as observed for products **3**-**6** (Figure 5B), although the
362 concentration of the lactones increase steadily rather than providing a maximum concentration
363 and/or plateau as observed for **3**-**6.** The high concentration of Cu(II), equimolar to Fe(II) and
364 Fe(III), provides the highest concentration of $\mathbf{U_1}$-$\mathbf{U_3}$ and the low concentration of Cu(II)
365 provides similar concentrations to Fe(II) and Fe(III). The combination of Cu(II) with Fe(II)

significantly increases **U_1-U_3** concentration, but further addition of Mn(II) leads to no further increase of production.

The rank order for production of the xanthylium cation (Figure 5D) and their esters for specific metals is similar to that for **3-6** and **U_1-U_3**, with the main difference being the greater comparative yield of xanthylium cations for Fe(II) and Fe(III) compared to Cu(II) than observed for **3-6** and **U_1-U_3**. This suggested that the conversion of the carboxymethine-linked (+)-dimer to the xanthylium cation was accelerated by the presence of Fe ions more so than by Cu(II). This was most likely due to Fe(III) accelerating the oxidation of the xanthene to xanthylium cation, an irreversible step in the model wine conditions (step 5) (Figure 1). Furthermore, although Cu(II) induces higher concentrations of **3-6** and **U_1-U_3** when combined with Fe(II), compared to Fe(II) alone, this was not the case for the xanthylium cation where no increase in concentration was provided by Cu(II) with Fe(II). Mn(II), on the other hand, for the first time demonstrates an influence on the mechanism of xanthylium cation production from (+)-catechin and glyoxylic acid (steps 2-5) (Figure 1). Mn(II) induces greater xanthylium cation production with Fe(II) and Cu(II), than the combination of Fe(II) and Cu(II). This suggests that Mn(II) aids Fe(III) in the conversion of the carboxymethine-linked (+)-catechin dimer to the xanthylium cation, and again, most likely due to accelerated oxidation of the xanthene (step 5).

Products from (+)-catechin and glyoxylic acid: concentration of individual isomers. The influence of the Cu(II) versus Fe(II) and Fe(III) on the relative concentrations of **1** and **2** were different (Figure 6A and B). Figure 6A shows that 1.14 mg/L Cu(II) induces the highest concentration of **1**, and the enhancement of concentration of **1** by 0.30 mg/L Cu(II) in the presence of Fe(II) is large (54% increase at day 6) compared to Fe(II) without Cu(II).

391 However, for product **2**, Figure 6B shows that its concentration is highest for Fe(II) and
392 Fe(III), and that the samples with Cu(II) provide either negligible or a minor increase of **2**
393 compared to the sample with no added metal. For Fe(II) and Fe(III), the concentrations of **1**
394 and **2** are similar to each other while for Cu(II), product **1** is at least 5-10 times higher in
395 concentration than for **2**. This can be seen in the average concentration ratios (days 2-10) for **2**
396 to **1** being lower for the Cu(II) samples compared to the Fe(II) and Fe(III) samples (Table 1).
397 The samples with Al(III), Zn(II), Mn(II) or no added metal had concentration ratios for **2** to **1**
398 between that of Cu(II) and Fe(II) or Fe(III) (Table 1). This result, in combination with those
399 from the previous section, suggested that Fe ions were biasing the reaction towards the C-6
400 connected isomer of (+)-catechin and Cu(II) biasing the glyoxylic acid/(+)-catechin reactions
401 towards the C-8 connected isomer. The samples with both Cu(II) and Fe(II) had a **2** to **1**
402 concentration ratio of 0.52 ± 0.05, intermediate between the results for samples with either
403 Cu(II) or Fe(II), and Mn(II) did not impact this ratio upon its inclusion in the Cu(II) and Fe(II)
404 sample. The inability of Mn(II) to provide a synergistic impact on the production of **1** and **2**
405 suggests that its contribution is to the tartaric acid degradation step (step 1) (Figure 1) rather
406 than in the addition reaction of glyoxylic acid to (+)-catechin. The fact that no difference was
407 observed between Fe(III) and Fe(II) also suggests the initial difference in their redox forms at
408 the start of the experiment is more critical to glyoxylic acid production.
409
410 The impact of the metal ions on the individual concentrations of **3-6** (Figure 6C-E) is clearly
411 evident and similar to that observed for products **1** and **2** (Figure 6A-B). Cu(II) dominated the
412 production of product **3**, the 8-8 connected isomer, at both high and low concentrations of Cu.
413 Alternatively, higher concentrations of product **6**, the 6-6 connected isomer, were evident for
414 both Fe(II) and Fe(III) over the sample with equimolar concentrations of Cu(II), and at the
415 wine-like concentrations of Cu(II) (Figure 6E). No further increase in product **6** was evident

when Cu(II) or Cu(II) and Mn(II) were combined with Fe(II). The production of **1-6** shows for the first time that Fe can accelerate the reaction between (+)-catechin and glyoxylic acid (steps 2 and 3) (Figure 1), as reported previously for Cu(II), and also that metal ions can influence the isomeric distribution of phenolic products. As for the samples without added glyoxylic acid, the 8-8 and 6-6 connected isomers of the carboxymethine-linked (+)-dimers (Figure 6C and E) appear more stable than the 8-6 and 8-6 connected isomers (Figure 6D).

Table 1 shows the ratio of different isomers of $\mathbf{U_1}$-$\mathbf{U_3}$ and xanthylium cations to each other for the samples with added glyoxylic acid. From this it is evident that Fe ions and Cu(II) can influence the isomeric distribution of these phenolic products relative to each other and to the sample without added metal ($\mathbf{U_3}/\mathbf{U_2}$ and $\mathbf{X_2}^+/\mathbf{X_1}^+$). Fe(II) and Fe(III) bias the isomeric production away from the 8-8 connected isomers ($\mathbf{U_1}$, $\mathbf{U_2}$ and $\mathbf{X_2}^+$) suggesting that an Fe(III)-(+)-catechin interaction may direct the attack of the electrophilic glyoxylic acid towards the C-6 of (+)-catechin. The octahedral arrangement of Fe(III) complexes may facilitate the specific substitution site favored on (+)-catechin (i.e., C-6) compared to the square planar co-ordination geometry favored by Cu(II). The lack of influence of the initial redox state of iron on this step suggests that its redox equilibration is not sufficiently limiting on catalyzing the reaction between glyoxylic acid and (+)-catechin or impacting the isomeric distribution. The fact that the overall reaction is accelerated by Fe, compared to no added metal, suggests that Fe must be activating either (+)-catechin and/or the aldehyde moiety of glyoxylic acid. The latter effect may involve a (+)-catechin-Fe(III)-glyoxylate complex that provides close proximity between glyoxylic acid and (+)-catechin in such a way that it facilitates attack of glyoxylic acid on C-6 of (+)-catechin. For Cu(II), a Cu(II)-glyoxylate complex may activate the aldehyde moiety of glyoxylic acid for reaction with (+)-catechin, and enhance substitution at C-8, the favored site for electrophilic addition on (+)-catechin.

441

442 The role of Fe is multi-fold in the mechanism for the production of xanthylium cations. Fe
443 can induce the production of glyoxylic acid from tartaric acid (step 1) (Figure 1), and the
444 results show that Fe(III) appears particularly efficient in this role compared to Fe(II). This
445 may be related to the ability of Fe(III) to initially induce more rapid oxidation of (+)-catechin
446 in the presence of molecular oxygen,[32] and hence production of hydrogen peroxide to bring
447 about the Fenton-degradation of tartaric acid. However, Fe(II) should be more efficient than
448 Fe(III) in the production of hydrogen peroxide from the reduction of molecular oxygen.[32]
449 Other possibilities include Fe(III) inducing photo-Fenton reactions,[11] despite the attempts to
450 limit light exposure to the samples, or Fe(III) increasing the yield of glyoxylic acid from the
451 tartrate radicals generated during Fenton chemistry.[33] The ability of Fe to mediate the reaction
452 of glyoxylic acid with (+)-catechin (step 2) (Figure 1) appears to be independent of the form
453 in which Fe was added (Fe(III) or Fe(II)), and both forms provided bias towards the C-6
454 connected isomers compared to the samples without added metal ions or those with Cu(II).
455 Furthermore, Fe(III) can accelerate the oxidative conversion of the carboxymethine-linked
456 (+)-catechin dimers to the xanthylium cation, most likely by enhancing xanthene oxidation.
457
458 The role of Cu(II) would appear to be predominantly in mediating the reaction between
459 glyoxylic acid and (+)-catechin (steps 1 and 2) (Figure 1) in a manner that enhances C-8 as
460 the preferred site of substitution on (+)-catechin. Mn(II) has a synergistic effect in aiding
461 production of glyoxylic acid from tartaric acid by Fe ions (step 1), and it also can aid the
462 conversion of carboxymethine-linked (+)-catechin dimers to the xanthylium cation in the
463 presence of Fe (steps 4a and 5). Finally, the results for Al(III) showed that it could also
464 mediate the reaction between glyoxylic acid and (+)-catechin (step 1 and/or 2), but only
465 marginally higher than the sample without added metal ions. The results show that the (+)-

466 catechin and glyoxylic acid reaction will be influenced by both Cu(II) and Fe activity in wine,
467 and also to a lesser extent, Mn(II). The results also show for the first time a tentative
468 identification of lactone isomers as products generated from glyoxylic acid and (+)-catechin.
469
470 In terms of wine coloration, Fe ions are critical in generating glyoxylic acid, the key precursor
471 to pigments, from tartaric acid, while both Cu(II) and Fe ions are most important for
472 enhancing the rate of production of the pigments from glyoxylic acid and (+)-catechin.
473 Consequently, the production of the carboxymethine-linked (+)-catechin dimers, **3**-**6**, in wine
474 conditions from added (+)-catechin and glyoxylic acid may provide a measure of Cu(II) and
475 Fe ion activity with negligible impact from Mn(II), Al(III), and Zn(II). Further work is
476 required to establish the ramifications of Cu(II) and Fe ions influencing the isomeric profile of
477 the phenolic products in wine as the taste response of bitterness and especially astringency are
478 known to be influenced by the size of phenolic compounds and their 3-dimensional structure.
479
480 **ABBREVIATIONS USED.** LC-HRMS Liquid Chromatography with High Resolution Mass
481 Spectrometric detection; **U** lactone; **X** xanthene; $\mathbf{X}^+$ xanthylium cation; $\mathbf{X}^+_{\mathbf{est}}$ ethyl ester of the
482 xanthylium cation.
483
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SUPPORTING INFORMATION

Supporting Table 1 presents the data supporting the assignment of chromatographic peaks to specific productions. Supporting Figure 1 provides the structures of the six isomeric xanthene and xanthylium cation structures. Supporting Figure 2 provides the HRMS extraction ion chromatograms associated with products. Supporting Figures 3 and 4 provide the proposed HRMS fragmentation mechanisms for the xanthene and lactone products. Supporting Figure 5 show the production of $\mathbf{X}_{\mathbf{est}}^{+}$ in experiments with and without glyoxylic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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591 **FIGURE CAPTIONS**

592

593 **Figure 1.** Production of xanthylium cations from tartaric acid and (+)-catechin.

594

595 **Figure 2.** The 280 nm (A) and 440 nm (B) chromatograms at day 28 for the 1.14 mg/L Cu(II)
596 samples with added glyoxylic acid. Also shown is the (C) 280 nm chromatogram after the
597 reduction of xanthylium cations with $NaBH_4$ to produce xanthenes. Peaks **1** and **2** are
598 glyoxylic acid-substituted (+)-catechin products, **3**-**6** are carboxymethine-linked (+)-catechin
599 dimers, $\mathbf{U_{1-3}}$ are tentatively assigned lactones, $\mathbf{X_{1-4}}^+$ are xanthylium cations, $\mathbf{X_{est}}^+$ are ethyl
600 esters of the xanthylium cations and $\mathbf{X_{1-4}}$ are xanthenes.

601

602 **Figure 3.** The combined isomer concentrations of (A) carboxymethine-linked (+)-catechin
603 dimers, **3**-**6**, (B) lactones, $\mathbf{U_{1-3}}$, and (C) xanthylium cations, $\mathbf{X_{1-4}}^+$, during the storage of
604 samples without added glyoxylic acid. The concentration units in (A) and (B) are mg/L (+)-
605 catechin equivalents.

606

607 **Figure 4.** The individual isomer concentrations for the carboxymethine-linked (+)-catechin
608 dimers during the storage of samples without glyoxylic acid: (A) product **3**, the 8-8 connected
609 isomer, (B) products **4** and **5** combined, the 8-6 and 6-8 connected isomers, and (C) product **6**,
610 the 6-6 connected isomer. The concentration units are mg/L (+)-catechin equivalents.

611

612 **Figure 5.** The combined isomer concentrations of (A) glyoxylic acid-substituted (+)-catechin,
613 **1**-**2**, (B) carboxymethine-linked (+)-catechin dimers, **3**-**6**, (C) lactones, $\mathbf{U_{1-3}}$, and (D)
614 xanthylium cations, $\mathbf{X_{1-4}}^+$, during the storage of samples with added glyoxylic acid. The
615 concentration units in (A)-(C) are mg/L (+)-catechin equivalents.

616

617 **Figure 6.** The individual isomer concentrations for the glyoxylic acid-substituted (+)-catechin
618 isomers and carboxymethine-linked (+)-catechin dimers during the storage of samples with
619 glyoxylic acid: (A) product **1**, 8-connected isomer, (B) product **2**, 6-connected isomer, (C)
620 product **3**, the 8-8 connected isomer, (D) products **4** and **5** combined, the 8-6 and 6-8
621 connected isomers, and (E) product **6**, the 6-6 connected isomer. The concentration units are
622 mg/L (+)-catechin equivalents.

623

Table 1. Concentration Ratio of Isomers for Glyoxylic Acid-Substituted (+)-Catechin, Carboxymethine-Linked (+)-Catechin Dimers, Lactones and Xanthylium Cations.[a]

Sample[b]	Glyoxylic acid -(+)-catechin[c] (Ratio)	Carboxymethine-linked dimer[d] (Ratio)		Lactones[e] (Ratio)		Xanthylium cation[e] (Ratio)		
	2/1	**(4+5)/3**	**6/3**	**U_1/U_2**	**U_3/U_2**	**X_2^+/X_1^+**	**X_3^+/X_1^+**	**X_4^+/X_1^+**
				without added glyoxylic acid				
Fe(III)	n.det.	0.66 ±0.26 a	0.60 ±0.15 b	0.51 ±0.05 b	3.18 ±0.22 a	0.35 ±0.00 a	0.30 ±0.01 a	0.09 ±0.02 a
Fe(II)	n.det.	0.94 ±0.42 a	0.87 ±0.16 a	0.40 ±0.03 c	3.09 ±0.18 a	0.35 ±0.07 a	0.26 ±0.07 a	n.det.
Fe(II)+Cu(II)	n.det.	0.53 ±0.25 a	0.41 ±0.05 b	0.41 ±0.01 c	1.63 ±0.09 b	0.43 ±0.04 a	0.30 ±0.03 a	n.det.
Fe(II)+Cu(II)+Mn(II)	n.det.	0.59 ±0.22 a	0.51 ±0.12 b	0.64 ±0.05 a	3.23 ±0.11 a	0.38 ±0.03 a	0.24 ±0.02 a	0.08 ±0.01 a
				with added glyoxylic acid				
No added metal	0.60 ± 0.05 b	0.25 ±0.12 ab	0.11 ±0.01 cd	0.53±0.01 de	0.70 ±0.03 e	0.53 ±0.03 b	0.24 ±0.01 c	0.10 ±0.01 b
Fe(III)	0.71 ± 0.08 a	0.45 ±0.26 ab	0.28 ±0.04 a	0.52 ±0.02 e	1.55 ±0.03 a	0.37 ±0.01 e	0.28 ±0.01 ab	0.09 ±0.01 b
Fe(II)	0.78 ± 0.07 a	0.47 ±0.27 a	0.28 ±0.04 a	0.54 ± 0.01 de	1.61 ±0.09 a	0.36 ±0.01 e	0.27 ±0.01 b	0.09 ±0.003 b
Fe(II)+Cu(II)	0.52 ± 0.05 b	0.34 ±0.20 ab	0.21 ±0.04 b	0.57±0.004 bc	1.11 ±0.03 b	0.44 ±0.01 d	0.29 ±0.003 ab	0.09 ±0.01 b
Fe(II)+Cu(II)+Mn(II)	0.52 ± 0.05 b	0.33 ±0.21 ab	0.20 ±0.03 b	0.55±0.01 cd	1.10 ±0.03 b	0.46 ±0.01 cd	0.29 ±0.01 ab	0.08 ±0.004 b
Cu(II) (4.7μM)	0.23 ± 0.04 de	0.23 ±0.12 ab	0.10 ±0.005 d	0.59±0.003 b	0.70 ±0.01 e	0.55 ±0.01 b	0.24 ±0.01 c	0.13 ±0.04 a
Cu(II)	0.17 ± 0.01 e	0.20 ±0.12 b	0.10 ±0.004 d	0.58±0.01 b	0.56±0.01 f	0.73 ±0.01 a	0.30 ±0.001 a	0.10 ±0.01 ab
Al(III)	0.33 ± 0.05 c	0.29 ±0.13 ab	0.14 ±0.01 c	0.54±0.004 de	0.85 ±0.01 d	0.46 ±0.03 cd	0.21 ±0.02 d	0.11 ±0.02 ab
Mn(II)	0.38 ± 0.08 c	0.26 ±0.11 ab	0.12 ±0.01 cd	0.67 ±0.03 a	0.95 ±0.04 c	0.49 ±0.02 c	0.24 ±0.01 c	0.10 ±0.01 b
Zn(II)	0.29 ± 0.08cd	0.26 ±0.12 ab	0.12 ±0.01 cd	0.54 ±0.02 de	0.75 ±0.04 e	0.49 ±0.03 c	0.23 ±0.02 cd	0.10 ±0.02 b

Significant level: different letters in the same column indicate significant difference (Duncan's test: $P<0.05$, performed by DPS software (version 7.55, China)).

a. The concentrations used for calculation of the ratios were obtained from quantitation of the products by UHPLC at 280 nm (for **1-6** and $\mathbf{U_{1\text{-}3}}$) or 440 nm (for $\mathbf{X_{1\text{-}4}^+}$).
b. All metals are 17.91 μM, apart from one Cu(II) sample with 4.72 μM (0.30 mg/L). This equates to 1.00 mg/L Fe(III), 1.00 mg/L Fe(II), 1.17 mg/L Zn(II), 0.48 mg/L Al(III), 0.98 mg/L Mn(II) and 1.14 mg/L Cu(II).
c. Ratio calculated over the period of elevated concentration (days 2-10) (Figure 6A).
d. Ratio calculated over the period of elevated concentration, which was days 10-28 for samples without added glyoxylic acid and days 6-28 for samples with added glyoxylic acid (Figures 4A-C, 6C-E).
e. Ratio on the final day of the experiment (day 28), which was the time of highest lactone and xanthylium concentration in the samples.

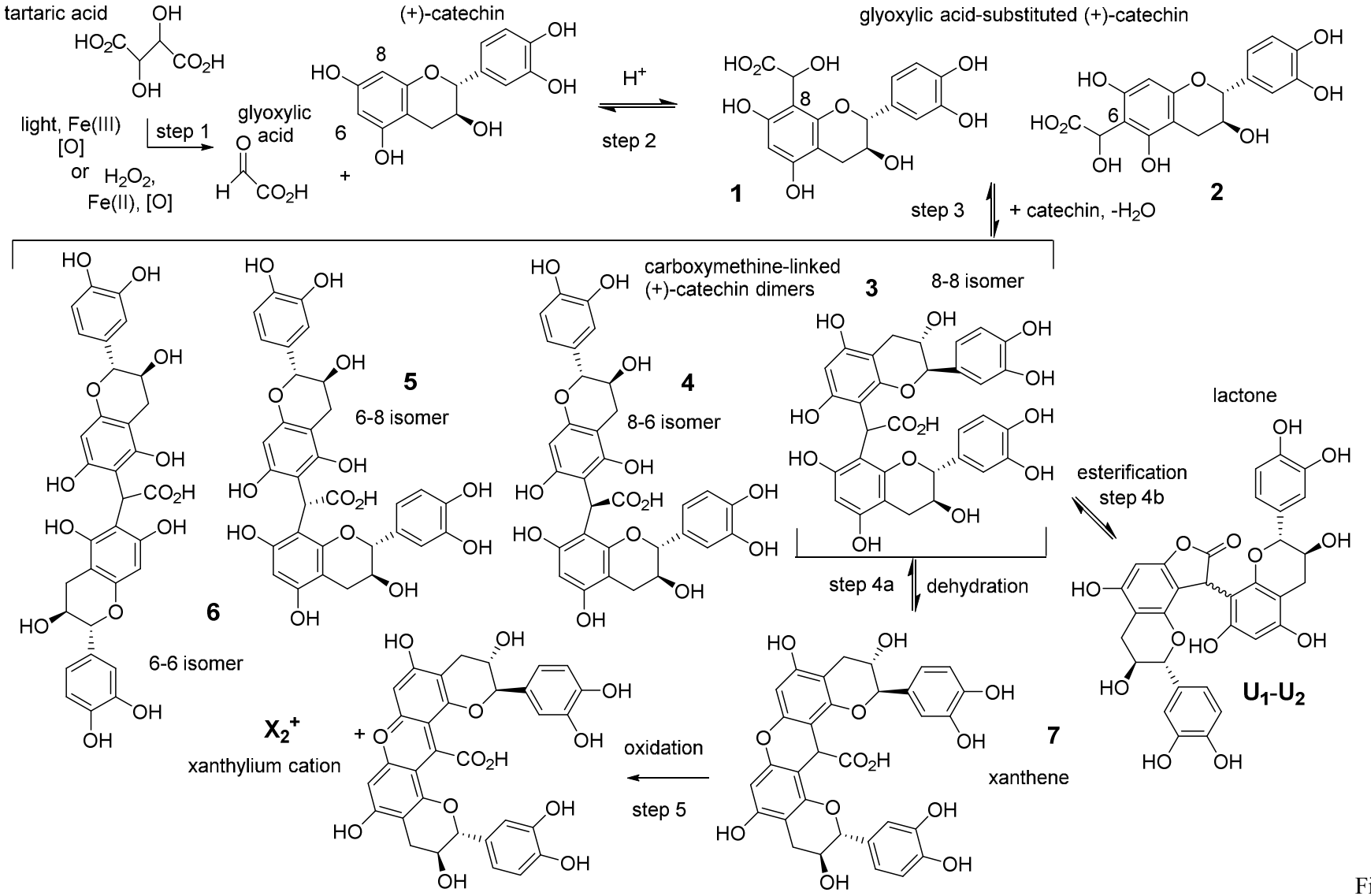

tartaric acid
light, Fe(III)
[O]
or H2O2,
Fe(II), [O]
step 1
glyoxylic acid
(+)-catechin
H+
step 2
glyoxylic acid-substituted (+)-catechin
1
2
step 3
+ catechin, -H2O
carboxymethine-linked
(+)-catechin dimers
3
8-8 isomer
4
8-6 isomer
5
6-8 isomer
6
6-6 isomer
step 4a
dehydration
esterification
step 4b
lactone
U1-U2
7
xanthene
oxidation
step 5
X2+
xanthylium cation

Figure 1

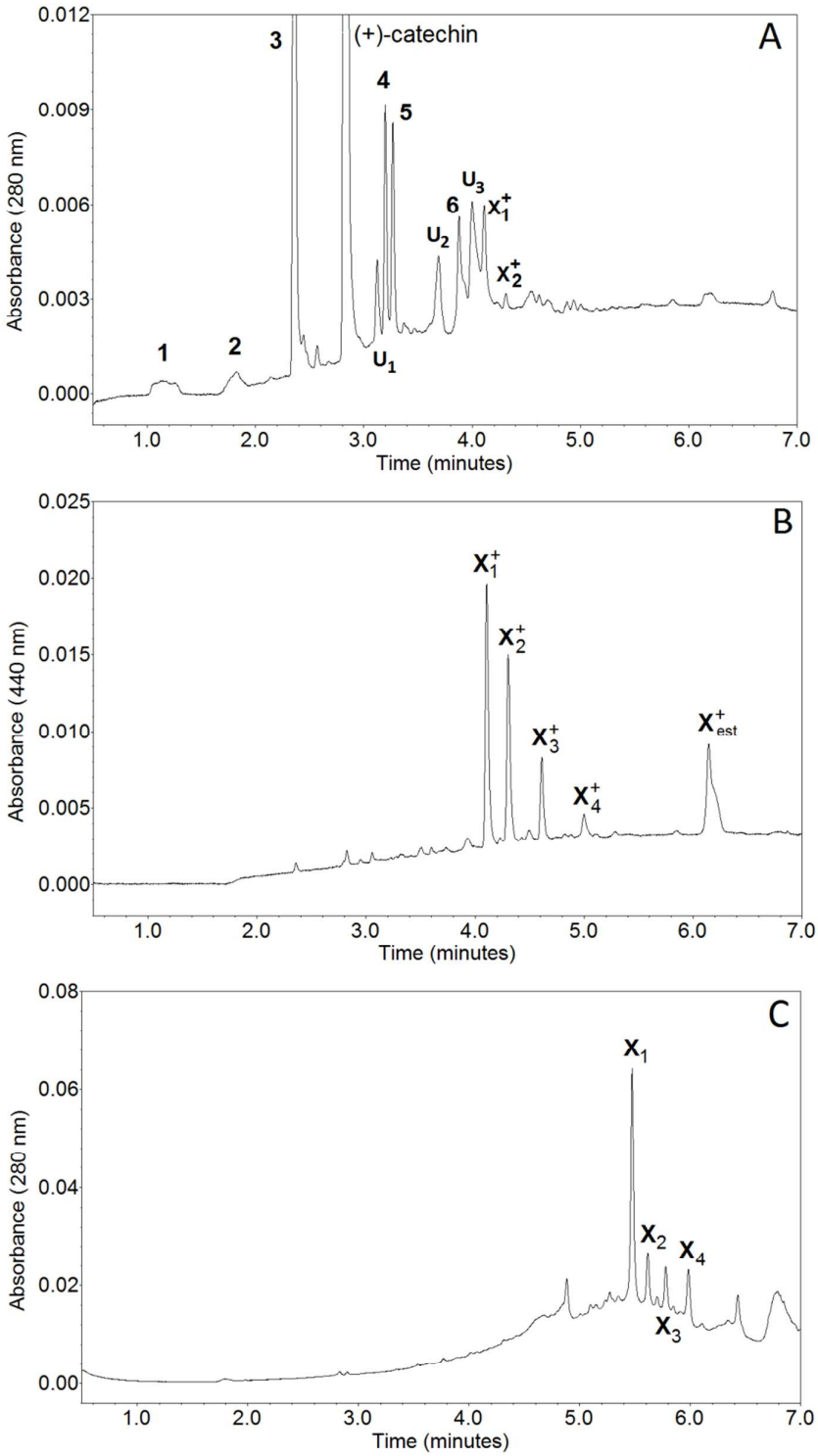


Figure 2, Guo et al. 2017

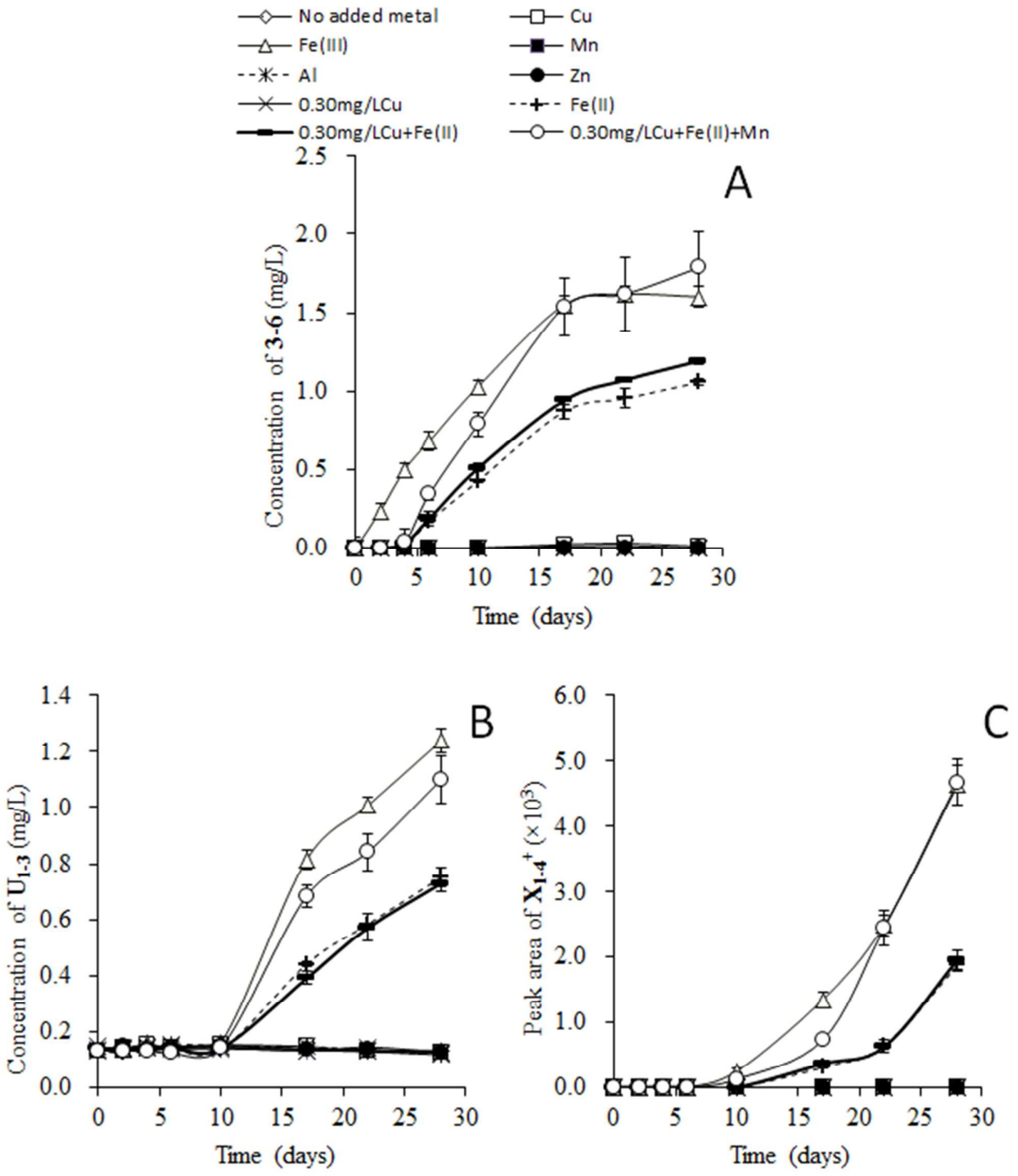


Figure 3

Guo et al. 2017

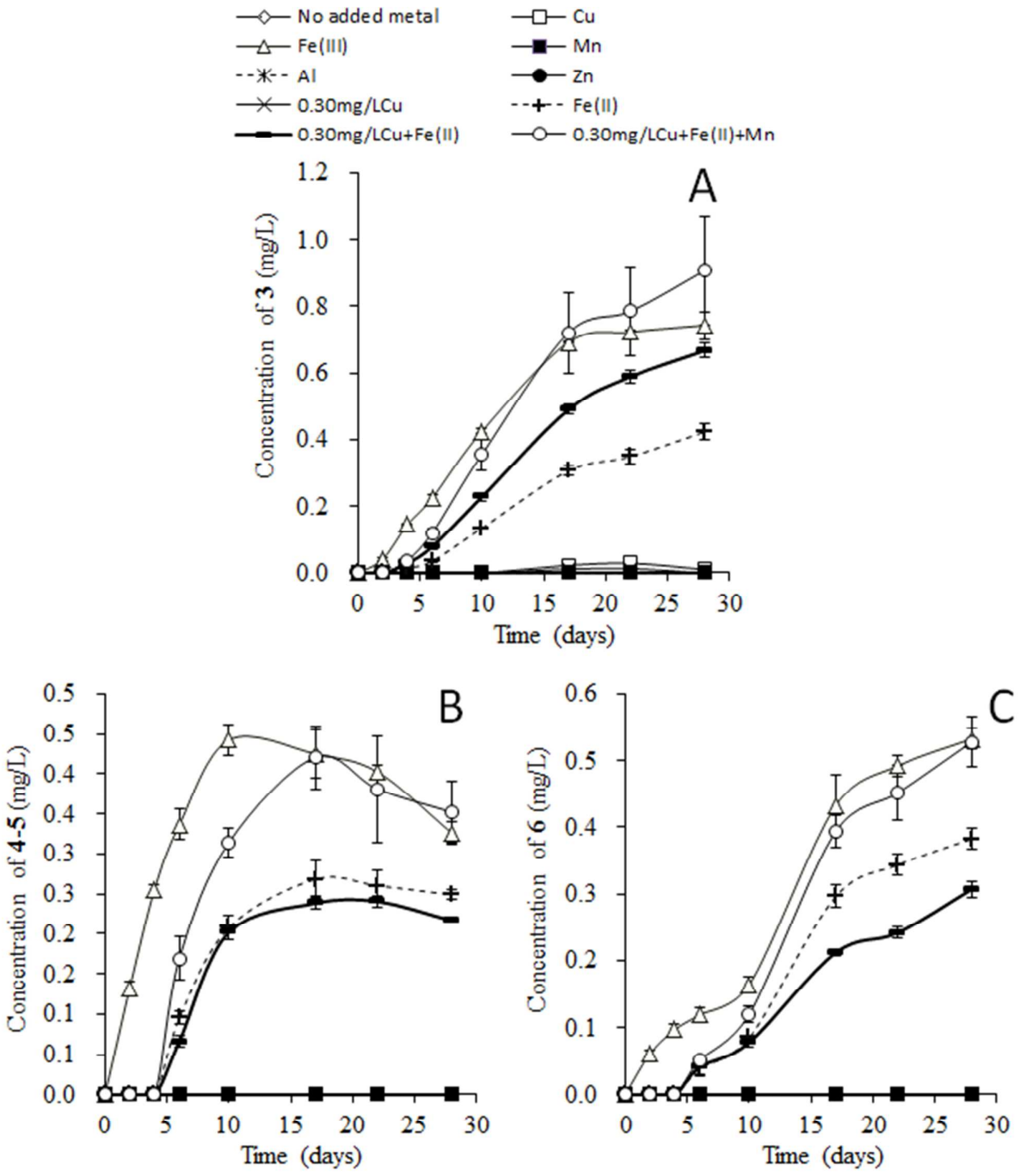


Figure 4

Guo et al. 2017

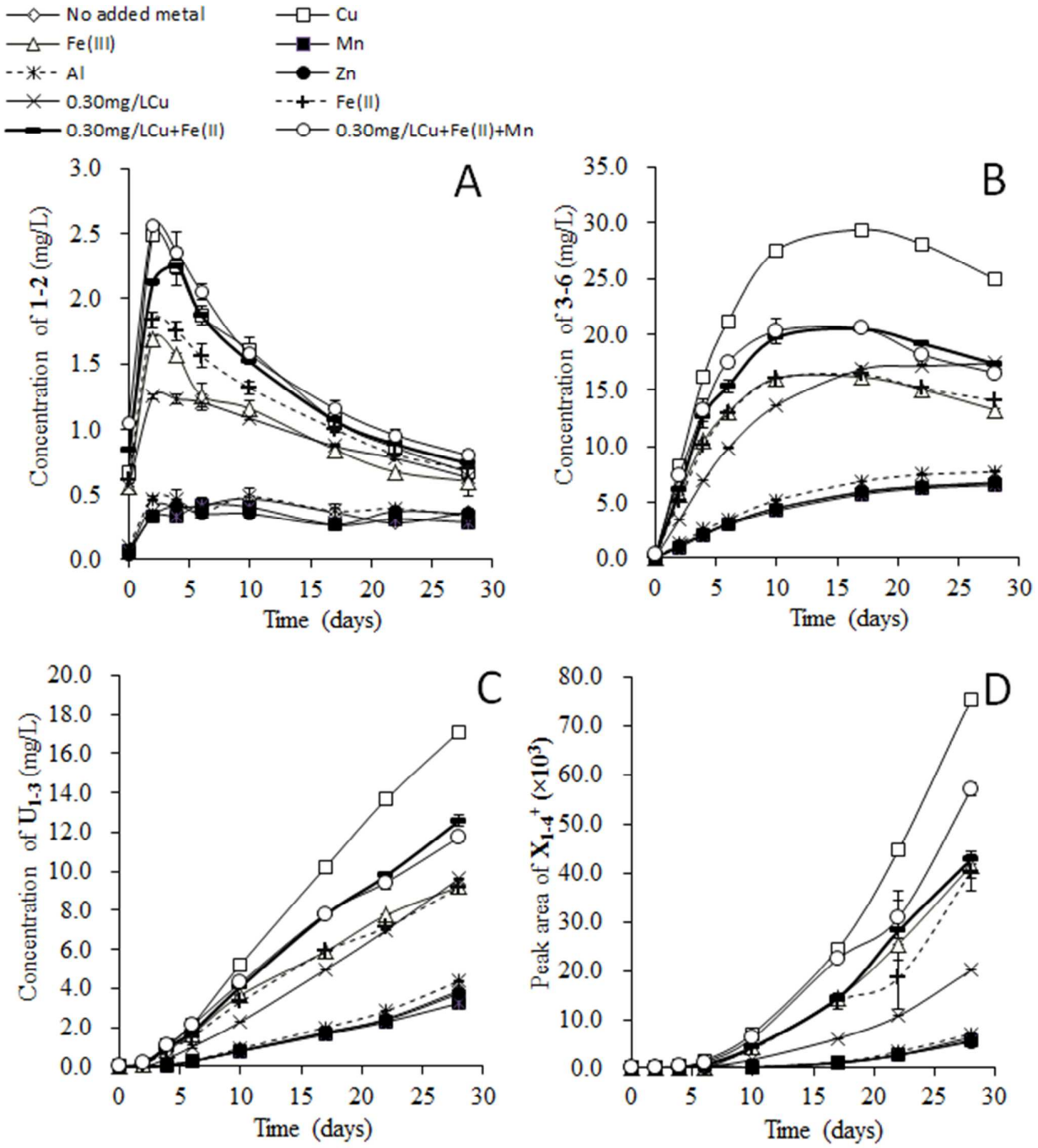


Figure 5, Guo et al. 2017

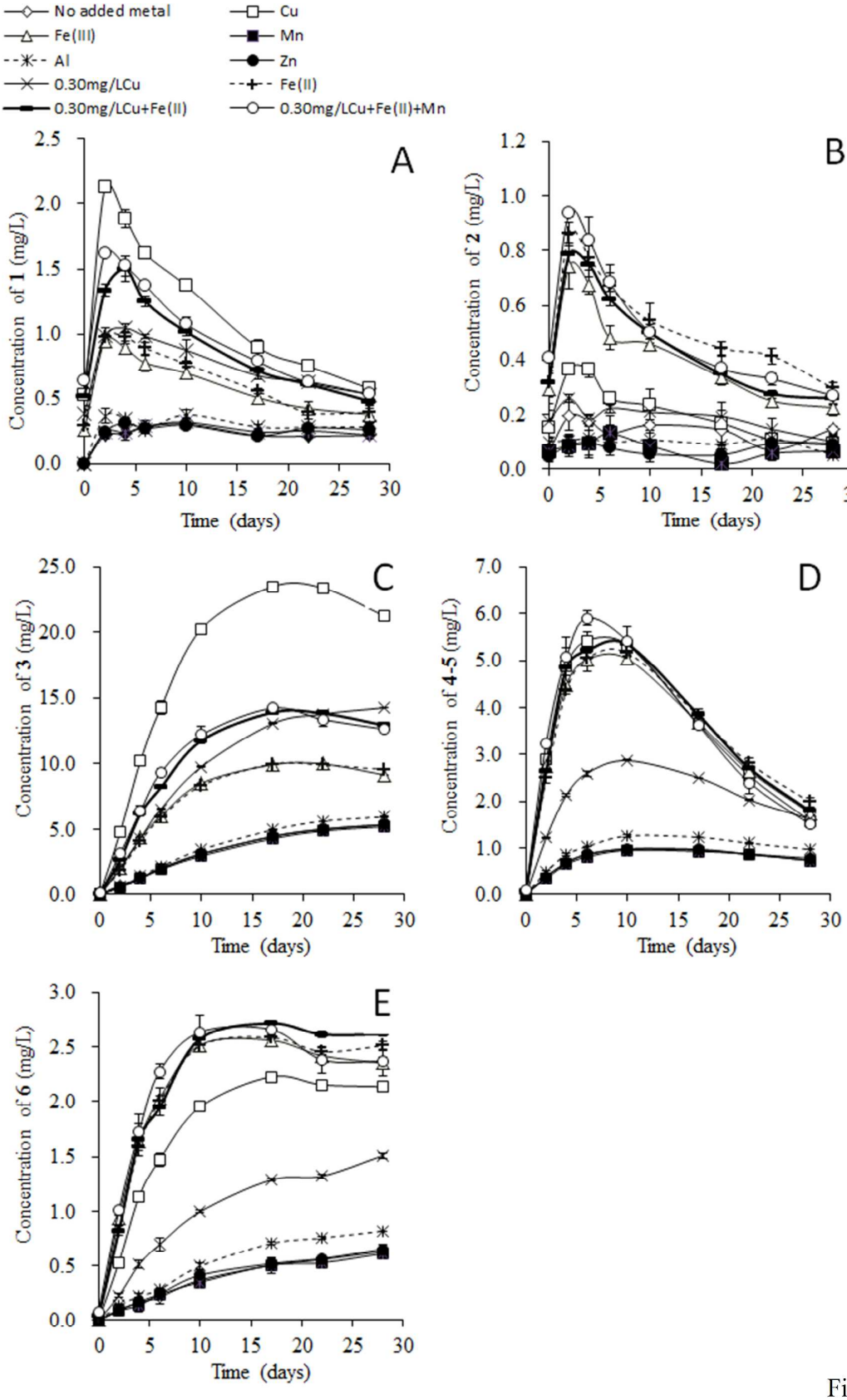
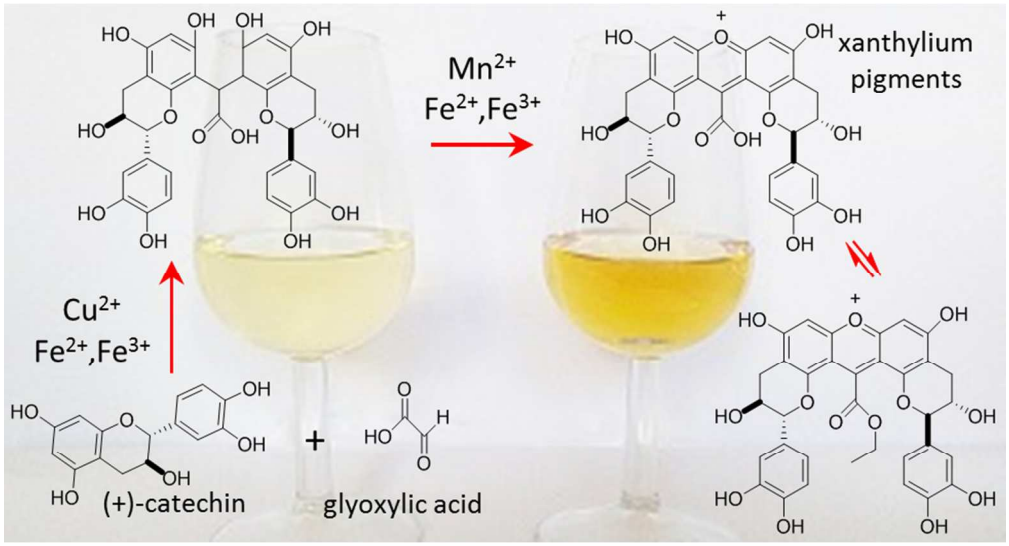


Figure 6, Guo et al. 2017



The impact of metals on the production of wine pigments from (+)-catechin and glyoxylic acid.

257x139mm (96 x 96 DPI)