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Interaction of Yeasts with the Products Resulting from the Condensation Reaction between (+)-Catechin and Acetaldehyde

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The condensation reaction between (+)-catechin and acetaldehyde was studied in model solutions in the presence and absence yeasts in order to evaluate its contribution to color changes in fermented drinks such as white wine. On the basis of the results, the yeasts retain the oligomers produced in the reaction, their retention ability increasing for higher polymerization degrees. As a result, the color of model solutions, measured as the absorbance at 420 nm, was found to decrease after the addition of yeasts. On the other hand, the yeasts exhibited no inhibitory effect on the condensation reaction, which took place at the same rate in their presence and absence. At acidity levels and reactant concentrations similar to those in wine, with acetaldehyde in high concentration as it is present in sherry wines, the reaction was found to occur very slowly. Taking into account that Yeasts are present during most of the winemaking process; consequently, they retain oligomers, and the studied reaction could mainly contribute to the alteration of the color of white wine after bottling.

KEYWORDS: (+)-Catechin; acetaldehyde; yeasts; browning; white wine

INTRODUCTION

Browning, which can be the result of enzymatic or chemical reactions, can cause serious alterations in vegetable foods. Enzymatic browning prevails in fruits, vegetables, and drinks where polyphenol oxidases (PPOs) are active, which is not the case of alcoholic beverages such as wine. Chemical browning, which is much slower, predominates when these enzymes are inhibited for some reason (e.g., thermal treatments, high ethanol content, or presence of SO_2). Browning of pale drinks restricts their market lifetime as their initial color gradually darkens, resulting in consumer rejection. Essentially, chemical browning can take place via three different pathways depending on the composition of the drink. Phenolic compounds are substrates for the development of the three ways, even though the flavan fraction has to date been the most widely studied. A first pathway is shared by all types of drink and involves the oxidation of phenols to quinones in variable degrees of polymerization, increasing the color in the yellow−brown region. This pathway is catalyzed by metals such as Fe and Cu, which alter the reaction rate to an extent dependent on their concentrations (*1*−*7*). A particular second pathway of browning for grape-based drinks involves the condensation of flavans with glyoxylic acid (*8*−*11*). The latter compound can be produced by oxidation of tartaric acid in musts and wines, and it acts as a bridge between phenol molecules. Depending on the degree of condensation, yellowish compounds that contribute to darkening of the drink color are formed. The third pathway involves the direct condensation of phenols with the acetaldehyde produced by yeasts in fermented drinks (*12*−*14*). The outcome of this in terms of color is similar to the previous one, increasing the color in the yellow spectral region as it does the condensation degree.

Currently available knowledge does not allow one to obtain a quantitative estimation of the contribution of each of the three above-described pathways to the alteration of wine color. However, one can reasonably expect the influence of each individual pathway to depend on the composition of the wine matrix and the production conditions. Specifically, pale sherry wines are known not to brown during their biological aging under flor yeasts, which develop an aerobic metabolism on their surface. Traditionally, the absence of browning in this type of wine has been ascribed to a protective effect of flor yeasts against oxygen. This effect might be exerted both by restricting the diffusion of atmospheric oxygen into the wine (the yeasts acting as an insulating surface layer) and by competition between yeasts and phenols for the dissolved oxygen in the wine. The latter possibility was demonstrated by Salmon et al. (*15*) using model solutions in contact with yeast lees, and it could contribute to the inhibition of browning in the presence of yeasts observed by Lopez-Toledano et al. (*16*) in model solutions of flavans. In any case, the oxidative browning pathways would be restricted to a great extent in sherry wines under biological aging. Conversely, the pathway involving condensation with acetaldehyde must be especially important in this type of wine, because this

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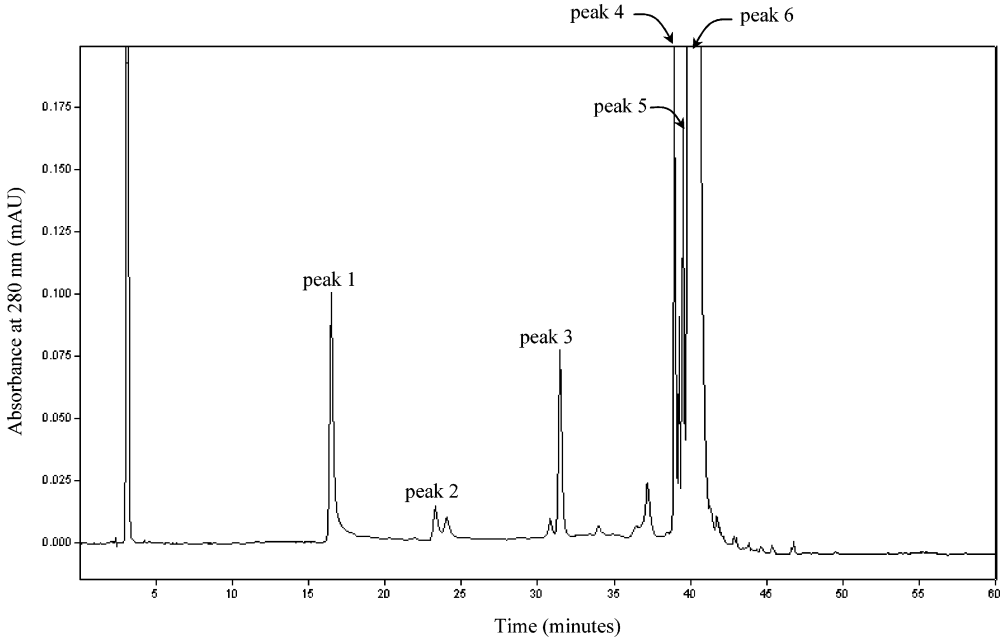


Figure 1. Chromatogram recorded at 280 nm after 90 min of reaction of (+)-catechin and acetaldehyde.

aldehyde is one of the main metabolites produced by flor yeasts (*17*), reaching concentrations up to 400 mg/L in some cases.

In addition to the above-described protective effect, yeasts exhibit the ability to retain browning compounds, so they are effective in correcting color in browned white wines (*18*). However, Razmkhab et al. (*19*) showed the efficiency of yeasts in this respect to depend on the degree of browning of the wine. This suggests that the ability to retain browning compounds depends on their chemical composition, which in turn will be a function of the prevailing chemical pathway. On the basis of the foregoing, the contribution of each chemical pathway to browning should be examined in the presence of yeasts, to better observe their inhibitory effect on the products formed and/or their ability to retain them.

In this work, the condensation reaction between (+)-catechin and acetaldehyde in relation to the presence/absence of yeasts is studied in order to increase the available knowledge on their interaction with browning compounds and their use to correct this alteration or delay its appearance in white wines.

MATERIALS AND METHODS

Reagents. (+)-Catechin was supplied by Sigma-Aldrich Chemical, S.A. (Madrid, Spain), whereas acetaldehyde, ethanol, acetic acid, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water system from Millipore (Bedford, MA). The yeast, *Saccharomyces cerevisiae*, was supplied in dehydrated form by Mauripan (Fleischmann, Canada).

Reactions. Two solutions containing 14.6 g/L (50.3 mM) of the (+)-catechin in 12% v/v aqueous ethanol were prepared and supplied with acetaldehyde at two different concentrations (2.12 M and 21.2 mM), their pH being adjusted to 2.2 with acetic acid. This acid was used rather than tartaric to avoid the oxidation of the latter in glyoxylic acid, which would lead to a competitive pathway with that of the acetaldehyde. The above-mentioned solutions of (+)-catechin were incubated at 20 °C for 48 h. The second solution (21.2 mM in acetaldehyde) was split into three batches that were used to develop the reaction in the absence of yeasts (a) and with yeasts added after 24 h (b) or from the beginning (c). The initial (+)-catechin/yeast ratio was 1:2 w/w.

A third hydroalcoholic solution (14% v/v) at pH 3.2 adjusted with acetic acid was prepared from 64.5 mg/L (0.222 mM) of the (+)-catechin and acetaldehyde (7.27 mM). This solution was incubated at 20 °C for 504 h.

Spectrophotometric Measurements and Determination of Acetaldehyde. Solutions were passed through Millipore filters of 0.45 μm pore size and their absorbance at 420 nm measured in a Beckman DU 600 spectrophotometer on 10 mm path length. Acetaldehyde was determined by direct GC injection according to the method of Muñoz (*20*).

HPLC/DAD Analyses. After filtration through Millipore filters of 0.45 μm pore size, samples were analyzed by direct injection into a TermoFinnigan Spectra System P4000 HPLC instrument equipped with a UV 6000 LP diode array detector. Eluted compounds were quantified as (+)-catechin at 280 nm, using a Merck C_{18} reversed-phase column (250 mm × 4.6 mm i.d., 5 μm particle size). The elution conditions were as follows: flow rate, 1 mL/min; solvent A, 98:2 v/v water/formic acid; solvent B, 80:18:2 acetonitrile/water/formic acid; elution from 5 to 30% B in 35 min, from 30 to 100% B in 5 min, held for 20 min, and followed by washing and re-equilibration of the column.

MS Instrument and HPLC/ESI-MS Analyses. ESI-MS measurements were made on a ThermoQuest Finnigan AQA quadrupole mass spectrometer with a mass range of 1634 amu and equipped with an electrospray source. The spectrometer was used in the negative ion mode, using an ion spray voltage of −4 kV and an orifice voltage of −60 V. HPLC separation (ThermoFinnigan Spectra System P4000 interfaced to the ESI-MS instrument) was performed after filtration of the samples (through Millipore filters of 0.45 μm pore size), using a Merck C_{18} reversed-phased column (250 mm × 4.6 mm i.d., 5 μm particle size). Elution was done using a flow rate of 200 μL/min, and the solvents and the elution conditions were the same as for the HPLC/DAD analyses. Mass data were acquired in two ways: in the scan mode (by scanning the *m*/*z* range 150−1066 using a 1.2 step size) and in the multiple ion mode (using mass ranges around specific *m*/*z* values).

RESULTS AND DISCUSSION

A first experiment was carried out to examine the reaction of (+)-catechin (50.3 mM) with acetaldehyde (2.12 M), therefore, in an aldehyde/flavan molar ratio of 42.1 and at pH 2.2. A slightly lower aldehyde/flavan molar ratio should be considered because a small fraction of the aldehyde (∼10%) inevitably reacts with ethanol to yield diethoxyethane (*21*). **Figure 1** shows the chromatogram obtained at 280 nm after a few minutes of the polymerization reaction. As can be seen, the peak for the (+)-catechin (peak 1) was accompanied by other major peaks at longer retention times corresponding to com-

pounds of greater molecular mass and lower polarity than the monomer. These reaction intermediates were identified by HPLC-MS in two ways: scan mode and multiple ion mode. The *m*/*z* values and their corresponding compounds were as follows: peak 2 (*m*/*z* 333), monomer adduct; peaks 3 and 4 (*m*/*z* 605), two of the four possible dimers; peak 5 (*m*/*z* 921), trimer; peak 6, oligomers of polymerization degree >3 units, which we called highly polymerized (HP). The presence of these compounds reveals that the polymerization process involves ethyl bridges and the formation of adducts as reaction intermediates, consistent with the mechanism initially proposed by Timberlake and Bridle (*22*) and later confirmed by Fulcrand et al. (*12*) and Es-Safi et al. (*14*):

acetaldehyde + catechin →
Cat−ethyl adduct monomer → Cat−ethyl−Cat dimer →
Cat−ethyl−Cat−ethyl dimer adduct → trimer → etc.

However, this reaction is too fast to allow the accurate examination of the interaction between yeasts and the oligomers formed. Thus, virtually all of (+)-catechin disappeared within 3.5 h after the acetaldehyde was added, the time at which the HP concentration peaked. As a result, the other compounds identified (dimers and trimer) appeared only in the chromatogram recorded within the first 90 min of reaction, after which their peaks decreased to reflect their rapid conversion into more polymerized oligomers that constituted the HP group.

The acetaldehyde concentration is known to influence the rate of flavan degradation, which invariably exhibits pseudo-first-order kinetics (*23*). According to these authors, the rate of this reaction increases with increasing acetaldehyde concentration at a constant flavan concentration. It is therefore logical to use higher than unity aldehyde/flavan molar ratios in works when the objective is to obtain quickly the end products. Thus, Fulcrand et al. (*12*) used a molar ratio of 51, and Saucier et al. (*13*) tested molar ratios of 130, 52, 26, and 13. However, to observe the interaction between the reaction products and the yeasts, it is necessary that the degradation process take place much more slowly, which can be accomplished by using a much lower aldehyde/flavan molar ratio.

On the basis of the above comments, a second polymerization experiment was carried out by using (+)-catechin at the same concentration (50.3 mM), but at a concentration in acetaldehyde 100 times lower (21.2 mM), so the aldehyde/flavan molar ratio was 0.421. **Figure 2** shows the changes in the contents of (+)-catechin and the dimers, trimer, and HP formed during the polymerization reaction under these conditions. As can be seen, both the degradation of the monomer and the production of the dimers were fast during the first few hours of reaction. The formation of the dimers and trimer peaked at 24 h, after which only the HP concentration increased because of the formation of more polymerized oligomers. These results are consistent with those of Es-Safi et al. (*14*): dimers accumulated during the first few hours of reaction and then decreased in content through conversion into more polymerized oligomers. The rate differences observed in the formation of the different oligomers may be explained by taking into account two different effects. On the one hand, as the reaction develops, the aldehyde/(+)-catechin molar ratio decreases due to the effect of the acetaldehyde being used to form all of the types of oligomers produced. In support of this hypothesis is the fact that the acetaldehyde concentration in the medium decreased by 62.5% (from 21.2 to 7.95 mM) within 24 h, whereas the (+)-catechin content decreased by only 32.2% (from 50.3 to 34.1 mM) over the same period. On the other hand, according to Dallas et al.

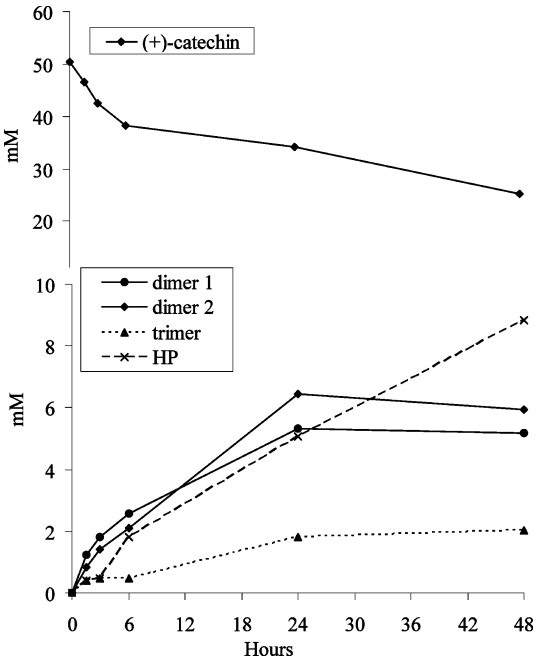
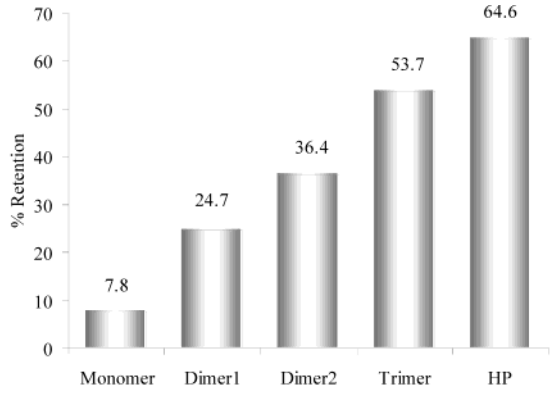


Figure 2. Changes in the contents of (+)-catechin and products formed during the condensation reaction.



solution composition after 24 hours (mM)	34.1	5.31	6.45	1.82	5.28

Figure 3. Composition of the model solution and the percent of retention in the contents of the different compounds after 90 min of contact with the yeasts.

(*23*), with equal concentrations of acetaldehyde, the rate of the condensation reaction increases with increasing polymerization of the oligomers, facilitating the formation of HP from the trimer.

To study the interaction of yeasts with the products of this reaction, a solution of (+)-catechin was supplied with dehydrated *S. cerevisiae* yeasts after 24 h of reaction. **Figure 3** shows the composition of the solution after this time and before the yeasts were added. Likewise, the percent retention of the contents in the compounds of interest before and after 90 min of contact with the yeasts is also shown. As can be seen, retention increased with increasing polymerization, that is, with the increase of the molecular mass. Thus, the (+)-catechin content decreased by only 7.8% while that of HP decreased by 64.6%.

Because of the important role of the flavan polymerization reaction in the browning of white wine, the variation of the absorbance at 420 nm throughout the studied period was recorded. It should be noted that, at the concentration used, the

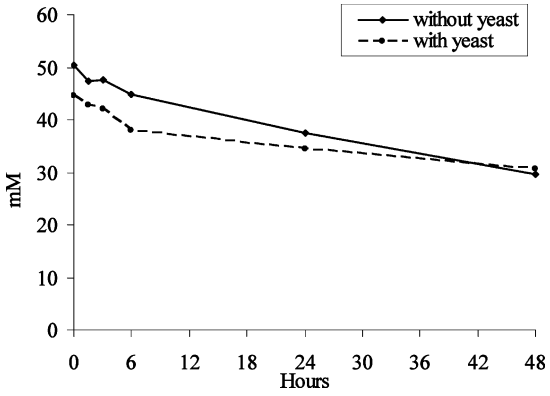


Figure 4. Changes in the (+)-catechin concentration during the condensation reaction in the absence and initial presence of yeasts.

(+)-catechin solution was colored, with an initial A_{420} value of 0.180 au, which increased to 0.218 au (17.4%) at 24 h of reaction. These changes reveal that the colored products absorbing in the visible region increased in parallel with polymerization. The addition of yeasts at 24 h decreased the absorbance to 0.122 au (43.9%) after 90 min of contact. These results show that the yeasts retained the products of the reaction to an extent that increases as the molecular mass of the oligomers formed does, yellow compounds corresponding to those with the largest masses.

To determine the potential effect of inhibition of the yeasts on the development of the polymerization reaction, a third experiment in which yeasts were added to the (+)-catechin solution before the acetaldehyde (in the same proportions as in the previous experiment) was performed. **Figure 4** shows the variation during the first 48 h of reaction of the (+)-catechin concentration in the presence and absence of yeasts. As can be seen, the solution containing the yeasts had a decreased initial flavan concentration, revealing its partial retention (~9%). Because the retention might also affect the aldehyde concentration, a solution containing only acetaldehyde at the same concentration as before was maintained in contact with the yeasts for 90 min. The microorganisms were found to retain ~10% of the aldehyde under these conditions. Therefore, the molar ratio was virtually the same in the absence of yeasts (0.421) and in their presence (0.428). As can also be seen from **Figure 4**, the (+)-catechin content changed in a similar way in both solutions throughout the studied period, so the yeasts did not show a clear inhibitory effect on the polymerization process. However, marked differences in the concentrations of the different reaction products (dimers, trimer, and HP) by the effect of the presence of the yeasts were observed (**Figure 5**). Taking into account that (+)-catechin reacted to a very similar extent in both solutions, the decreased oligomer contents in the presence of yeasts can reasonably be ascribed to their retention by the yeasts after their formation. Again, the selectivity of the yeasts toward specific molecular masses is reflected in the virtual absence of HP and the lower concentration of the trimer compared with those of the two dimers. On the other hand, the low concentration of HP is also the result of the retention of the lower oligomers by yeasts, which decreases the synthesis of the higher oligomers.

The results obtained in the previous experiments are difficult to extrapolate with a view to quantifying the contribution of the polymerization reaction to total browning in white wine. Thus, the flavan and acetaldehyde concentrations used were much higher than those typically present in any type of wine, but useful with a view to accurately identifying and quantifying the different compounds involved. In addition, the model solutions used were at lower pH than that of wine to expedite the reaction. Therefore, a final experiment using reactant concentrations and conditions similar to those of a sherry-type white wine was performed (*24*): 14% v/v ethanol, pH 3.2 (adjusted with acetic acid), 64.5 mg/L (0.222 mM) (+)-catechin, and 320 mg/L (7.27 mM) acetaldehyde (aldehyde/flavan molar ratio ~33).

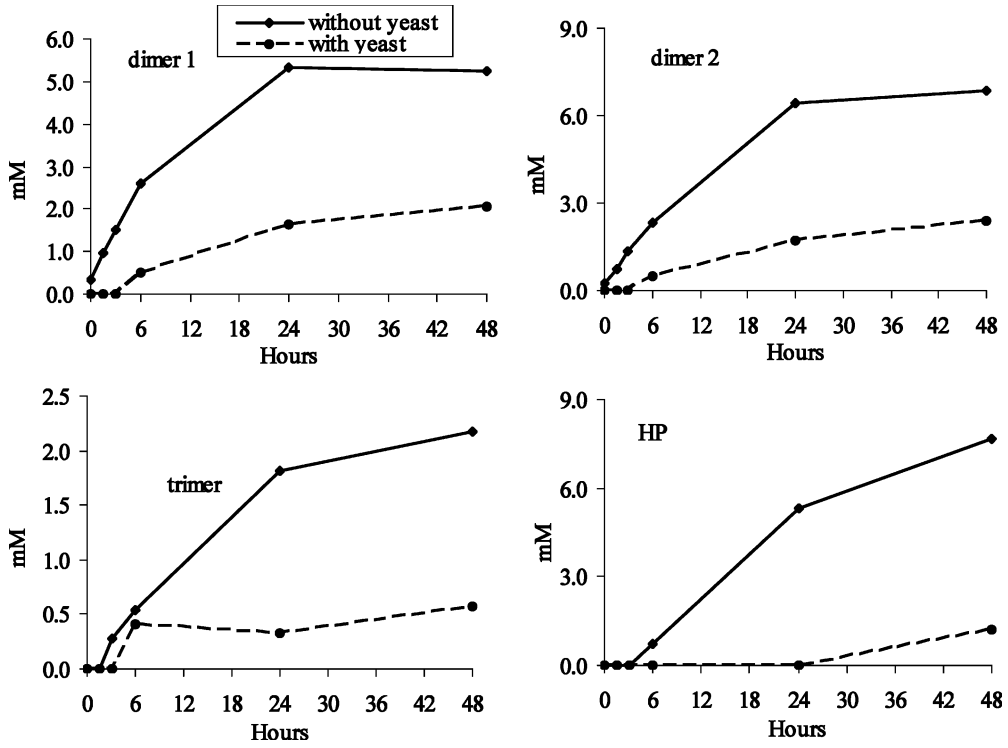


Figure 5. Changes in the dimer, trimer, and higher polymer (HP) concentrations during the condensation reaction in the absence and initial presence of yeasts.

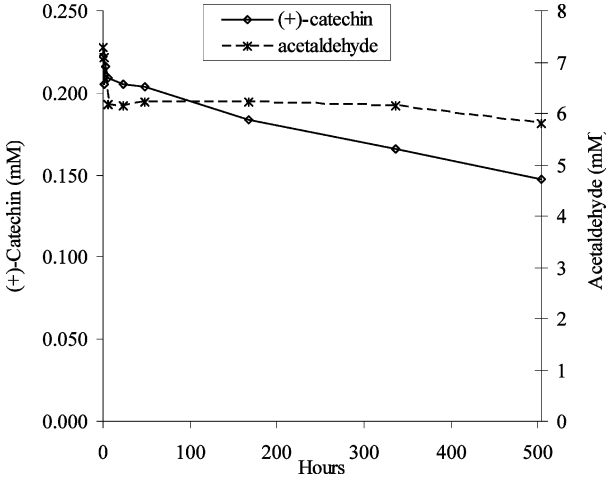


Figure 6. Changes in the (+)-catechin and acetaldehyde contents in the winelike model solution.

Figure 6 shows the changes in the (+)-catechin and acetaldehyde contents throughout the duration of the experiment (504 h). As can be seen, the degradation of (+)-catechin was much slower than in the previous experiments: its content decreased by only 8.2% within the first 48 h, with no appreciable rise in the oligomer concentration. Even after 3 weeks, these compounds were produced in very small amounts, so they could not be quantified with accuracy. In relation to the acetaldehyde concentration, it decreased during the first few hours, largely attributable to the formation of diethoxyethane. Subsequently, the content in this compound leveled off throughout the remainder of the process, with a slight trend to decrease. The results obtained in this experiment are consistent with the findings of other authors (*14*), which point out that the reaction rate decreases with decreasing acidity of the medium, possibly as a result of a decreased availability of the acetaldehyde carbocation. This has also been observed in the polymerization of several flavan oligomers with acetaldehyde (*23*), as well as in the condensation of malvidin 3-*O*-glucoside with (+)-catechin in the presence of this aldehyde (*25*).

On the whole, reasonably, the reaction studied should not contribute to a great extent to the browning of white wines. In this sense, it should be pointed out that acetaldehyde is produced throughout alcoholic fermentation, so the oligomers potentially formed during this process would be retained by fermentation yeasts and not contribute to increasing the color of the wine. This assertion can be extended to wines aged for a short time under yeast lees. Likewise, the retention of colored polymers would also occur during the biological aging of wines such as those of the sherry type. Although the flavan condensation reaction in such wines would be favored by the increased production of acetaldehyde by flor yeasts, again, the ability of the yeasts to retain the resulting products would prevent browning. Therefore, the studied reaction could contribute to the alteration of the color of the wine only after bottling, beyond which the protective effect of the yeasts would disappear. However, this reaction is very slow under the acidic conditions typical of wine, so only after very long storage, and depending on the relative proportion of acetaldehyde and flavans, could it exert an appreciable influence on the wine color.

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