Influence of Acetaldehyde Induction on Monomeric and Polymeric Polyphenols in Wine using the Polyphenol/Protein-binding Model

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Polyphenols make a substantial contribution to the sensory properties of wine, and their evolution is affected by the acetaldehyde present during fermentation and ageing. In this work, five typical monomeric phenolic standards and three different polymeric flavanol fractions separated from wine were tested for polyphenol/protein binding by means of circular dichroism measurement and fluorescence spectrum assay in the presence or absence of acetaldehyde, and the formation of new oligomeric compounds linked by ethyl bridges was observed through HPLC-MS analyses. The results show that the protein-binding ability of these monomers was in the order of gallic acid > caffeic acid > quercetin > (+)-catechin > (-)-epicatechin, while acetaldehyde exerted a stronger effect on (+)-catechin and (-)-epicatechin monomers. Moreover, different wine fractions had different responses when reacted with proteins with the participation of acetaldehyde, while the polymeric proanthocyanidins produced the largest value (84.67%) of the salivary protein precipitation index and the strongest fluorescence-quenching effect.

INTRODUCTION
The evolution of the taste of red wines during fermentation and ageing is thought to involve the function of acetaldehyde, which results from yeast metabolism and ethanol oxidation (Saucier et al., 1997; Danilewicz, 2003). Acetaldehyde can link oxygen with the wine phenolics, especially when it acts as a catalytic compound adjusting the reaction between phenolics generating macromolecule polymers to altering the astringency and stabilising colour, ultimately helping to change the organoleptic properties of red wine (Mateus et al., 2003; Sheridan & Elias, 2015).

There are a variety of polyphenols in grapes and wine that are particularly important in viticulture and oenology. The condensation reactions of polyphenols occur with the formation of acetaldehyde. Acetaldehyde can capture a proton, thereby becoming a carbocation, which can react with monomeric phenols in wine and start the process of forming ethylidene bridges, while at the same time producing a new monomeric phenol. The formation of acetaldehyde is associated with the wine's perceived features. We know that monomeric and polymeric flavan-3-ols induce both bitterness and astringency but, according to previous reports, physical and chemical features that change the organoleptic properties of wine colour and flavour (Boulton, 2001; Zhang et al., 2016). In particular, the wine astringency was shown to be lower than that unmodified by acetaldehyde (Vidal et al., 2004). To promote the yields of acetaldehyde and their beneficial effects, the micro-oxygenation (MOX) technique has been widely applied in the wine industry (Gómez-Plaza & Cano-López, 2011; Kontoudakis et al., 2011). To the best of our knowledge, phenolic compounds generally improve the sensory properties and oral mouthfeel perceptions of red wine through several reactions, such as the incorporation of anthocyanins into the tannin structure, which could decrease the perceived astringency and increase colour stability (Joaquín-Cruz et al., 2015). These compounds are divided into monomeric, oligomeric and polymeric phenols, depending on the degree of polymerisation (DP) (Sun et al., 1998). It is well known that the monomeric phenols comprise mainly flavanols, stilbenes and phenolic acids, specifically including (+)-catechin, (-)-epicatechin, epigallocatechin, quercetin, gallic acid, caffeic acid, p-coumaric acid and so on (Ren et al., 2016). It is easier to conduct qualitative or quantitative research with all of these monomeric phenols because they have a relatively smaller molecular weight. Moreover, the degree of polymerisation of polyphenols is associated with the wine’s perceived features. We know that monomeric and polymeric flavan-3-ols induce both bitterness and astringency but, according to previous reports,
the monomeric flavan-3-ols increase the wine’s bitterness faster than its astringency. In contrast, the oligomeric and polymeric proanthocyanidin fractions increase the astringency more rapidly than the wine’s bitterness (Gawel, 1998; Kennedy & Jones, 2001).

To study the effect of polyphenols on astringency in wine, sensory analysis was the most commonly used method before some of the more appropriate physical-chemical methods were proposed. Recent studies have attempted to mimic the physiological environment and apply the in vitro model of protein/polyphenol to explore the interaction of phenolic compounds and proteins. For example, when referring to the mechanism of astringency perception, it is generally accepted that the interactions between salivary proteins and tannin play a significant role in astringency (Lee et al., 2012). In previous studies, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was commonly used to evaluate the intensity of astringency, and the salivary protein precipitation index (SPI) is an index evaluating the precipitation abilities of phenolic compounds with protein (Gambuti et al., 2011). The bovine serum albumin (BSA) was commonly used as an alternative to human serum albumin (HSA) in science research, and conservative substitutions of BSA are found among different amino acids, therefore it is convenient to use to research many features of proteins, such as binding constants and binding sites. However, the SDS-PAGE method is more suitable for the interaction of polymeric phenolics with proteins than that of monomeric phenolics. Taking this into account, five monomeric phenols derived from three different classes of main phenol families were studied by means of circular dichroism (CD) and fluorescence spectroscopy techniques. These sensitive and efficient spectroscopic methods are frequently used to explore the various aspects of protein structure and their interaction with small-molecule phenolic compounds (Gorji et al., 2015). In addition, HPLC-MS has also been applied to detect the influence of acetaldehyde induction on polyphenols in red wine. This research further explored the effect of acetaldehyde on monomeric and polymeric phenols.

MATERIALS AND METHODS

Reagents

The standards of (+)-catechin, (-)-epicatechin, gallic acid, caffeic acid, quercetin, BSA, p-dimethylamino cinnamaldehyde (DMACA), Folin-Ciocalteu reagent and Waters C18 Sep-Pak columns were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetaldehyde, formic acid (MS grade), acetonitrile and formic acid (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). The SDS-PAGE kit was purchased from Shanghai Solarbio Bioscience Technology Co., Ltd. (Shanghai, China).

Red wine samples

Five millilitres of red wine sample (Cabernet Sauvignon, Xi’an, 2012) was evaporated at 30 °C and dissolved in 0.067 mol/L phosphate-buffered solution (PBS) to 20 mL (pH=7). Next, 20 mL of the wine sample was fractionated utilizing Waters C-18 Sep-Pak cartridges with the elution of 10 mL of distilled water, 25 mL of ethyl acetate and 15 mL of metha-
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The absorbance was measured at 765 nm, and the resulting values were expressed in gallic acid equivalents (GAE) using units of mg/L. The total flavanol (TFA) content of the samples was determined by spectral analysis, as previously described (Meng et al., 2012). The absorbance was measured at 640 nm, and the total flavanol content was calculated through a calibration curve using catechin as the standard, with the results expressed as catechin equivalents (CTE).

SDS–PAGE assays

Saliva was collected from four healthy volunteers between 10:00 and 11:00; the volunteers were not permitted to consume any beverages or food before saliva collection. These human saliva samples were mixed and centrifuged to remove any insoluble material. The obtained supernatant was referred to as human saliva (HS). Then, 300 μL of HS and 150 μL of sample were allowed to interact for 5 min at 25 °C, before being centrifuged at 10 000 g for 10 min. The supernatant was added to an equal volume of 2 x electrophoresis sample buffer (1 mol/L Tris–HCl, 4% SDS, 20% glycerol, 0.2 mol/L DTT, 0.1% bromophenol blue, pH 6.8), heated at 100°C for 5 min, and then analysed by SDS–PAGE (the stacking gel and resolving gel were run at 100 V and 120 V respectively). The gel was stained and destained as described by Gambuti et al. (2011). The percentage of reduction in the optical density of the two bands (at 55 kDa and 15 kDa) was obtained after interacting with the wine samples, and the SPI (salivary protein precipitation index) was calculated to evaluate the precipitation abilities of phenolic compounds with HS.

Statistical analysis

The results of total phenols and total flavanols are expressed as the mean ± standard deviation of three repetitions. One-way analysis of variance (ANOVA) was performed to verify significant differences at P ≤ 0.05 using DPS 7.05.

RESULTS

Interaction of monomeric phenols and BSA

The fluorescence intensity of BSA in the presence of different monomeric phenols is shown in Fig. 1A. These excitation spectra were characterised by a shoulder centred at approximately 285 nm to 450 nm and an emission maximum of approximately 345 nm. The maximum fluorescence intensity of BSA was 876.68 and, with the addition of monomeric phenols, there was a noticeable decrease in the fluorescence intensity, indicating that monomeric phenols had a quenching effect on BSA. In addition, the fluorescence intensity decreased in the order of gallic acid > caffeic acid > quercetin > (+)-catechin > (-)-epicatechin.

The far-UV CD spectra of BSA and the different concentrations of monomeric phenols are shown in Fig. 2. The region from 178 nm to 250 nm is the far-UV area, which was always used to investigate the secondary structure of protein (Pelton & McLean, 2000). Moreover, the CD spectrum of BSA exhibited characteristic features of the typical α-helix and β-sheet structure (Cliff et al., 2007). After the complexation of monomeric phenol with BSA, the far-UV CD spectra were similar to those of native BSA, but possessed a more markedly cotton structure (an α-helix structure with two negative minima at approximately 208 and 212 nm; there was a positive maximum at approximately 192 nm; the β-sheet structure had a positive CD spectral band at approximately 185 nm to 200 nm and a negative one at 216 nm), and the CD spectra changed by phenolic acid monomers (gallic acid) more significantly than flavanol monomers (-)-epicatechin.

Influence of acetaldehyde on monomeric phenols

The results of the acetaldehyde treatment showed that the intensity sequence of fluorescence-quenching ability was in accordance with that before acetaldehyde treatment. A comparison of the results of the CD spectra assays before and after the addition of acetaldehyde produced an equivalent order of the influence of monomeric phenol on the secondary structure of BSA. Nevertheless, the peak values of the spectra illustrate that the interaction of (+)-catechin with BSA was most strongly affected by the acetaldehyde. An unexpected finding was that the quenching effect of monomeric phenol on BSA was inhibited by acetaldehyde, but the CD spectra showed a more obvious cotton effect on the secondary structure of BSA after the acetaldehyde treatment. However, it is still not clear how acetaldehyde...
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Influenced the interaction of monomeric phenol with BSA.

These results were further demonstrated by HPLC-MS, as presented in Fig. 3 and Fig. 4. Three, five, zero, zero and two types of new compounds formed when (-)-epicatechin, (+)-catechin, gallic acid, caffeic acid and quercetin were incubated with acetaldehyde respectively. The identification of phenolic compounds with a low molecular weight was done by comparing their retention times and mass spectra features with data reported in the literature (Noureddine et al., 1999).

The figure for (+)-catechin represented typical HPLC chromatograms, showing that the first peak was (+)-catechin, the five others (CD1, CD2, CD3, CD4 and CD5) were potentially condensed products of (+)-catechin and acetaldehyde, and the newly formed compounds eluted later than their (+)-catechin precursor, indicating that they were less polar and/or larger molecules. The results obtained indicate that these products are oligomeric compounds consisting of monomeric phenol units [(-)-epicatechin, (+)-catechin and quercetin] bridged by ethyl groups.

As shown in Fig. 3, distinct (+)-catechin derivatives, CD1, CD3, CD4 and CD5, were formed and gave a mass signal at m/z 607 and m/z 629, which corresponds to a molecular weight of 606; however, the molecular weight of CD2 was 590, indicating that CD2 was not the isomer of catechin-ethyl dimer.

Taking the interaction of acetaldehyde with (-)-epicatechin, for example, fractions ED1, ED2 and ED3 formed when (-)-epicatechin was incubated with acetaldehyde and gave a signal at m/z 607 and m/z 629, which also corresponds to a molecular weight of 606 and thus a structure in which two (-)-epicatechin units are linked by an ethyl bridge.

When gallic acid and caffeic acid were incubated with acetaldehyde, their HPLC chromatograms showed that no new compounds were formed. As shown in Fig. 4B and Fig. 4C, the only peaks found were that of gallic acid at 2.89 min and caffeic acid at 6.23 min.

Investigation of three phenolic fractions incubated with acetaldehyde

Fig. 5 presents the total phenolic (TP) content and total flavonols (TFA) of the three fractions. The results clearly show that F3 possessed the highest TP and TFA values (963.19 ± 10.72 mg GAE/L and 341.13 ± 41.52 mg CTE/L respectively), while F1 had the lowest values of both TP and TFA (179.93 ± 3.97 mg GAE/L and 107.48 ± 3.46 mg CTE/L respectively).

There was a clear decrease in the optical density of all these three fractions, and the SPI values were obtained. As expected, these fractions showed different binding capacities, with F3 possessing the highest SPI with an average level of 87.15%. In addition, it was clear that, after interacting with HS, the optical density value of F3 was significantly small, as shown in Fig. 6.

The fluorescence emission spectra and the CD spectra of BSA in the presence of acetaldehyde were assessed, along with three types of fractions at 30°C. The CD spectra displayed a palpable cotton effect and a clear flute; moreover, these characteristics were most obvious in F3, which indicated

FIGURE 2
The circular dichroism spectra of BSA, with monomeric phenols at different reaction concentrations before and after acetaldehyde treatment. (A) (-)-Epicatechin. (B) Quercetin. (C) Gallic acid.
that F3 had a greater effect on the structure of BSA. There was a noticeable decrease in the fluorescence intensity as a result of binding to F1, F2 and F3. In comparison with other fractions, F3 exhibited a significantly stronger quenching of the fluorescence of BSA, as displayed in Fig. 1C. These results are in agreement with the findings of SDS-PAGE, namely that the affinity with proteins is in the order of F3 > F2 > F1.

DISCUSSION

Polyphenols are crucial substances that contribute to wine astringency and whose polymerisation reactions in wine ageing are chiefly induced by acetaldehyde. Many beneficial reactions may alter the perception of astringency and improve wine quality.

Acetaldehyde has been identified as one of the most important electrophiles with direct or indirect effects on the type of ethylidene catalytic reaction acting on monomeric and polymeric phenols (Wollmann & Hofmann, 2013). Our results on fluorescence spectroscopy and CD spectra show that acetaldehyde had a significant influence on the interaction of protein and polyphenol. Combined with the HPLC-MS assays of five types of monomeric phenols interacting with acetaldehyde, it was found that acetaldehyde could not only provide ethyl bridges and produce a new polyphenol family under the conditions of winemaking and ageing (25°C and pH 3.2), but also induce reactions among flavanol monomers ((+)-catechin, (-)-epicatechin) more easily than phenolic acid monomers (gallic acid, caffeic acid). Therefore, according to previous investigations (Escribano-Bailón et al. 1996; Drinkine et al., 2005), the major oligomers formed comprised two diastereomers of flavanol units linked by an ethyl bridge through their C-8 positions. This finding indicates that acetaldehyde is involved in the chain reactions and generates oligomeric (vinyl-dimers and vinyl-trimers) and polymeric proanthocyanidins, which could alter wine features such as astringency and colour.

Monomeric phenols constitute an important portion of the phenolic constituents of wine, and these monomeric phenols must have a complex evolution process owing to the induction of acetaldehyde during wine ageing. As described in a previous report (Li et al., 2009), flavan-3-ols are the predominant monomeric phenols in wine, and (+)-catechins and (-)-epicatechins are the main flavanol-3-ol components. However, our results on CD spectral and fluorescence quenching show that the quenching ability of (-)-epicatechin and (+)-catechin was weaker than that of gallic acid and caffeic acid. This result is in accordance with previous reports that have mentioned that the higher the content of gallic acid and caffeic acid in wine, the stronger the binding ability with salivary proteins in the oral cavity (Rudnitskaya et al., 2010; Saenz-Navajas et al., 2012; Gonzalo-Diago et al., 2014). The results of the acetaldehyde treatment show that acetaldehyde played a more significant role in (+)-catechin and (-)-epicatechin monomers than in gallic acid and caffeic acid monomers. Therefore, the flavan-3-ol substances exist mainly as oligomers and polymers in wine. This conclusion further proves that the abundant existence of gallic acid and caffeic acid, and even other phenolic acid monomers, could result in stronger binding with salivary proteins and possibly generate stronger astringent properties.

For the reasons given above, the flavonoid monomers ((+)-catechin and (-)-epicatechin) were influenced more greatly by acetaldehyde than the phenolic acid monomers,
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which is why flavonoids exist in wine as oligomers and polymers (Oszmianski & Sapis, 1989; Kovac et al., 2012). Moreover, the degree of polymerisation of polyphenols is associated with the perceived features of the wine. The different properties of monomeric and polymeric phenolics were evident among the three fractions, as our results show that, in contrast to F1 (phenolic acids) and F2 (monomeric flavan-3-ols and oligomeric proanthocyanidins), the F3 (polymeric proanthocyanidins) produced the highest TP and TFA values and salivary protein precipitation index. At the same time, in the presence of acetaldehyde, polymeric proanthocyanidins exhibited the greatest binding capacity with BSA, which

FIGURE 4
HPLC chromatogram of monomeric phenols interacted with acetaldehyde. (A) (-)-Epicatechin. (B) Gallic acid. (C) Caffeic acid. (D) Quercetin.

FIGURE 5
Total phenols and total flavanols of different fractions of wine samples.
Influence of Acetaldehyde Induction on Polyphenols was shown in the CD and fluorescence spectra. In summary, polymeric proanthocyanidins contribute greatly to red wine; in other words, a wine will display an intense affinity effect with human salivary protein in the presence of a higher content of polymeric proanthocyanidins.

There is a commonly accepted view that protein/polyphenol interactions cause the perception of astringency (Soares et al., 2017). In past investigations, proline-rich proteins (PRPs) and tannins were widely used to study the astringency perception at the molecular level (Wróblewski et al., 2001). In the latter study, a simplex protein (BSA) was selected to mimic the physiological environment in vitro. Moreover, circular dichroism and fluorescence spectroscopy techniques were used to study many features of protein, such as its binding affinity and binding constant. In the polyphenol/protein-binding model, monomeric phenolic solutions had no fluorescence emission under the conditions of the experimental concentration, while BSA solutions exhibited intense fluorescence emission, with a peak at 345 nm when excited at the appropriate wavelength (280 nm), and phenolic compounds played the role of quenchers to reduce the maximum fluorescence intensity of BSA. This behaviour of BSA concurs with the results of fluorescence-quenching studies described in the literature (Eswaran et al., 2015). The results demonstrate that there was a difference in the peak value of the fluorescence intensity among different polyphenols owing to the distinct affinity. This result could help to illustrate the contributions of different polyphenols to wine taste. In any case, apart from organoleptic evaluation, a polyphenol/protein-binding model is indeed a good method to further explore the complex perception mechanisms.

CONCLUSIONS
The results obtained in this work indicate that acetaldehyde could give rise to the polymerisation reactions of polyphenols. Specifically, (+)-catechin and (-)-epicatechin monomers were influenced by acetaldehyde more significantly than gallic acid monomers, despite having the strongest protein-affinity effect. In addition, the polymeric flavanols resulting from acetaldehyde induction possessed the highest values of TP, TFA and SPI, and exhibited the most intense affinity with proteins under the acetaldehyde-mediated influence, implying that polymeric proanthocyanidins to a large extent determine the fate of wine. In conclusion, acetaldehyde plays a vital role in the entire process of polyphenol and wine evolution.

LITERATURE CITED


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