

Article

EVALUATION OF COLOR AND STABILITY OF ETHYL-LINKED ANTHOCYANIN-FLAVANOL PIGMENTS IN MODEL WINE SOLUTIONS USING COMBINED CHEMICAL ANALYSIS AND 3D MOLECULAR SIMULATIONS

AVALIAÇÃO DA COR E ESTABILIDADE DE PIGMENTOS DE FLAVANOL-ANTOCIANINA COM LIGAÇÃO ETILO EM SOLUÇÕES MODELO DE VINHO UTILIZANDO ANÁLISE QUÍMICA COMBINADA COM SIMULAÇÕES MOLECULARES 3D

Jian Zhao¹, Min Guo², Ruoyao Wang³, Lingxi Li^{2,*}, Baoshan Sun^{2,4,*}¹ School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, 110016 Shenyang, China.² Faculty of Functional Food and Wine, Shenyang Pharmaceutical University, 110016 Shenyang, China.³ School of Pharmacy, Shenyang Pharmaceutical University, 110016 Shenyang, China.⁴ Instituto Nacional de Investigação Agrária e Veterinária, Polo de Inovação de Dois Portos, Quinta da Almoinha, 2565-191 Dois Portos, Portugal.

* Corresponding authors: +86 183 4143 52129; e-mail: lingxilee@163.com; sun.baoshan@iniav.pt

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SUMMARY

Ethyl-linked anthocyanin-flavanol pigments are one of the most important condensation products formed during the red winemaking and ageing period. They have great contribution to the color characteristics and stability of aged red wines. In this study, the color characteristics and stability of ethyl-linked anthocyanin-flavanol pigments and their precursor anthocyanins were evaluated by combined spectrophotometry and 3D molecular simulations. In model wine solutions, the condensation reactions between three anthocyanins and (-)-epicatechin, mediated by acetaldehyde, were conducted to produce ethyl-linked anthocyanin-flavanol pigments. The color was assessed by the CIELab method, and the concentration changes were analyzed by HPLC-DAD. On the other hand, the stability of these pigmented compounds was also calculated by the three 3D molecular simulation methods, that is molecular mechanics, molecular dynamics, and quantum chemistry simulation. The results obtained from CIELab analysis indicated that the formation of ethyl-linked anthocyanin-flavanol pigments resulted in a decrease of L*, a*, b* and C* values, and conversely, a rising of h* value. The 3D molecular simulations revealed that the stability of anthocyanins was as follows: Mv-3-O-glu > Pn-3-O-glu > Cy-3-O-glu. The *cis* or *trans* ethyl-linked anthocyanin-flavanol pigments were much more stable than their precursor anthocyanins. Among the pigments, ethyl-linked malvidin-3-O-glucoside-flavanol was more stable than ethyl-linked cyanidin-3-O-glucoside-flavanol and ethyl-linked peonidin-3-O-glucoside-flavanol.

RESUMO

Os pigmentos de antocianina-flavanol com ligação etilo são um dos mais importantes produtos de condensação formados durante a vinificação e o envelhecimento do vinho tinto. Estes compostos têm uma importante contribuição para a cor e estabilidade dos vinhos tintos envelhecidos. Neste estudo, as características da cor e a estabilidade de pigmentos antocianina-flavanol com ligação etilo, bem como os seus precursores (antocianinas), foram avaliadas através da combinação de espectrofotometria e de simulações moleculares 3D. Em soluções modelo de vinho, as reações de condensação entre três antocianinas e (-)-epicatequina, mediadas por acetaldeído, foram conduzidas para produzir pigmentos de antocianina-flavanol com ligação etilo. As características da cor foram avaliadas pelo método CIELab e as alterações de concentração foram analisadas por HPLC-DAD. Por outro lado, a estabilidade dos pigmentos foi também calculada pelos três métodos de simulação molecular 3D, ou seja, mecânica molecular, dinâmica molecular e simulação de química quântica. Os resultados obtidos pelo método CIELab indicaram que a formação de pigmentos de antocianina-flavanol com ligação etilo originou uma diminuição dos valores de L*, a*, b* e C* e, inversamente, um aumento do valor de h*. As simulações moleculares 3D revelaram que a estabilidade das antocianinas foi a seguinte: Mv-3-O-glu > Pn-3-O-glu > Cy-3-O-glu. Os pigmentos de antocianina-flavanol com ligação etilo (*cis* ou *trans*) apresentaram muito maior estabilidade do que os precursores. De entre os pigmentos, a malvidina-3-O-glucósido-flavanol com ligação etilo revelou-se muito mais estável do que a cianidina-3-O-glucósido-flavanol com ligação etilo e do que a peonidina-3-O-glucósido-flavanol com ligação etilo.

Keywords: Red wine, ethyl-linked anthocyanin-flavanol pigments, anthocyanins, color, stability.**Palavras-chave:** Vinho tinto, pigmentos de antocianina-flavanol com ligação etilo, antocianinas, cor, estabilidade.© Zhao *et al.*, 2023.

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INTRODUCTION

Color is an important sensory property of red wines and undergoes several changes during ageing from red to reddish-brown hues (Mano *et al.*, 2007; Yuan *et al.*, 2009; Yamagishi *et al.*, 2010; Zifkin *et al.*, 2012). Anthocyanins, as the color expression in wine, are extracted from grape skins through maceration during the winemaking process. However, anthocyanins from grape skins in a free state are unstable and tend to copigment with other compounds such as flavanols in red wine (Somers, 1971). Boulton (2001) reported that ethyl-linked anthocyanin-flavanol pigments formed by copigmentation can account for 50%-70% of the new wine. Ethyl-linked anthocyanin-flavanol pigments are formed essentially by condensation between anthocyanin and/or flavanols directly or mediated by aldehydes in the process of wine fermentation and ageing (Fulcrand *et al.*, 1996; He *et al.*, 2012). The biosynthetic pathways of these new pigments have been extensively studied in the last few decades (Timberlake and Bridle, 1976; Es-Safi *et al.*, 1999; Mateus *et al.*, 2002; Vivar-Quintana *et al.*, 2002; Dueñas *et al.*, 2006; Prat-García *et al.*, 2020). One of them is the condensation of anthocyanins and flavanols, including direct and indirect condensation reaction. The direct condensation reaction between anthocyanins (A) and flavanols (F) cause the formation of a anthocyanin-flavanol (A+F) adduct and a flavanol-anthocyanin (F-A+) adduct (Salas *et al.*, 2003). The indirect condensation reaction between anthocyanins and flavanols is mediated by an aldehyde linkage (Drinkine *et al.*, 2007; Pissarra *et al.*, 2003). The indirect condensation reaction begins with the nucleophilic addition of the flavanol to protonated acetaldehyde. The product thus formed loses a water molecule to give a new carbocation intermediate, which proceeds the nucleophilic addition by anthocyanins in the hemiketal form, to produce ethyl-linked F-Et-A or ethyl-linked F-Et-F adduct (Cheynier, 2002; Fulcrand *et al.*, 2004). Furthermore, indirect condensation as the main polymerization reaction in wine reaction occurs quickly and starts in the fermentation process (Es-Safi *et al.*, 2002; Dueñas *et al.*, 2006).

During fermentation and ageing, ethyl-linked anthocyanin-flavanol pigments gradually take a dominant role, giving the aged wine a brick-red tone and mellowing the taste from its original bitterness. Sun and Spranger (2005) reported that the majority of free anthocyanins transformed into ethyl-linked anthocyanin-flavanol pigments in 'Tinta Miúda' red wine after ageing in a bottle for two years. The result of UV spectrophotometric analysis showed that the absorption wavelength of the ethyl-linked anthocyanidin-flavanol pigments was slightly higher than that of anthocyanin, that is, a red shift, indicating

that the ethyl-linked anthocyanin-flavanol pigments may contribute more purple or red-purple to wine color. Besides, some studies showed that solutions containing such ethyl-linked anthocyanin-flavanol pigments (ethyl-linked malvidin-3-*O*-glucoside-flavanol pigment) have purple hues and are more stable against acid-base changes and bleaching by sulfur dioxide than anthocyanin precursor (Bakker and Timberlake, 1997; Casassa *et al.*, 2015; Pittari *et al.*, 2018).

Since the reaction between anthocyanins and flavanol is in a dynamic state, throughout, it is quite difficult to isolate and prepare abundant anthocyanin-flavanol pigments from wine to study their color or stability. Most of the studies on color change of anthocyanin-flavanol pigments were implemented in model wine solution, and the main anthocyanins investigated were malvidin-3-*O*-glucoside, while peonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and other monomeric anthocyanins, which were rarely involved (Es-Safi *et al.*, 2000; Escribano-Bailón *et al.*, 2001; Pissarra *et al.*, 2003; Sun *et al.*, 2008). Current studies revealed that anthocyanins can transform into anthocyanin-flavanol pigments to stabilize wine color during ageing (Sun *et al.*, 2008; Oliveira *et al.*, 2014), however, how their color transformation and stability change is still scarce. Molecular simulation, as a powerful compound analysis tool, may allow a multidimensional comparison of the compounds stability through various energy values (Zhao *et al.*, 2013). Therefore, the main objective of this work was to compare the color characteristics and stability of the free anthocyanins (malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside) and their indirect condensation products in model wine solution by using HPLC-DAD and CIELab method coupled with the three 3D molecular simulation methods (molecular mechanics, molecular dynamics and quantum chemistry simulation).

MATERIALS AND METHODS

Experimental reagents

The major free anthocyanins presented in red wine, that is malvidin-3-*O*-glucoside (Mv-3-*O*-glu), peonidin-3-*O*-glucoside (Pn-3-*O*-glu) and cyanidin-3-*O*-glucoside (Cy-3-*O*-glu), were isolated and purified in preparative scale by HSCCC as described in a previous work (Li *et al.*, 2018). (-)-Epicatechin and acetaldehyde were purchased from Aladdin (Shanghai, China). All organic solvents used in HSCCC (analytical grade) and HPLC (chromatographic grade) were purchased from the Chemical Branch of Shandong Yuwang Industrial Co., Ltd. (Shandong, China). L-tartaric acid, and hydrochloric acid were

also purchased from Chemical Branch of Shandong Yuwang Industrial Co., Ltd. (Shandong, China).

Condensation reaction between anthocyanins and flavanols mediated by acetaldehyde

The model wine solution for the indirect condensation reactions between anthocyanins and flavanols was prepared with 12% ethanol and 5 g/L of L-tartaric acid in water and adjusted to pH 3.2 with 1 mol/L HCl as previously reported (Sun *et al.*, 2008). In the presence of acetaldehyde, the reactions between anthocyanins and (-)-epicatechin were carried out in the model wine solution under the optimal conditions (molar ratio of anthocyanin/epicatechin/acetaldehyde: 1/6/10; reaction temperature 35°C) based on the previous reported study (Li *et al.*, 2018).

HPLC-DAD chromatographic conditions

The anthocyanins, (-)-epicatechin and their ethyl-linked anthocyanin-flavanol pigments were analyzed based on Waters e2695 Alliance HPLC including a quaternary pump, a controller, an autosampler, a 2998 PDA detector working in the range of 200-800 nm and a data processing work station. The optimized chromatographic conditions were demonstrated in the previously published research works (Li *et al.*, 2018). Briefly, the column was Innoval C₁₈ (5 μm, 4.6 × 250 mm) maintaining at 30°C. The flow rate was fixed at 0.7 mL/min. Solvent A (water:formic acid; 98:2, v/v) and solvent B (water:acetonitrile:formic acid; 68:30:2, v/v) were used with the following elution gradient: 0 min, 18% B; 42-48 min, 47% B; 78-110 min, 100% B; and flush and balance of the column for 10 min. The detection wavelength was 525 nm for the detection of anthocyanins and their derivatives, and 280 nm for all polyphenols.

Isolation, purification and structural identification of ethyl-linked anthocyanin-flavanol pigments

CIELab analysis

In the model wine solution system, reaction solutions of Mv-3-O-glu, Pn-3-O-glu, Cy-3-O-glu, and epicatechin in the presence of acetaldehyde were sampled three times each day. These samples were scanned in a quartz cell with a 2 mm path length under the visible spectrum (380-770 nm), using the CIE 1964 standard observer (10° visual field) and the CIE standard illuminant D65. Distilled water was used as a reference. L* (lightness), a* (green/red hue), b* (blue/yellow hue) and C* (saturation) values were calculated (Pessenti *et al.*, 2022). Hue (h*) and ΔE* values were determined by Equations 1 and 2.

$$h^* = \arctg b^*/a^* \quad \text{Eq. 1}$$

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad \text{Eq. 2}$$

Molecular mechanics method

ChemBioDraw Ultra 14.0 (CambridgeSoft Corporation, USA) was used to create the two-dimensional structure of the compounds. The two-dimensional constructions were converted into three-dimensional structures using Sybyl-6.91 (Tripos Corporation, USA). The maximum iteration was set to 10000, the force field charge was Gasteiger-Huckel, and small molecule mechanics optimization and simulation were carried out and the required parameters were obtained.

Molecular dynamics method

Molecular dynamics simulation was based on the optimal conformation. Simulated Annealing could find the global minimum energy. Polak-Ribiere algorithm was used to carry out the geometric optimization of single point calculation and obtain the optimized configuration. Simulated Annealing was selected for molecular dynamics simulation optimization based on the optimized configuration in this experiment to obtain the lowest global energy configuration and corresponding energy values. The reference values were as follows: time increment for dynamics computations was 0.5 fs; coupling time for temperature regulation was 2.0 fs; initial temperature for heating was 426.85°C; time to equilibrate at initial temperature was 1,000 fs; target temperature for annealing was -73.15°C; time to spend annealing was 1,000 fs. These steps were used to obtain a stable, balanced conformation with the lowest energy.

Quantum chemical method

The lowest energy and optimal three-dimensional conformation were obtained through molecular mechanics and molecular dynamics methods with HyperChem 8.0 software (Hypercube, USA). Single point calculations and structure optimization were carried out separately using semi-empirical PM3 quantum chemical methods.

Molecular simulations in aqueous solutions

A boundary condition of 20 × 20 × 20 was set to add 265 water molecules to simulate the spatial conformation and energy difference of anthocyanins and ethyl-linked anthocyanin-flavanol pigments in an aqueous solution. The minimum distance between the solvent and solute atom was set as 2.3 for all models.

Statistical analysis

All statistical analyses were performed using SPSS (Version 22.0, Chicago, IL, USA). The results were performed in three independent experiments for each sample. The results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Formation and identification of ethyl-linked anthocyanin-flavanol pigments in the indirect condensation reactions

Mv-3-*O*-glu, Pn-3-*O*-glu and Cy-3-*O*-glu were respectively used as a reactant to react with (-)-epicatechin to conduct the condensation reaction in model wine solution. The condensation reaction was monitored continuously for 224 hours. After the reaction started, samples were taken every two hours, filtered through a 0.22 μm filter membrane and analyzed under HPLC-DAD chromatographic conditions. At the beginning of the reaction, precursor free anthocyanins and (-)-epicatechin were detected by HPLC analysis as shown in Figure 1(A, D, G). The condensation reaction was carried out after 2 h, and the ethyl-linked anthocyanin-flavanol pigments were gradually formed as presented in Figure 1 (B, E, H). After the reaction proceeded to 224 h, there were not only predominantly ethyl-linked anthocyanin-flavanol pigments in the presence of the model wine solution system, but also formed of by-products, and presented in Figure 1 (C, F, I).

Formed ethyl-linked anthocyanin-flavanol pigments were identified based on primary and secondary fragment ions using HPLC-QTOF-MS as described in the previous study (Li *et al.*, 2018). The results showed that peaks 3 and 4, peaks 6 and 7, peaks 9 and 10 as isomers were consisted of (-)-epicatechin and anthocyanin linked by ethyl group, with the same molecular formula, molecular weight and cleavage pattern, but with different configuration and conformation. The absolute configuration and conformation of ethyl-linked anthocyanin-flavanol pigments were determined by ECD analysis. The results indicated that peak 3 was identified as *S*-configuration ethyl-linked cyanidin-3-*O*-glucoside-epicatechin (Cy-ethyl-EC), peak 6 was identified as *S*-configuration ethyl-linked malvidin-3-*O*-glucoside-epicatechin (Mv-ethyl-EC) and peak 9 was identified as *S*-configuration ethyl-linked peonidin-3-*O*-glucoside-epicatechin (Pn-ethyl-EC) in Figure 1. Peak 4 was named as *R*-configuration Cy-ethyl-EC, peak 7 was named as *R*-configuration Mv-ethyl-EC, and peak 10 was named as *R*-configuration Pn-ethyl-EC in Figure 1.

Content changes of anthocyanins and ethyl-linked anthocyanin-flavanol pigments during the indirect condensation reactions

As shown in Figure 2, the contents of Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu, as well as their associated ethyl-linked anthocyanin-flavanol pigments produced by interaction with (-)-epicatechin, were dynamically monitored by HPLC. Reactions of Mv-3-*O*-glu, Pn-3-*O*-glu and Cy-3-*O*-glu with (-)-epicatechin showed the same pattern of variation. The anthocyanin content decreased significantly from 0 to 40 h. The contents of Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu were reduced from 0.817 mg/mL to 0.017 mg/mL, 0.82 mg/mL to 0.004 mg/mL, 0.78 mg/mL to 0.0076 mg/mL, respectively. From 40 to 340 h, the decrease of the anthocyanin content tended to level off until below the limit of detection, indicating that they were basically completely transformed into ethyl-linked anthocyanin-flavanol pigments. Burtch *et al.* (2017) reported that wines containing high concentrations of diglucoside anthocyanins will form less polymeric pigment than wines containing primarily monoglucoside anthocyanins. In that study, monoglucoside anthocyanins were also converted to polymeric pigment approximately 7.5 times more quickly than diglucoside anthocyanins. Furthermore, monoglucoside anthocyanins reached nondetectable levels within 14 days (336 h), which are in agreement with the results of the present work, in which Cy-3-*O*-glu, Mv-3-*O*-glu, Pn-3-*O*-glu were almost completely converted into polymeric pigment after 340 h. For main ethyl-linked anthocyanin-flavanol pigments, Cy-ethyl-EC (*S*), Cy-ethyl-EC (*R*), Mv-ethyl-EC (*S*), Mv-ethyl-EC (*R*), Pn-ethyl-EC (*S*) and Pn-ethyl-EC (*R*) exhibited an increase followed by a decrease, reaching the highest level at 20, 18, and 16 h, respectively. The content of *S*-configuration of the ethyl-linked anthocyanin-flavanol pigments was lower than the *R*-configuration except for Cy-3-*O*-glu. After 340 h of reaction, the contents of Cy-ethyl-EC (*S*), Cy-ethyl-EC (*R*), Mv-ethyl-EC (*S*), Mv-ethyl-EC (*R*), Pn-ethyl-EC (*S*) and Pn-ethyl-EC (*R*) were 0.225, 0.105, 0.0144, 0.0178, 0.0306, and 0.0394 mg/mL, respectively. In this study, the pH 3.2 and normal oxygen level was used to simulate the matrix of real wine, and the content change of reactants and products was mainly investigated by reaction time. However, for a polymerization reaction, the presence of oxygen and lower pH values can promote it, since the formation of acetaldehyde and its protonated form is favored under such conditions (Rivas-Gonzalo *et al.*, 1995; Heredia *et al.*, 1998). Therefore, the influence of these factors on changes of ethyl-linked anthocyanin-flavanol pigment content requires further investigation.

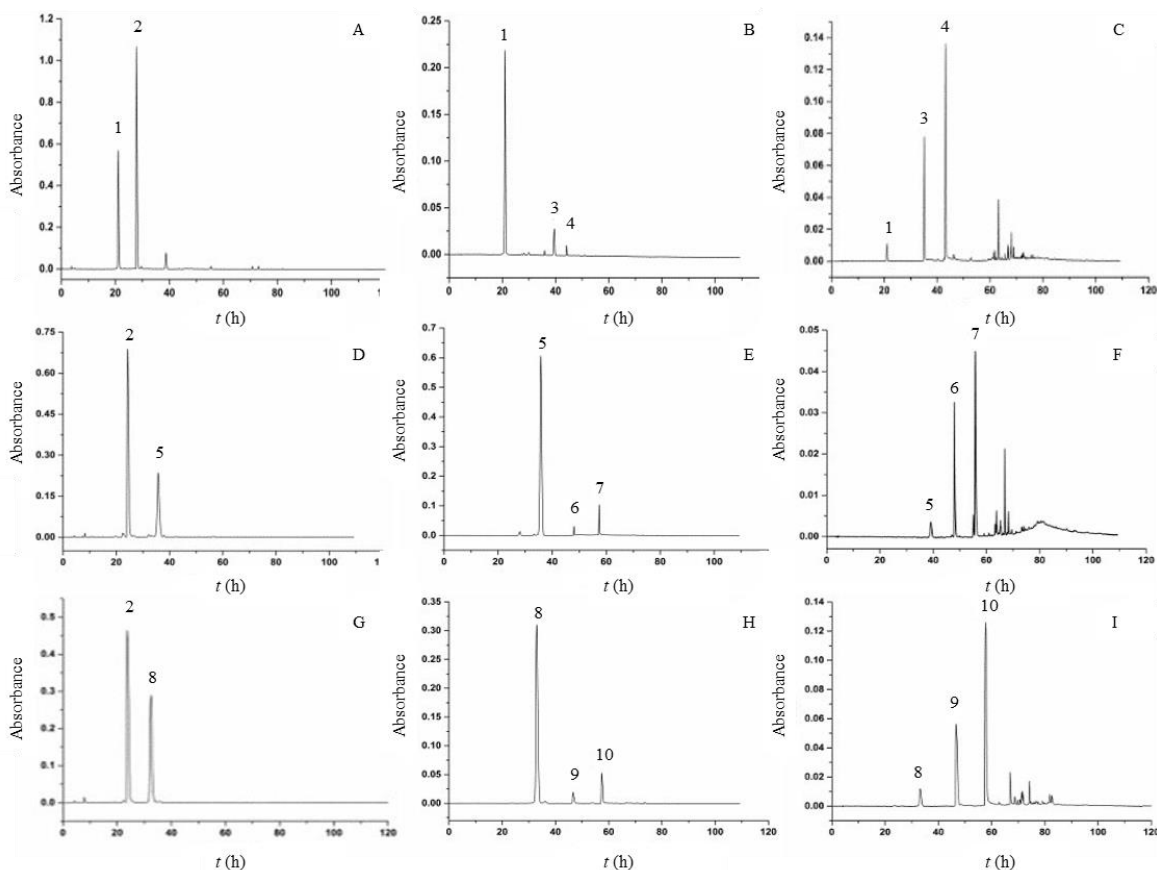


Figure 1. Chromatograms of HPLC-DAD analysis of the reaction between Cy-3-*O*-glu and (-)-epicatechin on 0h (A), 2 h (B), 224 h (C); Chromatograms of HPLC analysis of the reaction between Mv-3-*O*-glu and (-)-epicatechin on 0h (D), 2 h (E), 224 h (F); Chromatograms of HPLC analysis of the reaction between Pn-3-*O*-glu and (-)-epicatechin on 0 h (G), 2 h (H), 224 h (I); 1. Cy-3-*O*-glu, 2. (-)-epicatechin, 3. Cy-ethyl-EC (*S*), 4. Cy-ethyl-EC (*R*), 5. Mv-3-*O*-glu, 6. Mv-ethyl-EC (*S*), 7. Mv-ethyl-EC (*R*), 8. Pn-3-*O*-glu, 9. Pn-ethyl-EC (*S*), 10. Pn-ethyl-EC (*R*).

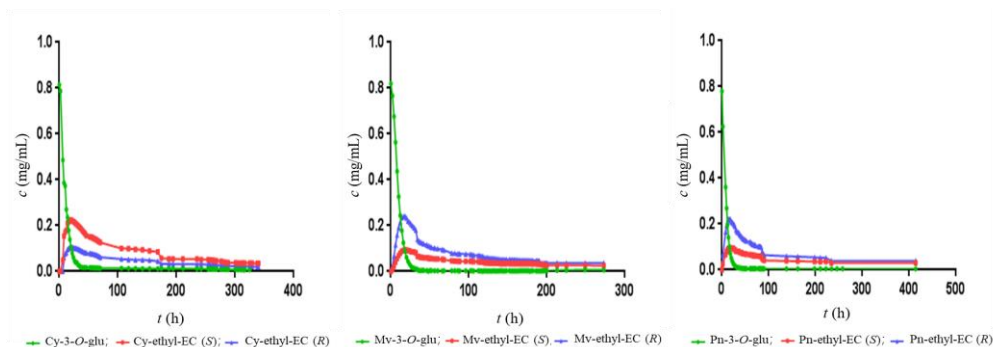


Figure 2. Concentration changes of anthocyanins and ethyl-linked anthocyanin-flavanol pigments in the reaction process.

CIELab evaluation of changes in color characteristics during the indirect condensation reactions

*Reaction system of Cy-3-*O*-glu and epicatechin in the presence of acetaldehyde*

For the precursor Cy-3-*O*-glu solutions, L^* value was 42.39, a^* value was 6.41, b^* value was 6.24, C^* value

was 8.95, h^* value was 52.3, and ΔE^* value was 5.67. Figure 3 (A) depicted the change in color characteristics during the reaction between Cy-3-*O*-glu and (-)-epicatechin. L^* value decreased from 42.39 to 31.48 in the 0-3 days, remained stable during 10 days, and then significantly decreased and remained stable; a^* , b^* and C^* values decreased significantly from 0-3 days, barely fluctuated from 3-10 days, and

significantly increased after 11 days; the h^* value showed a significant rise trend from 52.3 to 273.09 in 0-3 days, which was the same change trend as that of L^* value; the ΔE^* value decreased significantly in 0-3 days, then increased slowly in 3-9 days, followed by sustained fluctuations. As shown in Figure 3, the two-dimensional color parameters and color comparison between the initial and final reaction points for Cy-3-*O*-glu indicate a variation of an initial orange tone to a final brick red tone.

Reaction system of Mv-3-O-glu and epicatechin in the presence of acetaldehyde

For precursor Mv-3-*O*-glu solutions, L^* value was 43.62, a^* value was 4.79, b^* value was 5.26, C^* value was 7.12, h^* value was 51.75, and ΔE^* value was 4.2. Figure 3 (B) depicted the change in color characteristics during the reaction between Mv-3-*O*-glu and (-)-epicatechin. L^* value decreased from 43.62 to 31.64 in the 0-3 days, remained stable during 10 days, and then significantly decreased and remained stable; a^* and b^* values significantly decreased in the 0-3 days, and showed no fluctuation in the 3-10 days, followed by a great increase after 11 days; C^* value significantly decreased in the 0-1 day, remained stable in the 1-10 days, and decreased and remained stable after 11 days; the h^* value showed a significant rise trend from 51.75 to 272.9 in the 0-3 days, which was the same as that of L^* value; the ΔE^* value decreased significantly in 0-3 days, then slowly increased in 3-9 days, followed by continuous fluctuations. As shown in Figure 3, the two-dimensional color parameters and color comparison between the initial and final reaction points for Mv-3-*O*-glu indicate a variation of an initial red tone to a purple tone during the reaction.

Reaction system of Pn-3-O-glu and epicatechin in the presence of acetaldehyde

For precursor Pn-3-*O*-glu solutions, L^* value was 41.78, a^* value was 6.42, b^* value was 6.08, C^* value was 8.85, h^* value was 52.4, and ΔE^* value was 6.24. Figure 3 (C) depicted the change in color characteristics during the reaction between Pn-3-*O*-glu and (-)-epicatechin. The results showed that the L^* value decreased from 41.78 to 31.22 in the 0-3 days, and the change was stable in 3-10 days, and then a significant decrease occurred after 11 days; the a^* and b^* values showed a significant decrease in the 0-3 days, a more stable change with almost no fluctuation in the 3-10 days, and remained stable after a significant increase in the following 11 days; the C^* value showed a significant decrease in 0-1 day, remained stable in 1-10 days, and showed an increase and remained stable after 11 days; the h^* value showed a significant rise trend from 52.4 to 273.13 in the 0-3 days, which was the same change trend as that of L^* value; the ΔE^* value significantly decreased from 6.24 to 2.23 in the 0-1 day, and remained stable in the 2-13 days. As shown in Figure 3, the two-dimensional color

parameters and color comparison between the initial and final reaction points for Pn-3-*O*-glu indicate a variation of the initial orange tone to a brick red tone during the reaction.

The a^* and h^* values of Cy-3-*O*-glu and Pn-3-*O*-glu were both greater than those of Mv-3-*O*-glu. The order of L^* values was Mv-3-*O*-glu > Cy-3-*O*-glu > Pn-3-*O*-glu. This was the opposite order observed for ΔE^* . The order of both b^* and C^* values was Cy-3-*O*-glu > Pn-3-*O*-glu > Mv-3-*O*-glu. The color values of various monomer anthocyanins were influenced by their structures, substituents on the B-ring, acyl groups on the glucoside and the molecular steric structure (Han *et al.*, 2008). B ring of Cy-3-*O*-glu was linked to -OH (C3' positions), -OH (C4' positions), and -H (C5' positions). B ring of Mv-3-*O*-glu was linked to -OCH₃ (C3' positions), -OH (C4' positions), and -OCH₃ (C5' positions). B ring of Pn-3-*O*-glu was linked to -OCH₃ (C3' positions), -OH (C4' positions), and -H (C5' positions). Therefore, it can be concluded that hydroxyl groups contributed more to both the decrease of L^* values and the increase of a^* , b^* , C^* , h^* and ΔE^* values than the methoxyl groups. This result is consistent with those of the study of Heredia *et al.* (1998), in which it was concluded that the absorbance decreased when hydroxyl groups were substituted by methoxyl groups, according to the comparison of two (Cy-3-*O*-glu > Pn-3-*O*-glu) B-ring substituted anthocyanins. Therefore, it is suggested that to a large extent, the color of the three common anthocyanin depended on the number, position and type of substituents on B-ring.

Based on the results above mentioned, significant differences in color characteristics were observed among the different reaction systems. In the process of reaction between anthocyanins (Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu) and (-)-epicatechin, during 0-3 days, L^* values of Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu gradually decreased, remained stable for a while and decreased significantly after 11 days. C^* value presented a similar variation trend to that of a^* and b^* for Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu. In short, it mainly showed that a^* , b^* and C^* significantly decreased in the 0-3 days and remained stable in the 3-10 days, then rise and remain stable after 11 days. h^* values of Cy-3-*O*-glu, Mv-3-*O*-glu and Pn-3-*O*-glu presented an opposite change compared with a^* , b^* and C^* , which significantly increased in the 0-3 days and remained stable in the 3-10 days with no significant fluctuations in data. Escribano-Bailón *et al.* (2001) found that in the acetaldehyde-mediated condensation between Mv-3-*O*-glu and (+)-catechin, a decrease occurred in the L^* values and h^* values. The variation tendency of h^* values was opposite to the results of the present study. It was possible that *cis-trans* isomers of flavan-3-ol have an effect on h^* value. The decrease of ΔE^* value was fastest for Pn-3-*O*-glu, followed by Cy-3-*O*-glu and Mv-3-*O*-glu in the

0-3 days. This behavior was likely due to the slightly higher reaction rate of Pn-3-O-glu with (-)-epicatechin than that of the other two anthocyanins. To determine if the changes in chromatic parameters were noticeable, the color differences (ΔE^* values) among three reaction system were calculated. Previous research has shown that even an untrained human eye can distinguish between two colors with a ΔE^* value of 3.0 units (Pissarra *et al.*, 2003). The obtained results revealed that the ΔE^* value was consistently higher than 4 for each reaction system, indicating a noticeable color change above the perceptible threshold and making it always detectable (Spagna *et*

al., 1996). The noticeable change in color before and after the reaction in model wine solution was clearly presented in Figure 3, providing strong evidence for the reliability of the experimental results. The formation of ethyl-linked anthocyanin-flavanol pigments promoted a decrease of L^* , a^* , b^* and C^* values and, conversely, a rising of h^* value. The results were consistent with those of Dufour and Sauvaitre (2000), according to whom these ethyl-linked anthocyanin-flavanol pigments are more stable than monomer anthocyanins and contribute to stabilizing wine color.

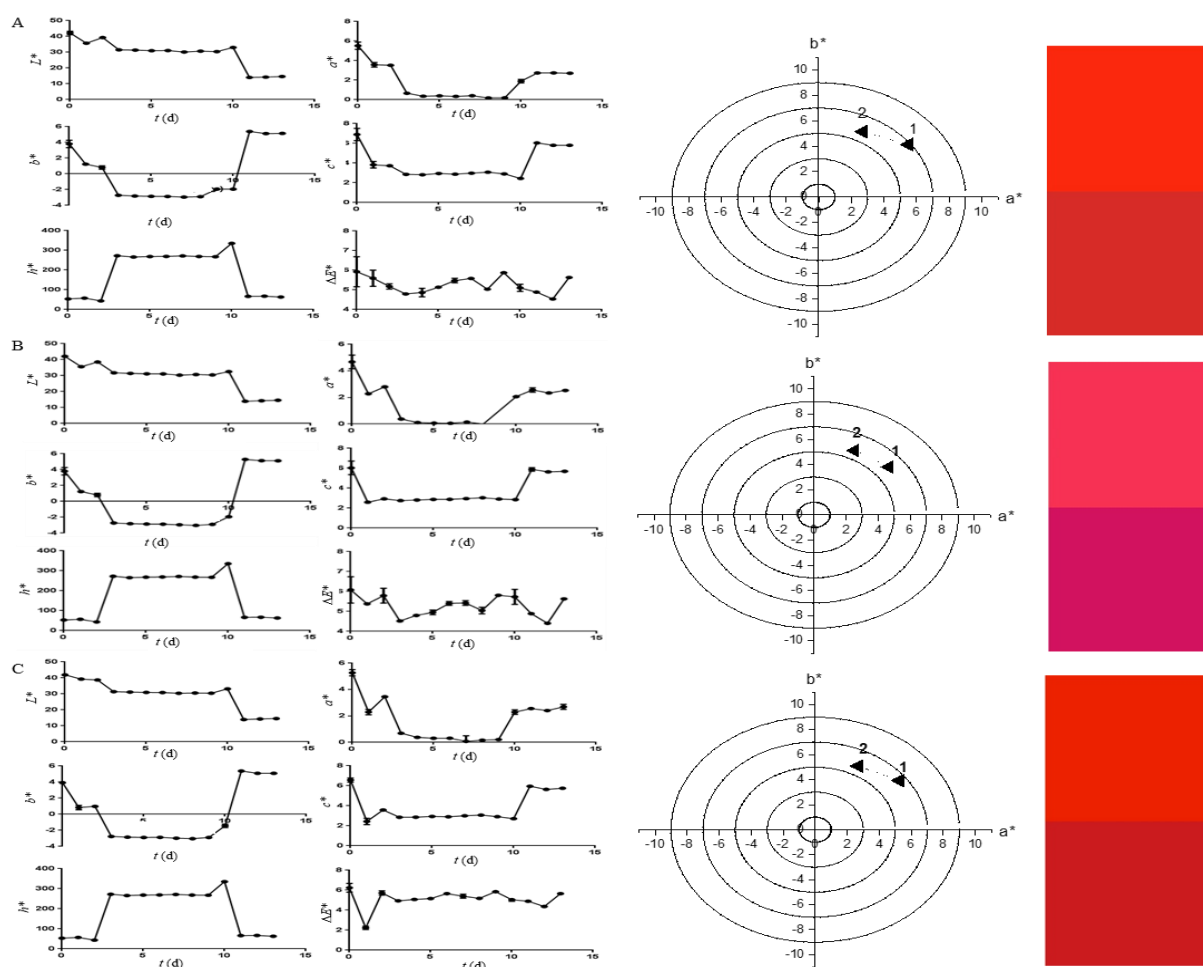


Figure 3. (A) Changes of color parameters, and cartesian representation of the CIE Lab coordinates a^* and b^* of the reaction of Cy-3-O-glu and (-)-epicatechin — (1) initial point as orange-red hue and (2) final point as brick-red hue; (B) Changes of color parameters, and cartesian representation of the CIE Lab coordinates a^* and b^* of the reaction of Mv-3-O-glu and (-)-epicatechin — (1) initial point as red hue and (2) final point as violet hue; (C) Changes of color parameters, and cartesian representation of the CIE Lab coordinates a^* and b^* of the reaction of Pn-3-O-glu and (-)-epicatechin — (1) initial point as orange-red hue and (2) final point as brick-red hue (Chromatic circle: $+a^*$ redness to greenness $-a^*$; $+b^*$ yellowness to blueness $-b^*$).

Molecular simulation of anthocyanins and ethyl-linked anthocyanin-flavanol pigments

Stability of three-dimensional structure model of anthocyanins and ethyl-linked anthocyanin-flavanol pigments by molecular mechanics method

2D structural models of anthocyanins and ethyl-linked anthocyanin-flavanol pigments were presented in Figure 4. The 2D structure was transformed into a 3D structure using Sybyl-6.91. Following molecular mechanics optimization, the simulated molecular structure was depicted in Figure 5, and the energy values are shown in Table I. The bond energy (bond stretching energy, angle bending energy, torsional energy and out of plane bending energy) represents the strength of the bond. It was important to consider bond energy value when estimating the stability of the compounds (Liu *et al.*, 2017). It was clear that the bond energy of the three anthocyanins was Mv-3-*O*-glu > Pn-3-*O*-glu > Cy-3-*O*-glu, therefore, it was speculated that the 3' and 5' methoxyl groups on anthocyanin B ring are more difficult to fracture than hydroxyl groups with the same position on anthocyanin B ring. The bond energy of either *cis* or *trans* ethyl-linked anthocyanin-flavanol pigments was higher than that of the corresponding anthocyanins. Hence, it indicated that the ethyl-linked anthocyanin-flavanol pigments

structure was more stable than the anthocyanin one. The high contribution of van der Waals energy leads to a general contraction and higher stability of compounds (Lan *et al.*, 2013). Regardless of Mv, Pn, and Cy, ethyl-linked anthocyanin-flavanol pigments have high van der Waals forces compared to monomer anthocyanins, indicating that ethyl-linked anthocyanin-flavanol pigments had good stability. In terms of total potential energy, the compounds were sorted as follows: Mv-3-*O*-glu (34.780 kJ/mol) > Pn-3-*O*-glu (27.956 kJ/mol) > Cy-3-*O*-glu (26.852 kJ/mol), Mv-ethyl-EC (*S*) (42.213 kJ/mol) > Mv-ethyl-EC (*R*) (39.900 kJ/mol) > Pn-ethyl-EC (*S*) (37.116 kJ/mol) > Cy-ethyl-EC (*S*) (36.481 kJ/mol) > Cy-ethyl-EC (*R*) (35.919 kJ/mol) > Pn-ethyl-EC (*R*) (34.111 kJ/mol). The total potential energy of the ethyl-linked anthocyanin-flavanol pigments was larger than that of anthocyanins. Further, the total potential energy of ethyl-linked malvidin-3-*O*-glucoside-flavanol was larger than ethyl-linked cyanidin-3-*O*-glucoside-flavanol and ethyl-linked peonidin-3-*O*-glucoside-flavanol. In short, it was shown that ethyl-linked anthocyanin-flavanol pigments had good stability, especially, the ethyl-linked malvidin-3-*O*-glucoside-flavanol.

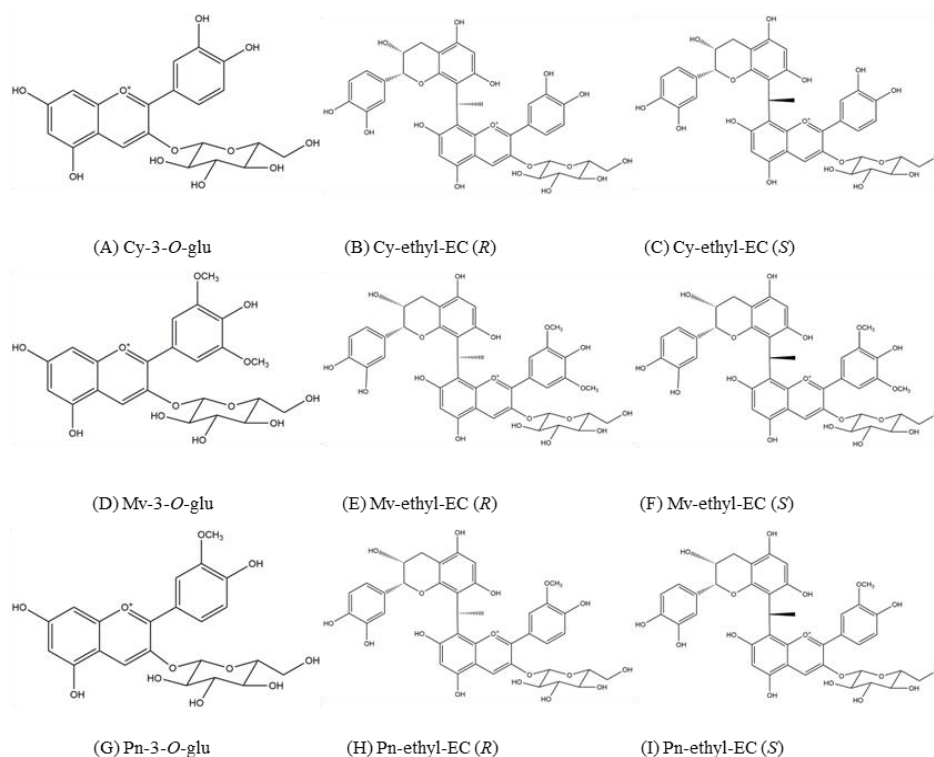


Figure 4. 2D structural models of anthocyanins and ethyl-linked anthocyanin-flavanol pigments.

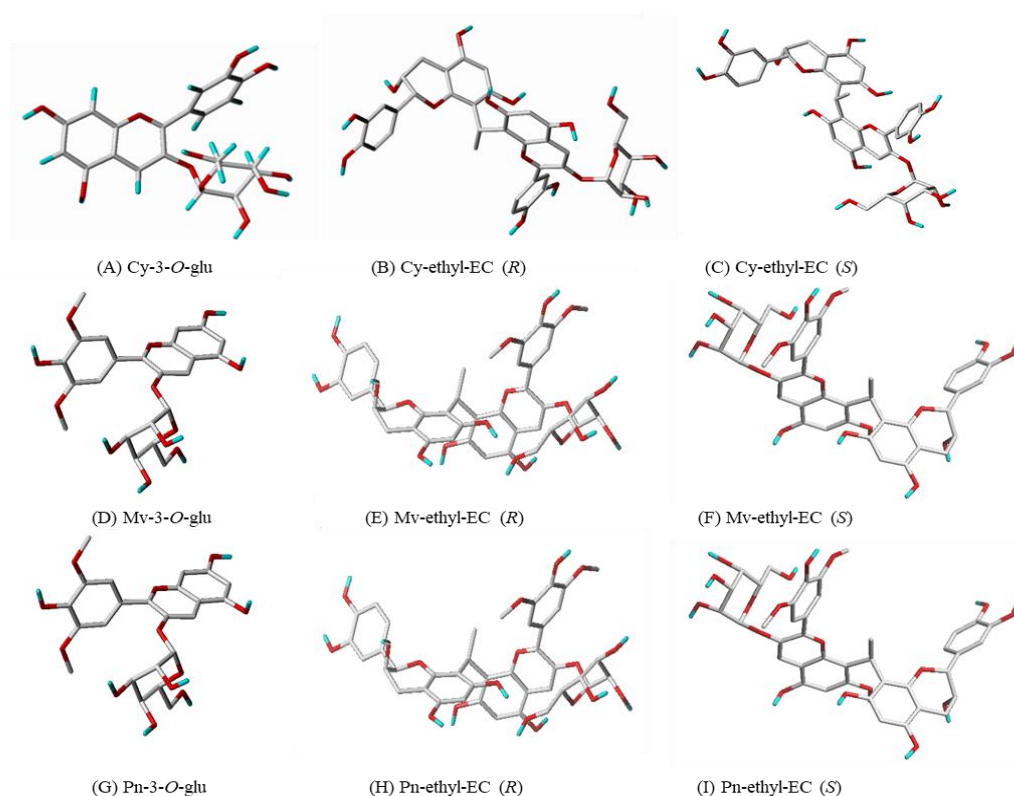


Figure 5. 3D structural model of anthocyanins and ethyl-linked anthocyanin-flavanol pigments obtained by molecular mechanics optimization.

Table I

Energy values obtained for the molecular modeling of anthocyanins and ethyl-linked anthocyanin-flavanol pigments with molecular mechanics

Compound	Bond stretching energy	Angle bending energy	Torsional energy	Out of plane bending energy	Van der Waals energy	Electrostatic energy	Total energy (kcal/mol)
Cy-3-O-glu	1.054	5.985	5.785	0.013	-8.239	-12.350	26.852
Cy-ethyl-EC (R)	1.761	10.627	6.797	0.014	-14.437	-18.687	35.919
Cy-ethyl-EC (S)	1.760	10.866	6.710	0.018	-14.459	-18.133	36.481
Mv-3-O-glu	1.222	9.905	5.648	0.012	-8.187	-13.480	34.780
Mv-ethyl-EC (R)	2.028	12.793	10.666	0.095	-18.073	-22.005	39.900
Mv-ethyl-EC (S)	1.931	13.294	8.521	0.038	-16.601	-19.207	42.213
Pn-3-O-glu	1.119	7.412	5.858	0.017	-8.544	-12.469	27.956
Pn-ethyl-EC (R)	1.896	10.869	10.848	0.090	-17.999	-21.017	34.111
Pn-ethyl-EC (S)	1.797	11.453	8.665	0.034	-16.434	-17.674	37.116

Stability of three-dimensional structure model of anthocyanins and ethyl-linked anthocyanin-flavanol pigments by molecular dynamics method

The 3D conformation of the anthocyanin and ethyl-linked anthocyanin-flavanol pigment after optimization by simulated annealing is shown in Figure 6, and the lowest energy values are presented in Table II. In molecular dynamics, there is a close correlation between the energy value of a compound

and its activity level, with higher energy values corresponding to more active compounds (Ai *et al.*, 2022). The energy of Mv-3-O-glu, Pn-3-O-glu, and Cy-3-O-glu were 99.443, 98.911 and 85.362 kcal/mol, respectively. Therefore, the energy values of the three anthocyanins were Mv-3-O-glu > Pn-3-O-glu > Cy-3-O-glu. The energy of six ethyl-linked anthocyanin-flavanol pigments was higher than those of monomer anthocyanins. Among them, the order of energy was: Mv-ethyl-EC (R) (158.767 kcal/mol) >

Mv-ethyl-EC (*S*) (152.349 kcal/mol) > Cy-ethyl-EC (*S*) (151.849 kcal/mol) > Pn-ethyl-EC (*S*) (145.956 kcal/mol) > Pn-ethyl-EC (*R*) (145.364 kcal/mol) > Cy-ethyl-EC (*R*) (135.130 kcal/mol). It demonstrated that, at the global minimum energy, the ethyl-linked anthocyanin-flavanol pigments were easier to form or

react with other compounds, comparing with monomer anthocyanins. Especially, ethyl-linked malvidin-3-*O*-glucoside-flavanol was more easily formed or reacted with other compounds than ethyl-linked cyanidin-3-*O*-glucoside-flavanol and ethyl-linked peonidin-3-*O*-glucoside-flavanol.

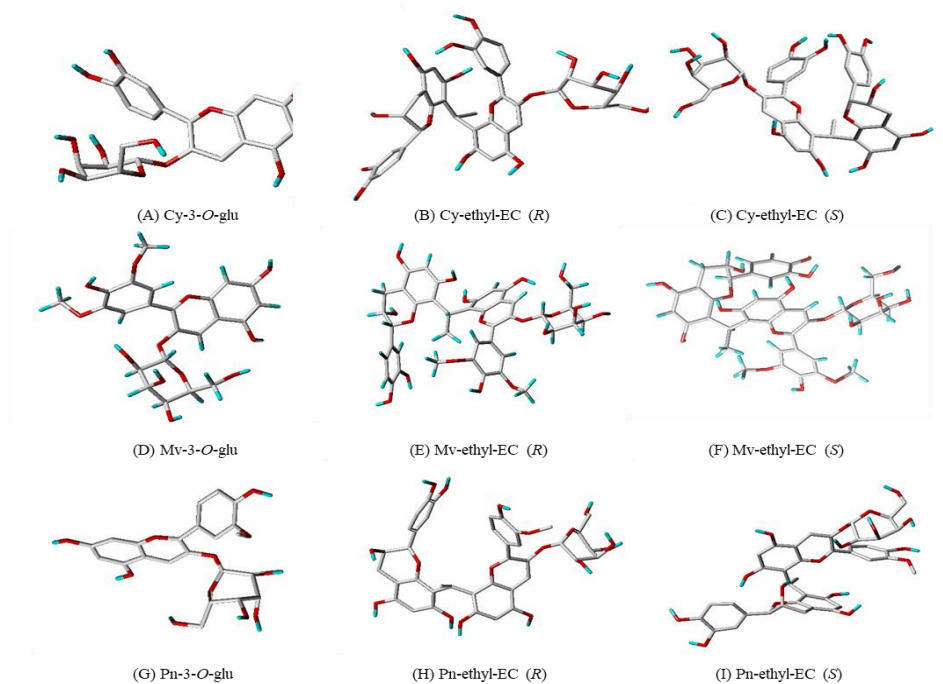


Figure 6. 3D structural model of anthocyanins and ethyl-linked anthocyanin-flavanol pigment obtained by simulated annealing.

Table II

Energy values obtained for the molecular modeling of anthocyanins and ethyl-linked anthocyanin-flavanol pigment with molecular dynamics

Compound	Energy (kcal/mol)
Cy-3-<i>O</i>-glu	85.362
Cy-ethyl-EC (<i>R</i>)	135.130
Cy-ethyl-EC (<i>S</i>)	151.849
Mv-3-<i>O</i>-glu	99.443
Mv-ethyl-EC (<i>R</i>)	158.767
Mv-ethyl-EC (<i>S</i>)	152.349
Pn-3-<i>O</i>-glu	95.911
Pn-ethyl-EC (<i>R</i>)	145.364
Pn-ethyl-EC (<i>S</i>)	145.956

Stability of three-dimensional structure model of anthocyanins and ethyl-linked anthocyanin-flavanol pigments by quantum chemical method

The computation of anthocyanins and ethyl-linked anthocyanin-flavanol pigments models was carried out by geometric optimization using the molecular

mechanic method in the MM⁺, AMBER and OPLS force fields of HyperChem 8.0. A parameterized model 3 (PM3) semi-empirical quantum chemistry method was used to calculate the heat of formation (Zhao *et al.*, 2013). Comparing the heat of formation for each conformation after optimized 3D conformations, low values showed a positive

correlation with structural stability (Zhao *et al.*, 2013). According to Table III, in the three force fields, no difference was found in the heat of formation for the same compound. The order of heat of formation was: Mv-3-*O*-glu < Pn-3-*O*-glu < Cy-3-*O*-glu. It indicated that the stability of anthocyanin was: Mv-3-*O*-glu > Pn-3-*O*-glu > Cy-3-*O*-glu. The heat of formation of the ethyl-linked anthocyanin-flavanol pigments was much less than that of the monomer anthocyanins, with the

following order: Mv-ethyl-EC (*S*) < Mv-ethyl-EC (*R*) < Pn-ethyl-EC (*R*) \approx Pn-ethyl-EC (*S*) < Cy-ethyl-EC (*S*) \approx Cy-ethyl-EC (*R*), revealing that the ethyl-linked anthocyanin-flavanol pigments were more stable than the anthocyanins, in which ethyl-linked malvidin-3-*O*-glucoside-flavanol was the most stable compared to ethyl-linked cyanidin-3-*O*-glucoside-flavanol and ethyl-linked peonidin-3-*O*-glucoside-flavanol.

Table III

Calculated properties for anthocyanins and ethyl-linked anthocyanin-flavanol pigments with different molecular mechanics methods in vacuum by the single energy of method PM3

Methods	MM ⁺ force field	AMBER force field	OPLS force field
Cy-3-<i>O</i>-glu			
Heat of formation (kcal/mol)	-5206.095	-5230.801	-5 179.792
Gradient (kcal/mol/Ang)	41.560	27.958	38.844
Cy-ethyl-EC (<i>R</i>)			
Heat of formation (kcal/mol)	-9480.691	-9528.646	-9510.169
Gradient (kcal/mol/Ang)	38.021	24.507	33.670
Cy-ethyl-EC (<i>S</i>)			
Heat of formation (kcal/mol)	-9491.860	-9548.608	-9483.998
Gradient (kcal/mol/Ang)	37.482	24.202	34.273
Mv-3-<i>O</i>-glu			
Heat of formation (kcal/mol)	-5848.599	-5880.124	-5875.277
Gradient (kcal/mol/Ang)	40.244	28.616	37.607
Mv-ethyl-EC (<i>R</i>)			
Heat of formation (kcal/mol)	-10131.814	-10558.893	-10528.381
Gradient (kcal/mol/Ang)	36.955	26.796	33.278
Mv-ethyl-EC (<i>S</i>)			
Heat of formation (kcal/mol)	-10137.330	-10182.839	-10035.393
Gradient (kcal/mol/Ang)	36.613	24.240	41.469
Pn-3-<i>O</i>-glu			
Heat of formation (kcal/mol)	-5477.244	-5507.523	-5480.420
Gradient (kcal/mol/Ang)	38.328	29.374	37.971
Pn-ethyl-EC (<i>R</i>)			
Heat of formation (kcal/mol)	-9756.098	-9804.458	-9770.035
Gradient (kcal/mol/Ang)	37.367	24.345	33.175
Pn-ethyl-EC (<i>S</i>)			
Heat of formation (kcal/mol)	-9757.821	-9796.700	-9770.404
Gradient (kcal/mol/Ang)	37.691	24.805	-33.784

Differences in the spatial conformation and energy of anthocyanins and ethyl-linked anthocyanin-flavanol pigments in molecular simulations of aqueous solutions

In the aforementioned quantum chemical optimization, there was no discernible variation in the heat of production under three different molecular force fields. Therefore, the MM⁺ force field was selected as the typically force field to optimize the conformation of anthocyanins and ethyl-linked anthocyanin-flavanol pigments under simulation aqueous medium and to compare their stability. Molecular mechanics, molecular dynamics and quantum chemistry was carried out in a vacuum condition, whereas anthocyanins and ethyl-linked anthocyanin-flavanol pigments were actually presented in aqueous conditions. Therefore, it was focused on investigating the effect of anthocyanins

and ethyl-linked anthocyanin-flavanol pigments on their conformation and stability under simulation aqueous medium. Figure 7 showed the optimized three-dimensional conformation of anthocyanins and their ethyl-linked anthocyanin-flavanol pigments in aqueous solutions. The solvation and non-solvation energy values were compared, as shown in Table IV. The electrostatic energy of all compounds changed from zero to a negative value, and the van der Waals energy and bond energy significantly increased, indicating that each compound was more stable in aqueous medium (Lan *et al.*, 2013; Liu *et al.*, 2017). The bond energies of anthocyanins and ethyl-linked anthocyanin-flavanol pigments increased under simulation in aqueous medium. The bond energy values were: Mv-3-*O*-glu > Pn-3-*O*-glu > Cy-3-*O*-glu. In other words, Mv-3-*O*-glu has the strongest stability, followed by Pn-3-*O*-glu and Cy-3-*O*-glu. The stability of ethyl-linked anthocyanin-flavanol pigments in an

aqueous solution was as follows: Mv-ethyl-EC (*S*) > Pn-ethyl-EC (*S*) > Cy-ethyl-EC (*R*) > Mv-ethyl-EC (*R*) > Pn-ethyl-EC (*R*) > Cy-ethyl-EC (*S*). It means that the configuration has no regular influence on the stability of ethyl-linked anthocyanin-flavanol pigments. Compared to the bond energy of ethyl-linked anthocyanin-flavanol pigments and precursor anthocyanins, it was found that the bond energy of the former was higher than that of the latter, indicating that ethyl-linked anthocyanin-flavanol pigments were more stable in aqueous solution. In short, compared to precursor anthocyanins, ethyl-linked anthocyanin-flavanol pigments showed an obvious stability.

In conclusion, three anthocyanins (Mv-3-*O*-glu, Pn-3-*O*-glu, Cy-3-*O*-glu) and six ethyl-linked anthocyanin-flavanol pigments (Cy-ethyl-EC (*S*), Cy-ethyl-EC (*R*), Mv-ethyl-EC (*S*), Mv-ethyl-EC (*R*), Pn-ethyl-EC (*S*), Pn-ethyl-EC (*R*)) were simulated by molecular mechanics, molecular dynamics and quantum chemistry. The results showed that these nine compounds were relatively stabilized in the force field.

By comparing bond energy, the heat of formation, and other energy parameters, it was found that ethyl-linked anthocyanin-flavanol pigments were more stable than their corresponding precursor anthocyanins. In particular, ethyl-linked malvidin-3-*O*-glucoside-flavanol had distinctive characteristics of stability. Conformational simulations of anthocyanins and ethyl-linked anthocyanin-flavanol pigments were carried out in MM⁺ force fields to study their stability under simulation in aqueous medium. It was found that the stability of anthocyanins and ethyl-linked anthocyanin-flavanol pigments in simulation aqueous medium was enhanced compare to that a vacuum condition in MM⁺ force field, and the ethyl-linked anthocyanin-flavanol pigments were more stable than the monomer anthocyanins in aqueous solution. It was confirmed that the ethyl-linked anthocyanin-flavanol pigments were more stable than the anthocyanins from the energy point of view.

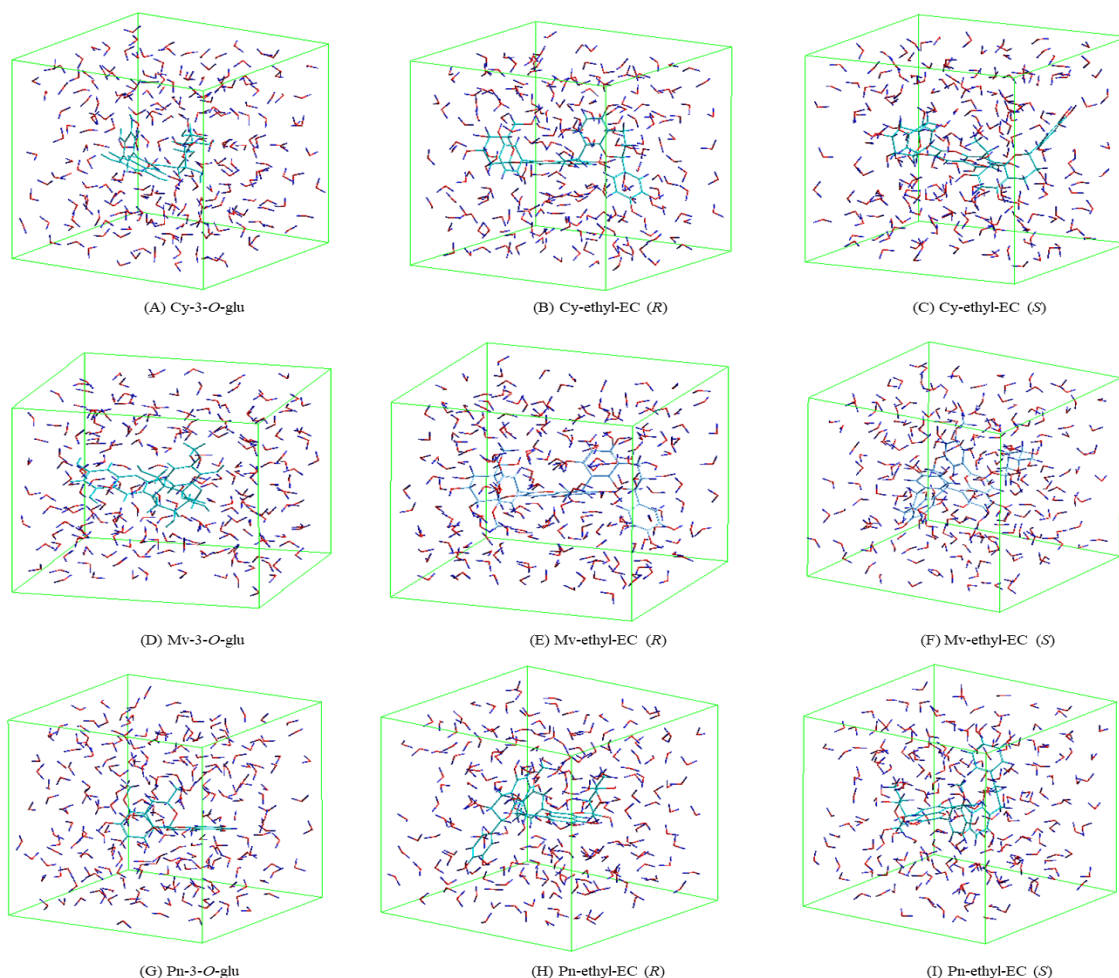


Figure 7. 3-D structure of anthocyanins and ethyl-linked anthocyanin-flavanol pigments obtained by geometry optimization in solution with MM⁺ molecular mechanics.

Table IV

Energy values of anthocyanins and ethyl-linked anthocyanin-flavanol pigments before and after solvation

	Bond	Angle	Dihedral	Vdw	Stretch-bend	Electrostatic	Energy
Cy-3- <i>O</i> -glu	1.711	5.077	4.666	20.130	0.207	0.000	31.791
Cy-3- <i>O</i> -glu in solution	1.743	10.080	5.569	-186.614	0.096	-436.975	-606.101
Cy-ethyl-EC (<i>R</i>)	3.932	10.908	2.185	36.159	0.389	0.000	53.573
Cy-ethyl-EC (<i>R</i>) in solution	4.255	14.789	2.937	-177.341	0.320	-410.688	-565.727
Cy-ethyl-EC (<i>S</i>)	3.754	10.023	2.665	35.377	0.351	0.000	52.169
Cy-ethyl-EC (<i>S</i>) in solution	3.926	14.453	2.569	-161.516	0.272	-395.706	-536.003
Mv-3- <i>O</i> -glu	1.757	6.563	5.052	20.614	0.242	0.000	34.228
Mv-3- <i>O</i> -glu in solution	1.898	10.751	5.261	-188.209	0.172	-436.741	-606.868
Mv-ethyl-EC (<i>R</i>)	4.086	11.695	2.788	35.765	0.443	0.000	54.777
Mv-ethyl-EC (<i>R</i>) in solution	4.182	16.636	2.076	-168.607	0.344	-400.865	-546.235
Mv-ethyl-EC (<i>S</i>)	4.149	12.036	2.659	36.823	0.417	0.000	56.083
Mv-ethyl-EC (<i>S</i>) in solution	4.416	17.023	2.150	-172.906	0.392	-407.598	-556.524
Pn-3- <i>O</i> -glu	1.663	5.717	6.389	18.702	0.215	0.000	32.686
Pn-3- <i>O</i> -glu in solution	1.767	10.602	5.508	-189.229	0.112	-441.114	-612.354
Pn-ethyl-EC (<i>R</i>)	3.963	11.526	2.685	35.314	0.401	0.000	53.888
Pn-ethyl-EC (<i>R</i>) in solution	4.031	15.036	1.813	-177.076	0.291	-404.727	-560.632
Pn-ethyl-EC (<i>S</i>)	4.063	11.746	0.643	37.401	0.422	0.000	54.274
Pn-ethyl-EC (<i>S</i>) in solution	4.376	15.543	2.866	-163.757	0.378	-398.236	-538.830

CONCLUSIONS

In this work, using model wine solution system, HPLC-DAD and CIELab analyses were performed to monitor changes in the contents and color parameters in the process of reaction between anthocyanins (Mv-3-*O*-glu, Pn-3-*O*-glu and Cy-3-*O*-glu) and (-)-epicatechin. The formation of ethyl-linked anthocyanin-flavanol pigments was responsible for the changes in color parameter values: decrease of L*, a*, b* and C*, and increase of h*. The color of the Cy-3-*O*-glu and Pn-3-*O*-glu reaction system shifts from the initial orange-red hue to a brick-red hue, and the color of the Mv-3-*O*-glu reaction system shifts from an initial red hue to violet hue. To the best of our knowledge, different 3D molecular simulation methods were used, for the first time, to calculate the stability of the three major anthocyanins in red wine (Mv-3-*O*-glu, Pn-3-*O*-glu, Cy-3-*O*-glu) as well as their indirect condensation products anthocyanin-flavanol pigments (Cy-ethyl-EC (*S*), Cy-ethyl-EC (*R*), Mv-ethyl-EC (*S*), Mv-ethyl-EC (*R*), Pn-ethyl-EC (*S*) and Pn-ethyl-EC (*R*)). It was shown that the stability of three anthocyanins was Mv-3-*O*-glu > Pn-3-*O*-glu > Cy-3-*O*-glu. Besides, ethyl-linked anthocyanin-flavanol pigments were found to be more stable than free precursor anthocyanins, particularly, the stability of the ethyl-linked malvidin-3-*O*-glucoside-flavanol was remarkable. Under simulation in aqueous medium, the stability of ethyl-linked anthocyanin-

flavanol pigments was higher than that of their precursor anthocyanins, and was as follows: Mv-ethyl-EC (*S*) > Pn-ethyl-EC (*S*) > Cy-ethyl-EC (*R*) > Mv-ethyl-EC (*R*) > Pn-ethyl-EC (*R*) > Cy-ethyl-EC (*S*). In conclusion, the present study used a combination of chemical analysis and 3D molecular simulations, confirming that the stability of the major anthocyanins in red wine can be significantly enhanced by indirect reaction with flavanols to form ethyl-linked anthocyanin-flavanol pigments during the ageing process.

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