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Technical Note

RED WINE COLOR: THE ROLE OF POLYMERIC PIGMENTS AND PYRANOANTHOCYANINS IN SO_2 BLEACHABLE METHODS

COR DO VINHO TINTO: O PAPEL DOS PIGMENTOS POLIMÉRICOS E PIRANOANTOCIANINAS NO MÉTODO DE DESCOLORAÇÃO COM SO_2

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SUMMARY

In this work, copigmentation assay described by Boulton and the “chemical age” indices proposed by Somers and Evans in wine were critically reviewed and updated. In particular, the influence of polymeric pigments and pyranoanthocyanins on color is reassessed. The current analytical understanding of these pigments should be taken into account when applying both methods, as some implicit considerations about SO_2 bleaching are presumably affected by their chemical structure. The differences between Anthocyanin-Tannin (AT) and Tannin-Anthocyanin (TA) polymeric pigments are carefully described, as well as the importance of pyranoanthocyanin contribution to the color. As a result, and considering current scientific knowledge, the true meanings of terms such as polymeric pigments and free anthocyanins in both analytical methods have been reviewed. Some possible research lines to improve these reliable and fast methods have been proposed.

RESUMO

O presente trabalho incide na revisão crítica e na atualização do ensaio de copigmentação descrito por Boulton e dos índices de “idade química” propostos por Somers e Evans no vinho. Em particular, foi reavaliada a influência dos pigmentos poliméricos e das piranoantocianinas na cor. A compreensão analítica atual desses pigmentos deve ser tida em consideração ao aplicar ambos os métodos, na medida em que algumas considerações implícitas são presumivelmente afetadas pela sua estrutura química. As diferenças entre pigmentos poliméricos Antocianina-Tanino (AT) e Tanino-Antocianina (TA) são descritas cuidadosamente, assim como a importância da contribuição das piranoantocianinas para a cor. Como resultado, e considerando o conhecimento científico atual, os verdadeiros significados de termos como pigmentos poliméricos e antocianinas livres em ambos os métodos analíticos foram revisatos. São propostas possíveis linhas de investigação para tornar os métodos mais robustos e rápidos

Keywords: Red wine, color, polymeric pigment, pyranoanthocyanin, SO_2.
Palavras-chave: Vinho tinto, cor, pigmento polimérico, piranoantocianina, SO_2.

INTRODUCTION

Red wine color is a sensory attribute highly valued by consumers. It is mainly due to anthocyanins, whose basic structure is exposed in Figure 1, which are colored molecules because of the delocalization of their positive charge in the flavilyum form. Changes in color depend on the localization of the charge and the resonating chemical structure; therefore, different colored forms are present in red wine at the same time (Castaneda-Ovando *et al.,* 2009). Sulfur dioxide (SO_2) is especially important in the production of wine and can loosely bind to anthocyanins for modifying their color (Pelonnier-Magimel *et al.,* 2023).

Wine anthocyanic forms are in equilibrium according to the mix of structures, which can be expressed as flavylium ions, quinoidal base, carbinol base and chalcones pseudobase (Figure 2). The preponderance of one or another chemical form depends on different factors, such as pH, temperature, or water activity (Wrolstad *et al.,* 2005). Copigmentation relationships lead the formation of tertiary colored structures with various stabilization mechanisms (Boulton, 2001).

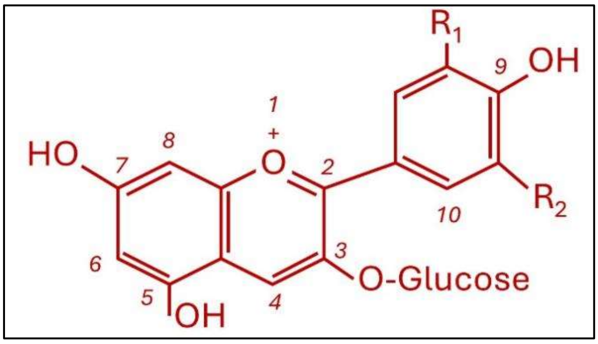


Figure 1. Anthocyanin A+ form or red flavilyum cation.

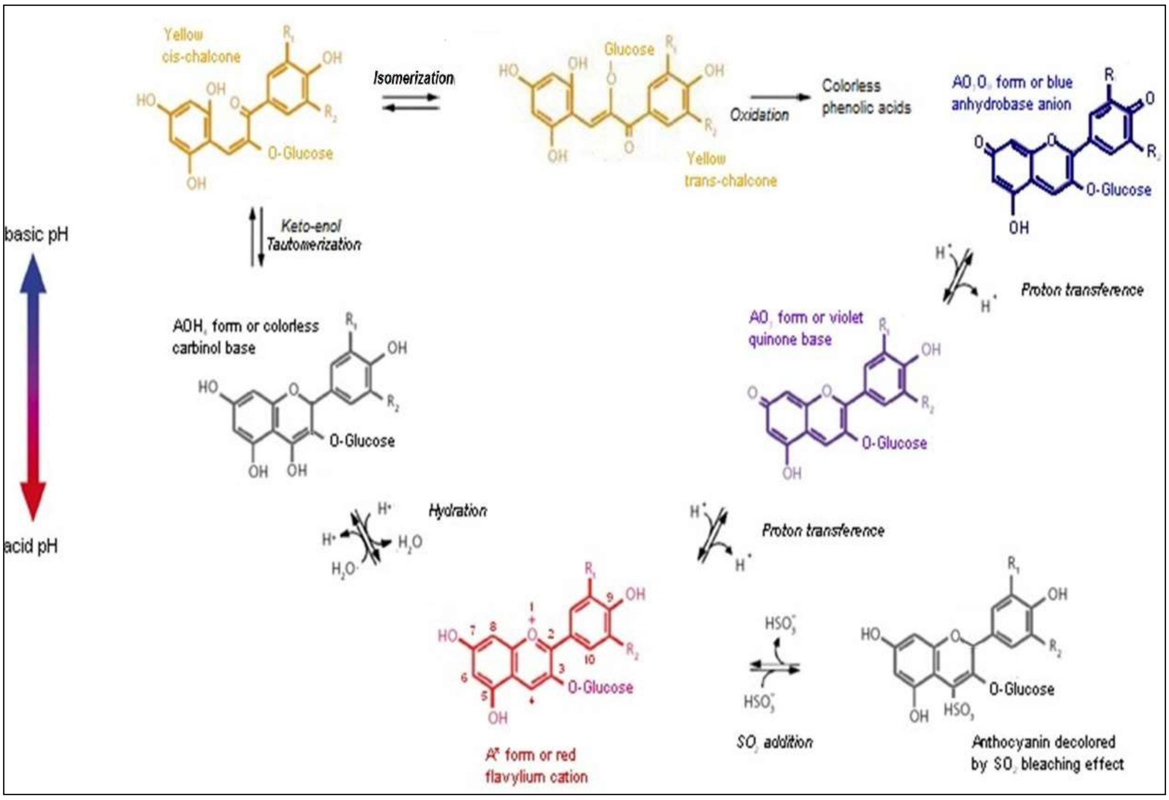


Figure 2. Anthocyanin stable forms according to pH and SO2 bleaching effect.

The internationally and well-known spectrophotometric method for copigmentation assessment proposed by Boulton (1996) was designed to quantify the different forms of pigments present in red wines. This assay is focused on copigmentation but it includes some considerations from the previous procedure for the evaluation of wine "chemical age" described by Somers and Evans (1977). Boulton´s method has widely been applied in order to evaluate the influence of the different anthocyanin forms on red wine color (Mollov *et al.,* 2007; Kopjar and Piližota, 2009; Pacheco-Palencia and Talcott, 2010; Heras-Roger *et al.,* 2014; Zhao *et al.,* 2020; De Oliveira *et al.,* 2024). On the other hand, the Somers and Evans procedure to evaluate "chemical age" of wines has also been extensively applied in food chemistry research (Harbertson *et al.,* 2003; Bindon *et al.,* 2014; Girschik *et al.,* 2017; Jorge *et al.,* 2021; Zhao *et al.,* 2023). Although both procedures are currently recognized, they fall short in accurately capturing the effects of complex pigment forms, such as polymeric pigments and pyranoanthocyanins. For instance, color changes in anthocyanin-tannin pigments using CIELab units have been recently studied in greater detail with 3D simulations (Zhao *et al.,* 2023).

The Boulton copigmentation assay focuses on the A_{520} wavelength to quantify the percentage of color due to three sources: free anthocyanins, copigmented anthocyanins and polymeric pigments (Boulton, 1996). Somers and Evans established indices of "chemical age" which are based upon the premises that anthocyanins are decolorized by SO_2 and the residual color is due to polymeric pigments, which are markers of wine aging (Somers and Evans, 1977).

It is usually assumed that polymeric pigments are resistant to the bisulfite discoloration described in Figure 2 for any anthocyanic form. This fact was applied in Boulton's copigmentation assay to estimate their contribution to color, and by Somers and Evans to calculate "chemical age" indices. To the best of our knowledge, this point is carefully discussed and revised, for the first time, in the present paper. Modern scientific research allows to include new structural considerations that should be taken in account when these assays are used in any anthocyanic rich product with polymeric pigments and/or pyranoanthocyanins. This study aimed to optimize these methods for integrating recent scientific advancements, thereby offering a more precise analysis and deeper understanding of wine pigments. Therefore, any previous conclusion established applying these methods can be relatively affected by the considerations expressed in the present study.

MATERIALS AND METHODS

Boulton method for the assessment of copigmentation

Boulton method firstly adjusts the wine pH to 3.6 in order to compare color at similar conditions regardless of pH effects. The adjustment is made with HCl if the wine pH is significantly greater than 3.6, or with NaOH when the wine is naturally more acidic. In both cases, these reagents are highly concentrated to minimize changes in wine volume and physicochemical equilibria. Since all adjustments and measurements are performed after the wine pH has been standardized to 3.6 by adding only a few drops, there is no need to apply volumetric factors or corrections in the equations related to the pH adjustment. Absorbance measurements at 520 nm are performed after the addition of chemicals and wine as follows:

- A^{acet}: 20 µL of 10% acetaldehyde solution is added to 2 mL of wine. The 520 nm measurement after adding acetaldehyde (A^{acet}) represents the total wine color. Acetaldehyde binds much more strongly with SO_2 than to the anthocyanins. Therefore, the addition of acetaldehyde allows to quantify the total color because it displaces SO_2 from the anthocyanins maximizing the presence of the colored flavylium cation. In these conditions, wine color would be maximal. The only way to enhance it would be a decrease of pH, since the colored equilibrium from carbinol and quinone bases would be displaced to the formation of the flavilyum cation (Figure 2).
- A^{20}: 100 µL of wine is placed into 1900 µL of buffer solution prepared by adding 24 mL pure ethanol to 176 mL distilled water with 0.5 g of potassium bitartrate dissolved into the solution and pH adjusted to 3.6. Wine color without copigmented anthocyanins is estimated by means of A^{20}, as wine dilution leads to the dissociation of the copigment complex while the contributions from free anthocyanins and polymeric pigments remain (Boulton 1996).
- A^{SO2}: 160 µL of 5% SO_2 solution is added to 2 mL of wine. The color due to polymeric pigments is supposed to be A^{SO2}, the absorbance at 520 nm measurement after addition of a SO_2 enriched solution to wine, because polymer pigments are considered completely resistant to bisulfite discoloration according to the analytical premises of this method.

Obviously, all absorbance measurements are converted to 10 mm pathlength and multiplied by the dilution factor prior to any calculation. Using these measurements, the color due to the different forms of pigments is estimated according to Equations 1 to 3.

Fraction of color due to free anthocyanins:

$$X_{\text{Free Anthocyanins}} = (A^{20}-A^{SO2})/A^{acet} \qquad \textbf{Eq. 1}$$

Fraction of color due to copigmented anthocyanins:

$$X_{\text{Copigmentation}} = (A^{acet}-A^{20})/A^{acet} \qquad \textbf{Eq. 2}$$

Fraction of color due to polymeric pigments:

$$X_{\text{Polymeric Pigments}} = A^{SO2}/A^{acet} \qquad \textbf{Eq. 3}$$

Somers & Evans method

Somers and Evans procedure considers the same reagents and approximations with an additional measurement at pH<1 adding HCl 1M (A^{HCl}), as in these conditions, anthocyanins are entirely in the colored flavylium form following acidification. There are two indices of "chemical age" which are obtained by Equations 4 and 5.

(i) A^{SO2}/A^{acet} **Eq. 4**

(ii) A^{SO2}/A^{HCl} **Eq. 5**

According to Somers and Evans (1977), both ratios are initially zero or close to zero in young red wines, but a progressive increase in each ratio during aging is observed, approaching 1.0 the index (i) and 0.9 the index (ii) in aged wines.

RESULTS AND DISCUSSION

Formation of polymeric pigments and color stability

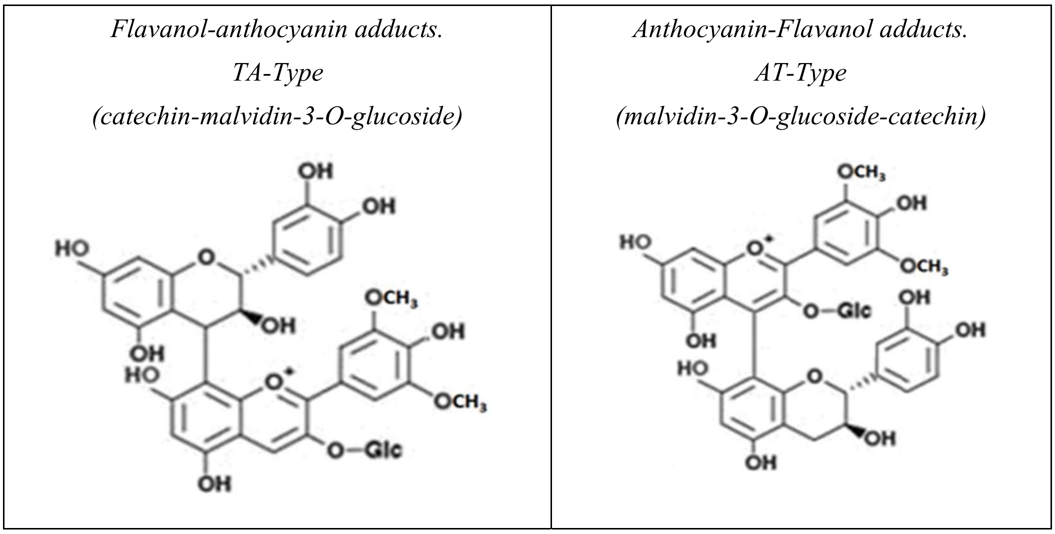
Research advances into the formation of polymeric pigments and the use of recent analytical techniques allow to describe structural details of these pigments.

They are formed in red wines by reactions between free anthocyanins and flavanols, usually known as tannins (Zhang *et al.,* 2022).

These pigments are formed by condensation processes in which anthocyanins (A) and tannins (T) can interact directly or via intermediate substances such as acetaldehyde (Wang *et al.,* 2023).

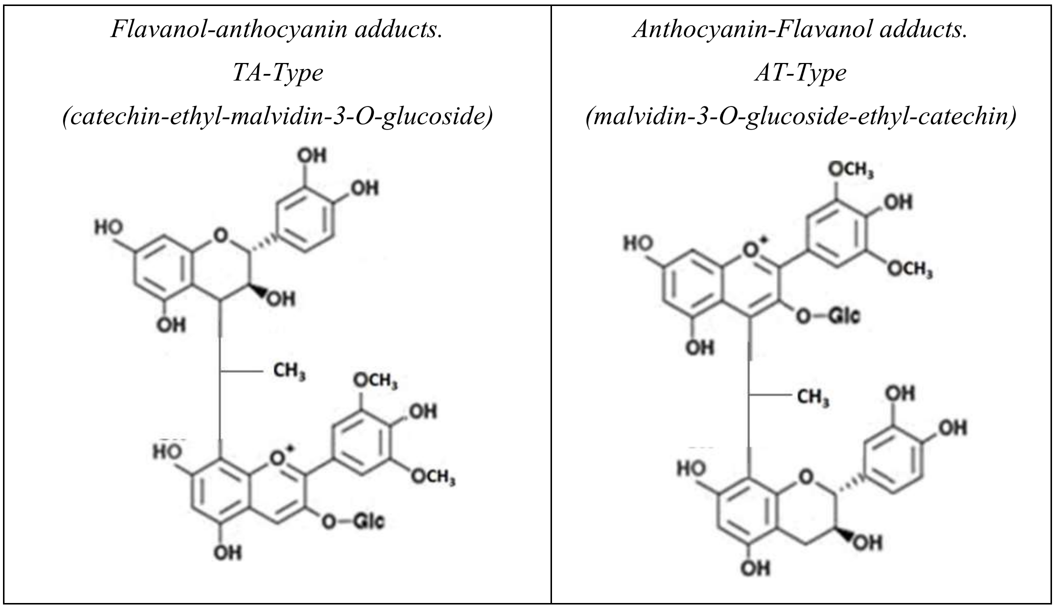
Anthocyanins might be located at the initial position of the polymer, known as AT-type, or at the end, TA-type (Figure 3 and 4).

Anthocyanin-flavanol adducts (AT) are formed by addition when the electrophile flavylium anthocyanin joins to a nucleophile flavanol molecule (Remy-Tanneau *et al.,* 2003). Flavanol-anthocyanin adducts (TA) are generated by direct condensation between anthocyanins and proanthocyanidins, traditionally known as condensed tannins. In this case, the reaction is based on the formation of a carbocation deriving from the flavylium junction rupture and proanthocyanidins in acidic medium (Salas *et al.,* 2004).


Flavanol-anthocyanin adducts.
TA-Type
(catechin-malvidin-3-O-glucoside)

Anthocyanin-Flavanol adducts.
AT-Type
(malvidin-3-O-glucoside-catechin)

Figure 3. Direct associations between anthocyanins and tannins.


Flavanol-anthocyanin adducts.
TA-Type
(catechin-ethyl-malvidin-3-O-glucoside)

Anthocyanin-Flavanol adducts.
AT-Type
(malvidin-3-O-glucoside-ethyl-catechin)

Figure 4. Indirect associations between anthocyanins and tannins bridged by acetaldehyde.

The formation of different types of polymeric pigments, including direct condensates (Figure 3) or indirect condensates (Figure 4), gives rise to less bright colors but stabilizes the color during the aging of the wine (Heras-Roger *et al.,* 2016). Polymeric pigments, both direct and indirect condensed, are considered more stable pigments than free anthocyanins because their colored equilibrium is not as sensitive to pH and to bisulfite discoloration (Somers and Evans, 1977).

Nevertheless, there are some polymeric pigments which are sensitive to the addition of bisulfite, contrasting with the considerations detailed in this study. Considering the current scientific knowledge, only AT polymeric pigments, whether directly or indirectly connected via acetaldehyde bridges, are insensitive to discoloration by SO_2 addition.

Therefore, the $X_{\text{Polymeric Pigments}}$ fraction proposed by Boulton does not estimate every polymeric pigments present in wines, but only those from AT-type. Similarly, the indices of "chemical age" proposed by Somers and Evans do not include any TA polymeric pigment influence in their current forms.

Sensitivity to bisulftie discoloration

The different sensitivity of polymeric pigments to discoloration by bisulfite is related to their chemical structure. Glories (1984) studied the equilibrium between SO_2 and the anthocyanin-bisulfite complex, proposing a mechanism in which a chalcone leads to a chalcone bisulfite adduct in which SO_2 joins C-2 position (Figure 1). This configuration was also hypothesized by Somers and Evans (1977).

Nevertheless, if this initial consideration would be true every polymeric pigment would be bleachable by SO_2 because C-2 position is accessible in all of them (AT- and TA-type), and the resonating colored structure from the anthocyanin would disappear. However, discoloration does not occur in all these pigments as they have been traditionally considered not bleachable by SO_2.

In fact, research using NMR disregard the proposed bisulfite adduct's structure (Berké *et al.,* 1999). These authors proved that these colorless compounds were produced by the reaction of SO_2 with anthocyanins in the C-4 position (Figure 1) because of its lower steric hindrance with respect to C-2 position. This confirms previous proposals reported by other researchers (Brouillard and Delaporte, 1977), who suggested the binding site in the C-4 position rather than the C-2 position.

Moreover, even before the development of these methods some researchers observed that the addition of a bulky group around the C-4 position would prevent the addition of SO_2 in flavylium derivatives (Timberlake and Bridle, 1968).

Therefore, anthocyanic pigments need to have the C-4 position of their molecule free in order to be sensitive to bisulfite additions. In other terms, C-4 position is where the bisulfite anion changes the charge equilibrium and therefore decolorizes the pigment molecule. This means that AT polymeric pigments are effectively insensitive to SO_2 discoloration as the anthocyanic C-4 position is covalently linked to another phenolic compound. Nevertheless, TA polymeric pigments have the C-4 position accessible, and therefore, they could be effectively decolorized by SO_2 addition, which contrasts with the original assays' considerations described by Boulton (1996) and Somers and Evans (1977).

Both bisulfite discoloration and color changes associated with pH variation (at least that from the flavylium form starting with hydration) need to have C-4 position of the anthocyanic form free, which is where the bisulfite anion or hydroxyl anion of water can react.

Following this study, Somers and Evans chemical age index (i) would never achieve a value of 1, as at least TA polymeric pigments cannot be quantified by means of A^{SO2}. In fact, for the best of our knowledge an index of 1 has never been obtained, being 0.93 the highest value described in a fifteen years old wine (Somers and Evans, 1977). Furthermore, some studies (data not published) with ten years old wines do not proportionate any value higher than 0.82 in terms of chemical age factor.

Even these chemical age indices are relatively high assuming the considerations explained herein until now, as AT polymeric pigments would be the only pigments quantified after SO_2 addition. This could be explained by the presence of pyranoanthocyanins also known as vitisin type pigments, which have become thoroughly studied in the last decade but were previously unknown (De Freitas and Mateus 2010; He *et al.,* 2012; Huang *et al.,* 2023; Zhao *et al.,* 2024).

Pyranoanthocyanins are products of cyclo addition characterized by a D pyran ring between the OH group of the C-5 and C-4 from the original flavylium cation (Figure 5). They derive from the reaction of anthocyanins at the C-4 position with pyruvic acid and other compounds (Casassa and Catania, 2006).

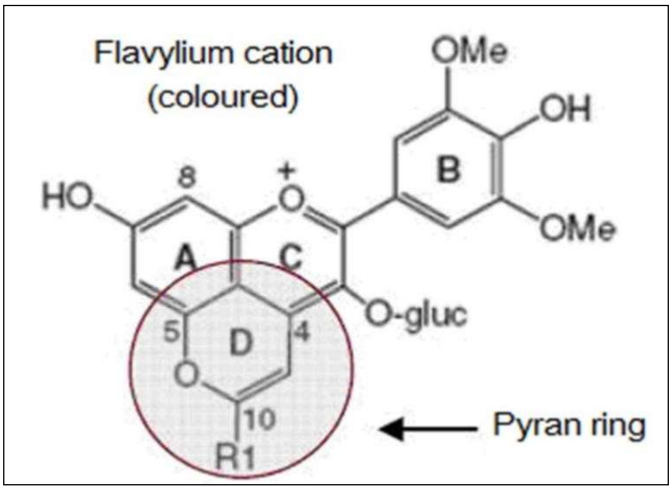


Figure 5. Pyranoanthocyanin structure.

Pyranoanthocyanin concentrations are usually low in young wines and they have been described as insensitive to pH changes, bleached with SO_2 or oxidative degradation because the pyran ring causes a steric hindrance (Asenstorfer *et al.,* 2006), in a similar way to that described in the present article for AT-type polymer pigments.

Therefore, these pigments cannot be bleached with bisulfite, and as consequence most of these compounds participate in wine color and they will be measured as "polymeric color" in both assays when quantifying A^{SO2}.

Challenges and potential methods for pigment quantification

An interesting challenge rises from these observations, as any fast and reliable spectrophotometric estimation of pyranoanthocyanin and polymeric pigments (TA or AT) separately would be highly useful to determine the specific contribution to wine color of each types of these pigments.

In fact an easy differentiated spectral identification of these groups directly in the wine matrix is highly challenging, as both polymeric pigments and pyranoanthocyanins are well known by their ability to provide the great color palette characteristics from aged red wines. Therefore, it is difficult to estimate them by direct measurements in the visible spectrum of wine. For instance, acetaldehyde links anthocyanins and tannins by an ethyl bridge, producing violet pigments, while direct associations between anthocyanins and flavanols result in stable red/orange pigments. Some anthocyanic polymeric pigments have been described with bluish-red shades and violet hues (Escribano-Bailón *et al.,* 2001) while pyranoanthocyanins are supposed to produce an hyperchromic and hypsochromic displacement in comparison to the initial anthocyanin colors, therefore generally lead to red and orange hues (Morata *et al.,* 2007).

Some research direction to quantify pyranoanthocyanins would be exposed them to the gradual but irreversible nucleophilic attack of water as they become neutral pyranone-anthocyanins, known as oxovitisins (He *et al.,* 2010). These compounds exhibit a yellowish or colorless hue in acid medium, which could be used as a possible path to obtain a spectrophotometric estimation of their presence in wine and somehow differentiate their contribution to color. However, this theoretical possibility needs further research to confirm its suitability for the purpose of fast and reliable color estimation.

Interestingly, the novel implications highlighted in this paper have been already confirmed by experimental results available in the literature (Bakker and Timberlake, 1997; Versari *et al.,* 2007, Versari, *et al.,* 2008). Nevertheless, for the best of our knowledge, no connection between the structural details presented herein and the characteristics of polymeric pigments has been previously made.

Indeed, Somers and Evans already described the likely presence of "intermediate pigment forms" in wines with consequently uncertain response to SO_2 and to pH changes. They considered polymeric pigments much less affected by decrease of pH. So, they introduce an experimental factor (5/3) in some of their equations to estimate the response of these components to acidification. The factor was based upon of the properties of polymeric pigments, varying from 3/2 to about 2 for any particular wine (Somers and Evans, 1977).

Moreover, some authors found that the total free anthocyanin content of red wines measured by Somers and Evans method is higher than those estimated by HPLC, considering the presence of polymeric pigments responsible somehow of this discrepancy (Rivas-Gonzalo *et al.,* 1992). These results agree with the considerations explained in the present work, as the use of this method implies a joined quantification of free anthocyanins with TA-type polymeric pigments because of the structural details already described.

In the same sense, another study (Versari *et al.,* 2008) showed that between 5% and 22 % of polimeric pigments from 20 young wines were partly bleached by SO_2. This fact supports the considerations herein, meaning that the wines analyzed by these authors contained between a 5% and 22% of TA-type polymeric pigments.

Furthermore, considering the chemical age indices published and according to the details explained above, it could be confirmed that polymeric pigments (TA and AT, indistinctly) are more important for wine color in relatively young wines, while in the case of highly aged wines the pyranoanthocyanin factor would have a higher contribution to the final wine color.

Polymeric pigments may form first in wine and may be related to the subsequent formation of pyranoanthocyanins, resulting in a significant decrease in anthocyanins. This diverse anthocyanic profile during wine ageing was recently and accordingly described by authors who analyzed Ports ranging from young to 30 years old (Mesquita *et al.*, 2024).

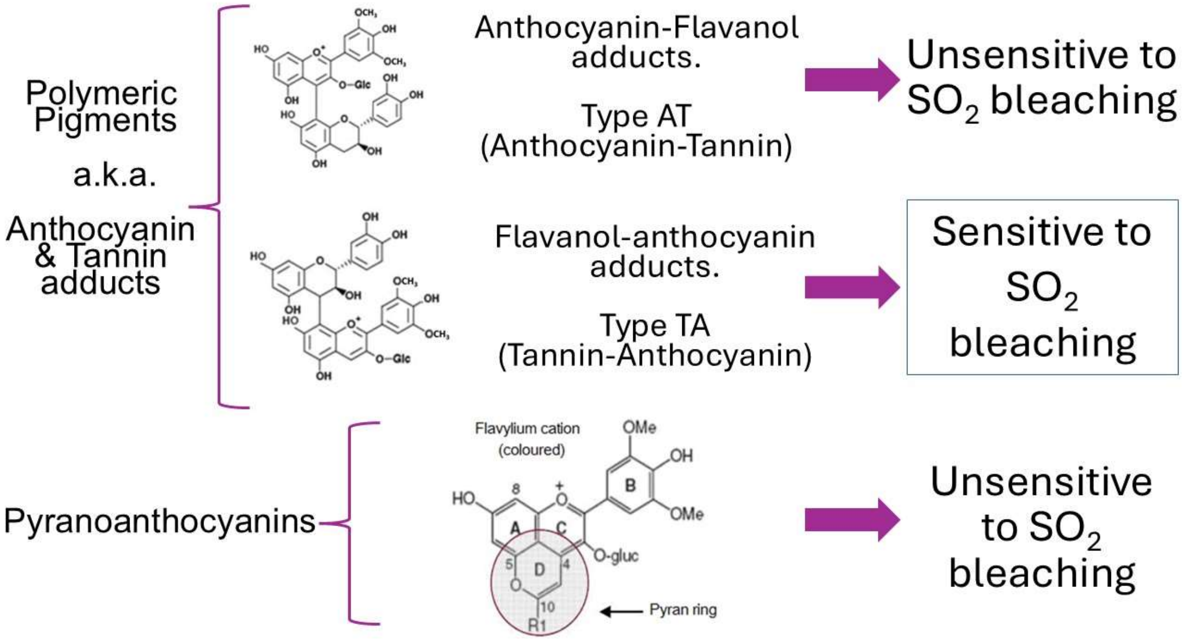


Figure 6. Detailed bisulfite bleaching diagram.

CONCLUSIONS

According to the structural considerations presented, the meaning of some terms in the Boulton and Somers equations need to be reconsidered, as A^{SO2} factor is exclusively evaluating the addition of AT polymeric pigments and pyranoanthocyanins as summarized. Thus, the determination of free anthocyanins is actually quantifying the free anthocyanins plus the TA Polymeric pigments, and the determination of polymeric pigments is quantifying the AT-type polymeric pigments plus the pyranoanthocyanins. However, the determination of copigmented anthocyanins remains unchanged. Thus, the calculations from both methods would be more appropriately expressed as follows.

Boulton method for the assessment of copigmentation (Equations 6 to 8).

$$X_{(\text{Free Anthoc +TA Polym Pigments})}=(A^{20}–A^{SO2})/A^{acet} \qquad \textbf{Eq. 6}$$

$$X_{\text{Copigmentation}}=(A^{acet}– A^{20})/A^{acet} \qquad \textbf{Eq. 7}$$

$$X_{(\text{AT Polymeric Pigments + Pyranoanthocyanins})} = A^{SO2}/A^{acet} \qquad \textbf{Eq. 8}$$

Somers & Evans method (Equations 9 and 10).

Chemical age index (i) excluding TA polymeric pigments = A^{SO2}/A^{acet} **Eq. 9**

Chemical age index (ii) excluding TA polymeric pigments = A^{SO2}/A^{HCl} **Eq. 10**

More research is needed in order to design a suitable method to discriminate between free anthocyanins, AT polymeric pigments, TA polymeric pigments and pyranoanthocyanins with spectrophotometric measurements. A better understanding of the behavior from these natural pigments is addressed in this article. The considerations and mechanisms presented allow to develop new lines of research focused on the formation of these colored pigments in wine and the improvement of these useful colorimetric assays. So, the following research directions are proposed:

(1) Development of improved spectrophotometric methods - Given the complexity of identifying and quantifying different types of polymeric and pyranoanthocyanin pigments, the need for more advanced and precise spectrophotometric methods is suggested. In this context, a potential research direction could involve using model solutions combining flavanols and anthocyanins to study their specific bisulfite decoloration by adding SO_2 under certain conditions, using a combination of chemical analysis and 3D molecular simulations. This approach has previously been proposed for studying the effects of pH on ethyl-linked pigments (Zhao *et al.,* 2023).

(2) Study of pyranoanthocyanin and polymeric pigments (TA or AT) separately - There is an opportunity identified for further research on these pigments, particularly regarding their specific contribution to wine color and stability during aging. A potential research direction could involve the synthesis of both types of polymeric pigments by combining anthocyanins and flavanols, followed by their separation using sensitive chromatographic techniques that achieve different elution times. If both types of polymeric pigments can be eluted separately, their bisulfite discoloration sensitivity could be spectrophotometrically assessed, and an experimental correction coefficient could be included in the equations presented.

(3) Investigation of the chemical structure of pigments and its influence on wine coloration - It is suggested to delve deeper into the study of the chemical structure of different pigments and how this influences the color properties of wine, especially in aged wines. Some suitable approaches for continuing this research in the near future could involve the use of advanced analytical techniques, such as NMR spectroscopy or X-ray crystallography, which can directly visualize the bisulfite attachment to these molecules.

These proposals open up new possibilities for a more detailed and accurate analysis of wine pigments, potentially leading to a better understanding and optimization of wine aging and quality.

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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