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# Analysis of aged red wine pigments by capillary zone electrophoresis

Rubén Sáenz-López, Purificación Fernández-Zurbano, María Teresa Tena*

*Department of Chemistry, University of La Rioja, Madre de Dios 51, E-26006 Logroño (La Rioja), Spain*

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**Abstract**

Red wines of different ages (1–14 years) were analysed by Capillary zone electrophoresis (CZE). Pigments were separated using a fused silica capillary with an effective length of 56 cm and an internal diameter of 75 cm. Disodium tetraborate at 50 mM with a pH of 9.4 was used as buffer solution with methanol as modifier. Electrophoregrams were recorded at 280, 520 and 599 nm and spectra were measured from 200 to 599 nm using a diode array detector. Several differences in the CZE signals obtained for different aged wines were observed, as well as between red and white wines. The signals of a group of seven peaks increased in mature wines. A decrease in free anthocyanins was also identified in aged wine. The migration times of these compounds corresponded to charge/size ratios higher than those of free anthocyanins, and their spectra suggested combinations of anthocyanins with flavanols or tannins. The global CZE response of these pigmented polyphenolic polymers was linearly correlated with the spectrophotometric determination of polymeric pigments (96%) and age (92%).


*Keywords:* Red wine; Polymeric pigments; Anthocyanins

## 1. Introduction

The colour of young red wine is mostly due to the presence of grape anthocyanins. During the maturation and ageing process, anthocyanins, catechins, proanthocyanidins and other wine components are believed to react with each other to produce pigmented polymeric compounds, and this process is thought to stabilise wine colour [1], soften astringency [2] and bitterness [3].

The literature describes different mechanisms involved in the formation of these polymeric pigments, either by direct reaction between anthocyanins and flavanols [1,4,5], or by formation of acetyl bridges from acetaldehyde [6], which is usually the result of yeast metabolism or ethanol oxidation. The chemical structure of the main pigmented polymers is shown in Fig. 1. These combinations depend on the conditions of the medium (temperature, oxidation, . . .), the nature of tannins and the tannin/anthocyanins ratio. However, the structure of the flavilium cation (red species) is preserved in the new pigments, whose colour ranges from orange to mauve depending on the bounds involved [7]. Several of these reactions have been studied in model wine solutions [8–11] and the presence of the resulting products has been demonstrated in wine [12–16]. The recent findings concerning the structure of anthocyanins and tannin derivatives and their formation processes in wine have been recently reviewed [17].

The reactions of anthocyanins cause a decrease in their number, whereas colour intensity is preserved or even increases because, for the same pH level, there are more total coloured forms when flavanol–anthocyanin combinations occur than when anthocyanins are free [7,18]. Free anthocyanins have a limited contribution to colour in aged wines and thus colour will depend, to a great extent, on the polymeric pigments derived from free anthocyanins. This, together with the fact that anthocyanins are much more unstable when they are free compounds than when they are part of polymeric structures [7] makes colour stability in aged wines to depend basically on the formation of these new pigments.

Changes in astringency is another relevant organoleptic issue in aged wines. It is well-known that condensed tannins or procyanidins can bind to proteins. When wine is drunk,

* Corresponding author. Tel.: +34 941 299627; fax: +34 941 299621.
*E-mail address:* maria-teresa.tena@dq.unirioja.es (M.T. Tena).

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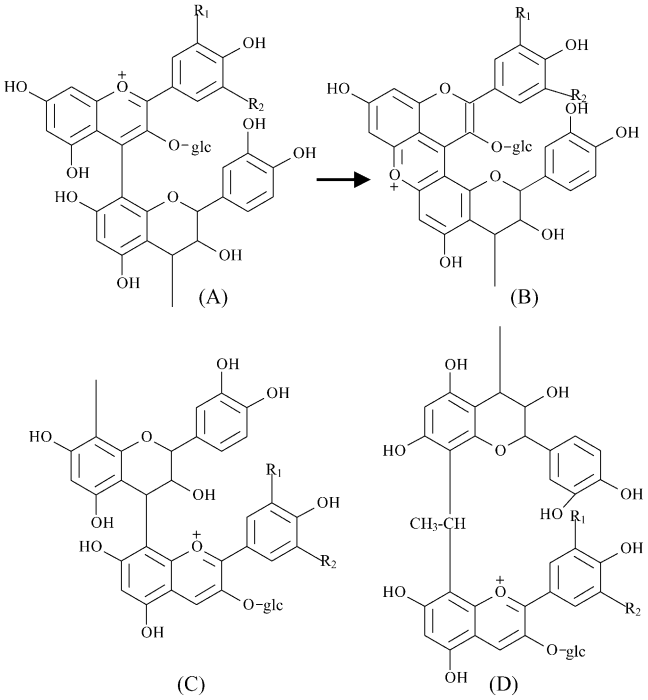


Fig. 1. Chemical structures of polymeric pigments: (A) flavylium $A^+$–T anthocyanin–tannin adduct; (B) yellow xanthylium salt; (C) flavylium T–$A^+$ tannin–anthocyanin adduct; (D) ethyl-linked T–$A^+$ tannin–anthocyanin adduct (glc means glucose).

tannins come into contact with the proteins present in saliva, which together cause an interaction that can precipitate and result in astringency. Positively charged polymeric pigments (Fig. 1) cannot bind to saliva proteins, which implies that the formation of these compounds will decrease the perception of astringency in wine [2,7,19].

The combination of high-performance liquid chromatography (HPLC) with diode array detection and mass spectrometry has been essential for the identification of anthocyanin derivatives in wine [13,20–25].

The analysis of polymeric pigments is quite complex at this moment in time thus global indexes based on particular characteristics of these molecules are used to monitor and control them. Somers and Evans [26] developed a spectral method for estimating the extent of pigmented polymer formation in red wines. The colour of red wine at its natural pH due to pigmented polymers was considered to be the residual wine colour after bleaching the anthocyanins with a large concentration of sodium metabisulphite. Total pigments were measured as wine colour when diluted with 1 M hydrochloric acid. The aforementioned authors proposed a chemical age index calculated as the ratio of these two absorbance measures after correcting dilution.

Bakker et al. [27] suggested the constant rate of anthocyanin loss determined by HPLC as a true measure of anthocyanin aging in port wines. Anthocyanins can be directly determined in red wines because they appear as discrete peaks without interference from pigmented polymers in young wines. In contrast, pigmented polymers elute as diffuse humps with long retention times [28].

Recently, a reverse phase HPLC method has been developed for the quantitative determination of pigmented polyphenolic polymers in red wine [29]. Pigmented polymers were properly separated from monomeric anthocyanins and eluted as a single peak using a polystyrene divinylbenzene column. The polymeric and polyphenolic nature of this peak was confirmed by its precipitation with proteins and its behaviour during ultrafiltration and chromatography on Sephadex LH20.

Capillary zone electrophoresis (CZE) features a higher separation efficiency than HPLC and has been successfully applied to the separation of charged macromolecules in other scientific fields. Our group has recently developed a capillary zone electrophoresis method for the quantitative analysis of wine anthocyanins in order to reduce analysis time and solvent consumption [30]. Anthocyanin monoglucosides and their acylated derivatives present in wine and grape extracts were separated in less than 14 min. In this paper, the separation of pigmented polymers by capillary electrophoresis is proposed for the first time. Anthocyanin-derived pigments were separated from free anthocyanins in less than 20 min by CZE compared to HPLC, where elution took more than 50 min [23].

The capillary zone electrophoretic signals obtained for aged red wines correlate well with the traditional spectrophotometric Chemical Age Index determinations and can be used to estimate wine age.

## 2. Experimental

### 2.1. Reagents and samples

HPLC grade methanol and ethanol supplied by Merck (Darmstadt, Germany) and MilliQ (Millipore, Molsheim, France) ultrapure water were used. Phosphoric and hydrochloric acid were supplied by Carlo Erba (Rodano, Italy), disodium tetraborate and potassium metabisulphite by Merck, sodium hydroxide by Prolabo (France), acetaldehyde by Fluka (Buchs, Switzerland), and tartaric acid by Sigma (St. Louis, MO, USA). All the solutions were filtered through a 0.45 μm filter, and CZE buffer solutions were sonicated for 15 min before use.

The samples used were red wine from the Qualified Origin Denomination Rioja (D.O.Ca. Rioja) with different degrees of maturation: four young wines (three from the 2003 vintage and one from the 2002 vintage), five *Crianza* wines (four years old, 1999 vintage), five *Reserva* wines (eight years old, 1995 vintage) and five *Gran Reserva* wines (twelve years old, 1991 vintage). *Crianza* wines have to be at least in their third year, having spent a minimum of one year in casks. *Reserva* wines have to have been aged for a minimum of three years, with at least one year in casks. *Gran Reserva* wines have to

spend at least two years in oak casks and three years in the bottle.

Two white wines were also analysed in order to compare CZE signals: a 2000 vintage *Crianza* white wine and a 1996 vintage white wine (not aged in casks).

### 2.2. Sample preparation

Wine samples were centrifuged at 5000 rpm for 5 min at room temperature using a 5804 Eppendorff centrifuge (Hamburg, Germany). Part of the supernatant was transferred to a topaz bottle, kept at 4 °C under nitrogen and filtered through a 0.45 μm filter before use.

Potassium metabisulphite (250 mg $l^{-1}$ of $SO_2$) was added to the samples before the CZE analysis in order to avoid differences in the anthocyanin analytical response caused by $SO_2$ [30].

### 2.3. CZE separation

Capillary zone electrophoresis was performed using an Agilent CE instrument (Waldbronn, Germany) equipped with a standard cassette containing an uncoated fused-silica capillary and a diode array detector.

The capillary was conditioned before injection by a first washing with 0.1 M sodium hydroxide for 2 min, then with ultrapure water for 2 min, and finally with running buffer for 5 min. The buffer vials were automatically replenished after each run in order to use fresh buffer solution each time and improve the reproducibility of migration times.

Fifty mM sodium tetraborate buffer solutions of pH 8.4 and 9.4 with 15% and 10% methanol (v/v) content, 46 and 56 cm (effective length) capillaries were used to separate anthocyanins and polymeric pigments, respectively. The remaining CZE conditions were the same for anthocyanins and polymeric pigments: voltage, 25 kV; capillary temperature, 10 °C; capillary internal diameter, 75 μm; and hydrodynamic injection, 50 mbar × 6 s (30 nl sample volume or 6 mm plug length).

Electrophoregrams were recorded at 280, 520 and 599 nm, and the spectrum from 200 to 599 nm was also collected for each peak. All the analyses were performed in duplicate and the results were expressed as mean values. The polymeric pigments were detected at 280 and 520 nm, and anthocyanins at 599 nm because at pH 8.4 they were presented as a blue quinoidal base.

The identification of free anthocyanins and pyranoanthocyanins peaks was based on the migration times of these compounds in the electrophoregrams and on the previous works of our research group [30,31]

### 2.4. Spectrophotometric analysis of the polymeric pigments

In order to evaluate the amount of polymeric pigments, the Chemical Age Index (CAI) was calculated according to Somers and Evans [26]. A Hewlett Packard diode array spectrophotometer (Palo Alto, CA, USA) equipped with 1 and 10 mm path length quartz cells was used.

Absorbance of red wine at 520 nm was measured in a 1 mm path length cell 2 min after adding approximately 7.3 mg $SO_2$ $l^{-1}$. Absorbance at 520 nm was also measured after 4 h in a 10 mm path length cell of a 50-fold dilution of red wine into 1 M hydrochloric acid. Absorbance values were corrected by the dilution factor and referred to a 10 mm path length cell in order to obtain the $E_{520}^{+SO_2}$ and $E_{520}^{HCl}$ values, which were used to calculate the CAI as a ratio ($E_{520}^{+SO_2}/E_{520}^{HCl}$). For young wines, the CAI was close to zero but it gradually increased during ageing, and in aged wines (fifty years) it approached unity.

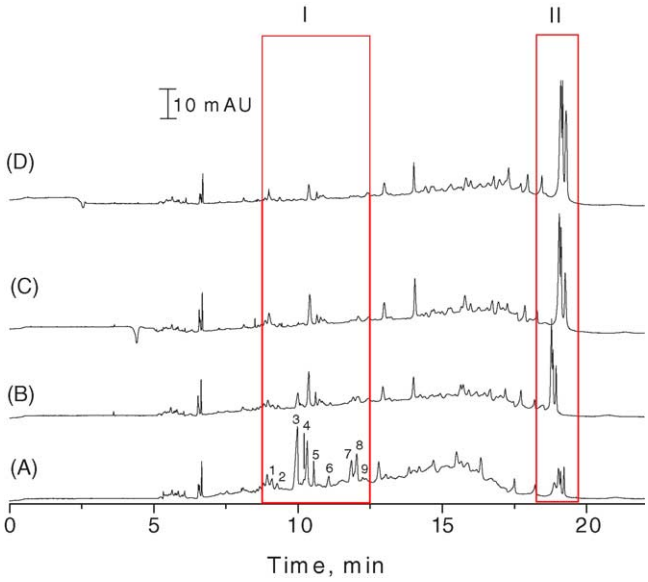


Fig. 2. Electrophoregrams at 280 nm of: (A) a 2002 vintage young wine; (B) a 1999 vintage *Crianza* wine; (C) a 1995 vintage *Reserva* wine; and (D) a 1991 vintage *Gran Reserva* wine. See text for CZE conditions. The signal zones for (I) anthocyanins: (1) malvidin-3-*O*-(6-coumaroyl)-glucoside; (2) malvidin-3-*O*-(6-acetyl)-glucoside; (3) malvidin-3-*O*-glucoside; (4) peonidin-3-*O*-glucoside; (5) malvidin-3-*O*-glucoside catechin dimer; (6) malvidin-3-*O*-glucoside and pyruvic acid derivative; (7) petunidin-3-*O*-glucoside; (8) delphinidin-3-*O*-glucoside; (9) cyanidin-3-*O*-glucoside) and (II) polymeric pigments are shown in the electrophoregrams.

## 3. Results and discussion

### 3.1. CZE analysis of wines with different aging times

Nineteen red wines were analysed by CZE using the conditions described for anthocyanins in Section 2. Fig. 2 shows the electrophoregrams at 280 nm obtained for four of them (with different aging times: young, *Crianza*, *Reserva* and *Gran Reserva* wines) as an example. As it can be seen, anthocyanin signals decreased with aging, and almost disappeared in the oldest samples. The same was observed at 599 nm.

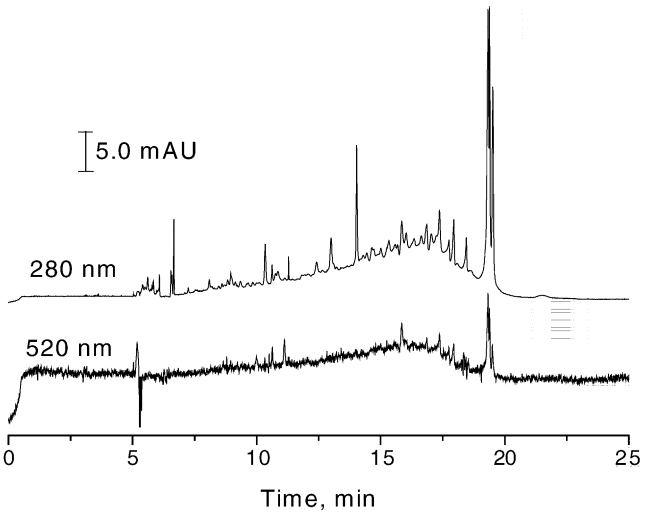


Fig. 3. Electrophoregrams at 280 and 520 nm of a *Gran Reserva* wine. For CZE conditions see text.

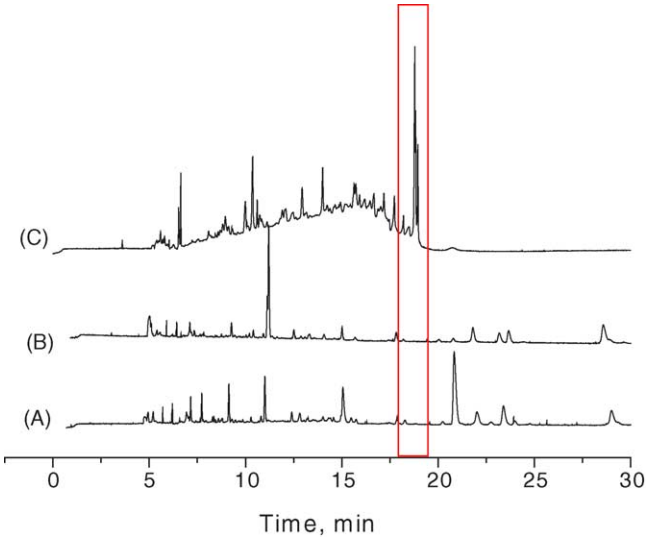


Fig. 4. Electrophoregrams at 280 nm of: (A) a 2000 vintage *Crianza* white wine; (B) a 1996 vintage white wine (non aged in casks); and (C) a 1989 vintage *Reserva* red wine. For CZE conditions see text.

Moreover, a group of peaks increased with age with migration times of around 19 min. This suggests the formation of compounds such as polymeric pigments during wine maturation.

### 3.2. Chemical nature of the CZE signals

Different tests were performed in order to elucidate the chemical nature of the compounds responsible for the CZE signals at a migration times of around 19 min.

Firstly, the electrophoregrams of a *Gran Reserva* wine at 280 and 520 nm are shown in Fig. 3. The signal of this group of peaks at 520 nm with migration times of around 19 min justifies the claim that these compounds are red. Thus, the relationship of these compounds to anthocyanins was postulated.

Secondly, white wine contains flavanols (albeit at a lower concentration than red wine) that can polymerise during the aging process giving tannins, and in order to verify that compounds with migration times longer than 19 min do not correspond to polymerised tannins, electrophoregrams of aged red and white wines were compared. Fig. 4 shows the electrophoregrams obtained. As it can be seen, there were no signs corresponding to these pigments in white wine.

Thirdly, the impact of a pH change on the spectra was studied. Polymeric pigments preserve their absorbance at 520 nm even at pH 8.4 but free anthocyanins do not [1,32]. One of the features of free anthocyanins is that their colour is strongly dependent on pH. In wine, anthocyanins behave as a different species and if wine pH increases, the equilibrium results in the formation of a blue quinoidal base and a colourless carbinol pseudobase, and therefore the typical absorption band at 520 nm is displaced towards a longer wavelength, of around 600 nm. This colour change with pH is easily observed in young red wines because free anthocyanins are the compounds responsible for wine colour. In contrast, colour is preserved in aged wines because it is mainly due to polymeric pigments, which are more stable to pH changes. These effects can be seen in Fig. 5, which shows the spectra of a young and a mature red wine with an acid and basic pH. Note that mature wine absorbance is similar with both pH levels, which reveals that the basic conditions of the CZE method are also appropriate for polymeric pigments.

### 3.3. Migration times

The migration order of anthocyanins can be explained by the charge/size ratio and the possibility of complex formation with buffer molecules [30]. Since the CZE method used implied a positive polarity (cathode detection) and positive

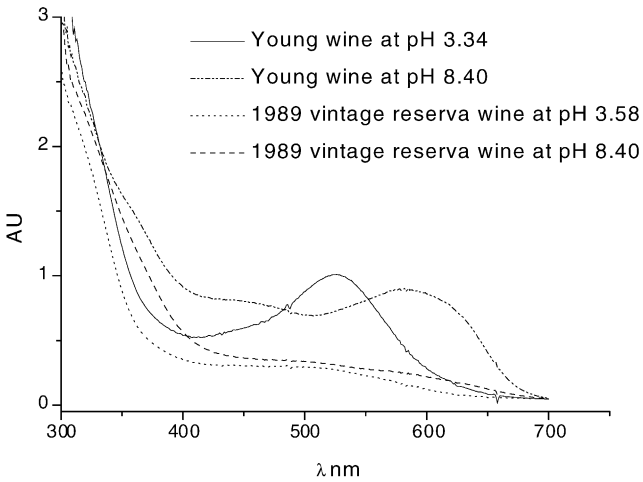


Fig. 5. Effect of a pH change in the spectra of different age wines: young and 1989 vintage *Reserva* red wines.

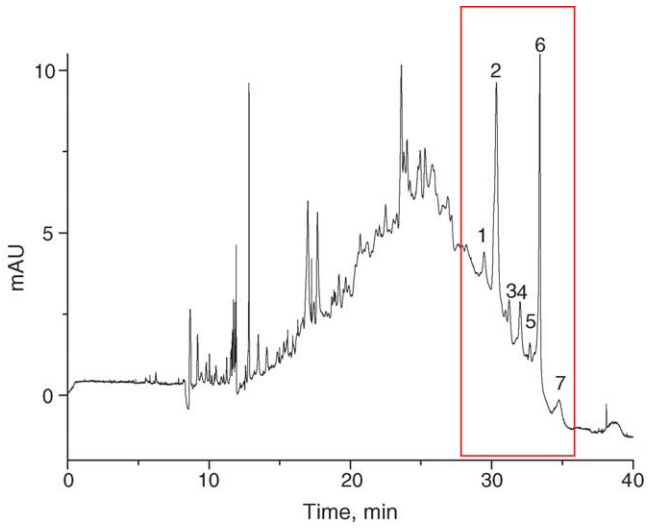


Fig. 6. Electrophoregram at 280 nm of a 1991 vintage *Gran Reserva* red wine. See text for CZE conditions for polymeric pigment separation.

electroosmotic flow, anthocyanins (negatively charged at working pH) with higher charge/size ratios displayed longer migration times. Moreover, note that petunidin-, delphinidin- and cyanidin-3-*O*-glucoside, which have *ortho*-hydroxyl groups, can form complexes with tetraborate and thus increase their charge/size ratios compared to malvidin- and peonidin-3-*O*-glucoside (Fig. 2).

Polymeric pigments consist of an anthocyanin molecule and one or more flavanol molecules. Although they have a higher molecular mass, they can also acquire several negative charges. Therefore, their charge/size ratio is higher than that of free anthocyanins. As a consequence of this, they migrate slower, presenting longer migration times than free anthocyanins (Fig. 2).

### 3.4. CZE separation of polymeric pigments

Since the CZE conditions optimised for free anthocyanin separation were not efficient enough to separate polymeric pigments individually, the influence of the CZE variables on the resolution of the group of peaks at 19.0–19.5 was also studied. The optimal conditions were as follows: pH, 9.4; methanol percentage, 10%; and capillary length, 56 cm. The rest of conditions were the same as those for free anthocyanins and can be seen in the Section 2. For optimisation, a 12-year-old wine sample was used. The electrophoregram obtained with the optimised method for a 1991 vintage *Gran Reserva* red wine is shown in Fig. 6. A total of seven peaks were found and marked in the electrophoregram.

### 3.5. Correlation between CZE signals and CAI

The CZE signals obtained for polymeric pigments were compared to Chemical Age Index values for the 19 wines analysed. The sum of the peak area values of the polymeric pigments obtained under CZE conditions for the separation

Table 1
Correlation coefficients $R^2$ between CZE peak areas and Chemical Age Index.

| Peak number | Correlation coefficient $R^2$ |
|---|---|
| 1 | 0.3 |
| 2 | 0.4 |
| 3 | 0.03 |
| 4 | 0.004 |
| 5 | 0.0007 |
| 6 | 0.02 |
| 7 | 0.7 |

of free anthocyanins were consistent with the results provided by the usual non-$SO_2$-bleachable pigment assay proposed by Somers and Evans [16]. The level of polymeric pigments estimated by CZE was linearly correlated to the Chemical Age Index ($R^2$ = 0.92) as shown in Fig. 7A. This value was better than that reported by Peng et al. [29] for the percentage of pigment in the polymeric form estimated by HPLC and the Chemical Age Index ($R^2$ = 0.86) or the relationship between non-$SO_2$-bleachable colour ($E_{520}^{+SO_2}$) and concentration of pigmented polymers ($R^2$ = 0.83).

The sum of the areas of the seven peaks separated by the optimised method for polymeric pigments (migration times between 29 and 34 min) showed an acceptable linear correlation of 86% ($R^2$ = 0.74) to CAI, see Fig. 7B (the point corresponding to a *Reserva* wine was rejected as an outlier because wine went off during the storage period between the two analysis). However, individual peak areas and CAI were not linearly correlated at all, as shown in Table 1. This could be due to the fact that, apart from having a different initial polyphenolic composition, pH, ethanol content and maturation conditions, such as temperature, lighting, oxygen intake, winemaking treatment, etc. were extremely varied for the wines studied and this probably affected the preferential reactions of polymeric pigment formation that take place during wine aging. In fact, ethanol content strongly affected the formation of ethyl-linked polymeric pigments [24,9]. It can therefore be concluded that although the initial wine composition, maturation conditions and treatments determine the polymeric pigment profile, the total amount of polymeric pigments can be calculated as the sum of the CZE signals as it is well-correlated to CAI (96%).

### 3.6. Correlation between CZE signals and wine age

Polymeric pigments CZE signals were also correlated with wine age and a good linear correlation was observed (92%, $R^2$ = 0.85). The equation was: area = (23 ± 5) × age + (14 ± 4) × 10, where age and area are expressed in years and mAU s, respectively. This equation can be used only to estimate wine age by CZE analysis (at least for the types of wines studied, being a maximum of 12-years old, and provided that colorant matter has not precipitated) because the values of slope and intercept standard deviations are higher than 20%. The limit of sensitivity (calculated as ($B$ + 3$s_b$

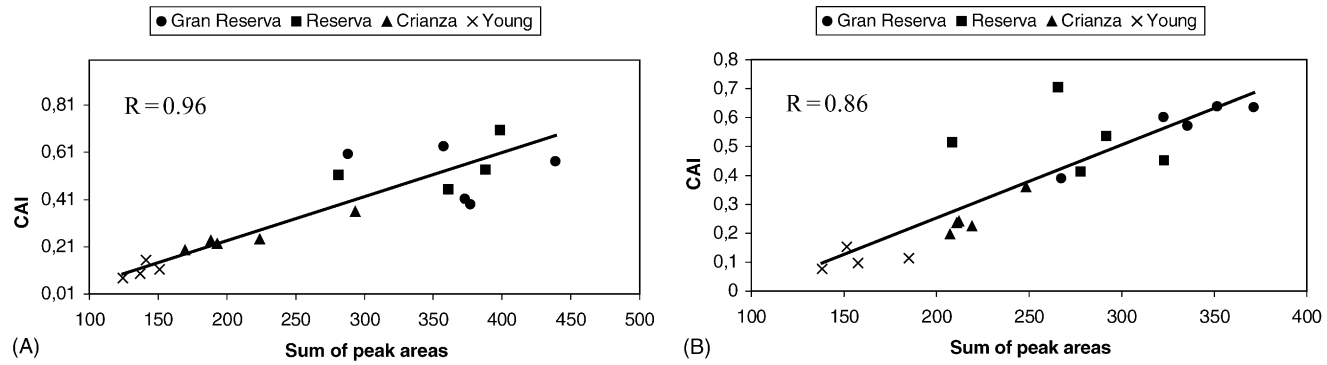


Fig. 7. Correlation between the sum of polymeric pigment peak areas and the Chemical Age Index of the wine. The peak areas were obtained by the CZE method developed for: (A) free anthocyanins; and (B) polymeric pigments.

$- b)/m$, where $B$ and $s_b$ are the mean and standard deviation of young wine signals, $b$ is the intercept and $m$ is the slope) was 1.1 years. The minimal difference in wine age required to distinguish between two wines with a confidence of 95% was around 3.0 years. This has been estimated as $2(t/\sqrt{M})(s_r/m)\sqrt{((1/M)+(1/N))}$, where $s_r$ is the standard deviation of the residuals, $M$ = 3 is the number of sample replicates, $N$ = 19 is the number of calibration points and $t$ is 2.093 for $\alpha$ = 0.05 and $M + N - 3$ = 19 degrees of freedom.

The same correlation (92%) was obtained for the polymeric pigment/total pigment signal ratio. Total pigment signal was calculated as the sum of polymeric pigment and anthocyanin signals. The equation was: Area ratio = (0.059 ± 0.006) × age + (0.39 ± 0.05), with age expressed in years and with relative standard deviations of slope and intercept lower than 13%. The limit of sensitivity and the minimal difference in wine age using this equation were 0.63 and 2.9 years, respectively.

## 4. Conclusions

A CZE-based method for the evaluation of the proportion of polymeric pigments in red wines is proposed in this paper. Unlike the classical technique based on HPLC, polymeric pigments can be easily separated by CZE. In addition, the proposed method involves a minimal set-up time, implies fewer costs and less reagent consumption and provides a better separation efficiency with an analysis time shorter than HPLC-based methods.

In our work, the signals of a group of seven peaks increased in mature wines. The migration times of these compounds corresponded to a charge/size ratio higher than that of free anthocyanins, and their spectra suggested that they were anthocyanin-derived polymeric pigments. Moreover, a decrease in free anthocyanins was observed with age.

The global CZE response of these pigmented polyphenolic polymers was linearly correlated with the spectrophotometric determination of polymeric pigments (96%) and wine age (92%).

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