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Journal of Chromatography A, 1112 (2006) 112–120
JOURNAL OF CHROMATOGRAPHY A
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Analysis of polymeric phenolics in red wines using different techniques combined with gel permeation chromatography fractionation

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Available online 19 December 2005

Abstract

A multiple-step analytical method was developed to improve the analysis of polymeric phenolics in red wines. With a common initial step based on the fractionation of wine phenolics by gel permeation chromatography (GPC), different analytical techniques were used: high-performance liquid chromatography–diode array detection (HPLC–DAD), HPLC–mass spectrometry (MS), capillary zone electrophoresis (CZE) and spectrophotometry. This method proved to be valid for analyzing different families of phenolic compounds, such as monomeric phenolics and their derivatives, polymeric pigments and proanthocyanidins. The analytical characteristics of fractionation by GPC were studied and the method was fully validated, yielding satisfactory statistical results. GPC fractionation substantially improved the analysis of polymeric pigments by CZE, in terms of response, repeatability and reproducibility. It also represented an improvement in the traditional vanillin assay used for proanthocyanidin (PA) quantification. Astringent proanthocyanidins were also analyzed using a simple combined method that allowed these compounds, for which only general indexes were available, to be quantified.

Keywords: Red wine; Anthocyanins; Polymeric pigments; Proanthocyanidins; Astringent proanthocyanidins; GPC/HPLC/DAD/CZE

1. Introduction

Polyphenolic compounds are widely known for their role in the organoleptic properties of wines and are extremely important for determining the final quality of the product, especially in red wines. The quality of these organoleptic characteristics of red wines will depend not only on the quantity of phenolic pigments, but also on their type, composition and distribution.

Two important families of polyphenolic compounds present in grapes are known to influence final wine quality: proanthocyanidins (condensed tannins with a polymerization degree over 5) and anthocyanins. Proanthocyanidins (PAs) are important for providing wine with bitterness and astringency. Polymeric anthocyanins are known to be responsible for the stable colour of red wines. The colour of these types of pigments is more stable to pH increases than that of monomeric anthocyanins; these pigments are also less sensitive to oxidation and to bleaching by sulphur dioxide than monomeric anthocyanins [1–3].

Colour changes in red wine from the initial red–purple to a more brick hue, occurring continuously during winemaking and ageing, are supposedly due to irreversible mechanisms and prompt the formation of new and stable pigments, with either high or low molecular weights [4–10]. The pigments in aged red wine appear to be primarily large polymeric compounds and are formed due to reactions of direct or indirect condensation between anthocyanins and proanthocyanidins [11].

Grape proanthocyanidins are responsible for some major wine organoleptic properties, as well as for the physiological effects associated with its consumption [12]. The mechanism of astringency perception is commonly ascribed to interactions of proanthocyanidins with salivary proteins. This property is known to vary both with PA structure and degree of polymerization, the larger PA molecules being the most important in the astringency sensation [13]. Kallithraka et al. [14], in an attempt to compare taste-panel results with analytical data, suggested that perceived astringency could be closely correlated to the amount of flavanols not precipitated by salivary proteins.

Many chromatographic methods have been developed for analyzing grape and wine phenolics. High-performance liquid chromatography (HPLC) techniques with photodiode array or mass spectrometry (MS) detection are widely used for the separation and quantitative determination of individual monomeric and oligomeric flavonoids from red wines. However, these

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doi:10.1016/j.chroma.2005.11.100

techniques are limited in their ability to analyze high molecular weight polyphenols. The analysis of polymeric pigments is relatively complex at present; their complex structure and low quantity makes their detection and separation difficult. Many methods have been used so far for the fractionation of polymers from red wine. Most of these methods are based on the separation of red wine pigments by gel permeation chromatography (GPC), solid-phase extraction (SPE) on C_{18} cartridges or more recent techniques such as countercurrent chromatography. These methods have been mainly developed to fractionate, isolate and identify new anthocyanin-derived pigments [3–5,7–9,15,16]. However, there have only been a few attempts to separate and quantify more complex polymeric pigments [11,17–19]. Recently, a method based on capillary zone electrophoresis (CZE) proved to be efficient enough to separate red wine polymeric pigments [20].

Whereas only a few studies of polymeric pigments from red wine have been published, many publications deal with proanthocyanidins from grape or grape skin. Different chromatographic techniques (HPLC–DAD, HPLC–EIS–MS) have been applied in the analysis of individual PAs, dimers, trimers and oligomers [21]; however, polymers of higher molecular weight cannot be resolved by these techniques. The methods used to estimate these polymers differ in terms of basic principles and specificity, and none of them can be considered totally satisfactory [21]. Traditionally, wine astringency is estimated using the gelatin index method proposed by Glories [22,23]. This method, based on the reaction between proanthocyanidins and gelatin, seems to be appropriate for estimating astringency; however, it only provides an index and not an absolute quantity.

The aim of this paper is to describe a versatile method that allows different families of polyphenols in red wines to be analyzed and quantified. Given the importance of polymeric phenolics in wine quality and the difficulties encountered in analyzing them, this method has been mainly developed for the analysis of these polymeric compounds. For this reason, a combination of techniques is proposed, all of them with a common initial step, namely the fractionation of red wine phenolics by GPC in order to separate monomeric and polymeric compounds. Thereafter, different analytical techniques were used: (a) identification and quantification of monomeric phenolic compounds of red wines by HPLC–DAD; (b) analysis of polymeric pigments by CZE; and (c) quantification of proanthocyanidins by reaction with vanillin. The interactions between polymeric phenolics and gelatin were also analyzed. Taking into account these results, a method for quantifying astringent proanthocyanidins is proposed. The properties of the described method were also studied and a validation study was performed.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical reagent grade. All chromatographic solvents were of HPLC grade. Malvidin-3-glucoside, peonidin-3-glucoside, ferulic acid, syringic acid, caffeic acid, *p*-coumaric acid, catechin, epicatechin, myricetin, quercetin, isorhamnetin, kaempferol and rutin were purchased from Extrasynthèse (Lyon, France), and gallic acid from Sigma (St. Louis, MO, USA). Formic acid and acetonitrile supplied by Sigma and MilliQ (Darmstadt, Germany) ultrapure water were used. Acetone was obtained from Riedel-deHäen (Sigma), and pure methanol, ethanol and disodium tetraborate from Merck. Trifluoroacetic acid and toluene-α-thiol (benzyl mercaptan) were supplied by Fluka (Sigma), phosphoric acid, hydrochloric acid, and sulfuric acid by Carlo Erba (Rodano, Italy), sodium hydroxide by Prolabo (France), and tartaric acid by Sigma. Vanillin was obtained from Aldrich (Sigma) and gelatin 80–100 blooms from Panreac (Montcada i Reixac, Barcelona, Spain). All the solutions were filtered through a 0.45 μm filter and sonicated for 15 min before use in HPLC or CZE.

The samples used were red wine from the Qualified Origin Denomination Rioja (D.O.Ca. Rioja). The wines were selected at several stages of vinification and maturation, presenting different organoleptic characteristics.

2.2. Fractionation of wine phenolics by GPC

TSK Toyopearl gel HW-50F (Tosohaas, Montgomery-ville, PA, USA) was suspended in the mobile phase and, after swelling, it was packed in a Millipore (Bedford, MA, USA) Vantage L column (120 mm × 12 mm i.d.) at atmospheric pressure. Gel permeation chromatography data were analyzed by connecting the column to a diode array detector (Agilent, G1315B). Two milliliters (2 ml) of wine were directly applied to the column and flow rate was regulated at 1 ml min^{-1} using a peristaltic pump. A first fraction (F1) was eluted with 60 ml of ethanol/water/trifluoroacetic acid (55:45:0.05, v/v/v). A second fraction (F2) was recovered by elution with 50 ml of acetone/water (60:40, v/v). The two fractions collected were taken to dryness under vacuum. All the wines were fractionated three times.

2.3. HPLC–DAD analysis

HPLC–DAD was performed in an Agilent modular 1100 liquid chromatograph (Waldbronn, Germany) equipped with a G1313A injector, a G1311A HPLC quaternary pump, an online G1379A degasser, a G1316A oven, a G1315B photodiode array detector, and an Agilent Chemstation software. The column was a reversed-phase Kromasil 100-C18 (5 μm packing, 200 mm × 46 mm i.d.) protected with a guard column of the same material (Teknokroma, Barcelona, Spain). Phenolic compounds were eluted under the following conditions: 1 ml min^{-1} flow rate; oven 30 °C; solvent A: formic acid/water (2:98, v/v); solvent B: acetonitrile/water/formic acid (80:18:2, v/v/v); gradients: isocratic 2% B in 3 min, from 2 to 10% B in 2 min, from 10 to 15% B in 10 min, from 15 to 30% B in 10 min, from 30 to 50% B in 10 min, from 50 to 60% B in 5 min, from 60 to 90% B in 5 min, followed by washing and reconditioning of the column.

Fractions were dissolved in 2 ml of solvent A and 50 μl was directly chromatographed. UV–vis spectra were recorded every second from 250 to 600 nm, with a bandwidth of 1.2 nm. The chromatograms were acquired at 515 nm for anthocyanins, 365 nm for flavonols, 310 nm for phenolic acids and 280 nm for flavan-3-ols. The different compounds were identified on the basis of their UV–vis spectra, retention times and by comparison with commercial standards. Unknown peaks were identified by mass spectrometry. The calibration curves were obtained by injecting different concentrations of standards: malvidin-3-glucoside for anthocyanins and anthocyanin derivatives, caffeic acid for phenolic acids, quercetin for flavonols, and catechin for flavan-3-ols. The range of the linear calibration curves ($r^2 > 0.99$ in all the cases) was from 0.01 (limit of detection) to 1 mg l^{-1} for the lower concentration compounds and from 1.0 to 100 mg l^{-1} for the higher concentration compounds. Unknown concentrations were determined from the linear regression equations. Each measurement was run in triplicate.

2.4. HPLC–MS analysis

MS analysis was performed by coupling the Agilent 1100 liquid chromatograph described above to a MS detector (Hewlett-Packard, Palo Alto, CA, USA). The mass spectrometer was equipped with an electrospray ionization source and a quadrupole mass analyzer, which were controlled by the MS Agilent 1100 software. Chromatographic separation was performed under the same conditions described above. The flow was split into a ratio of 35:100 between the HPLC detector and the MS detector in order to introduce the optimal flow-rate (35 μl min^{-1}) into the electrospray ionization interface. The eluted compounds were mixed with nitrogen at a 30 l min^{-1} flow-rate and 225 °C in the electrospray ionization interface. The mass spectrometer was operated in the positive ion mode for anthocyanins (*m/z* 100–600) and in the negative ion mode for flavonols (*m/z* 100–600), phenolic acids (*m/z* 200–600) and flavan-3-ols (*m/z* 200–600). Cone voltage was a linear function of the relative function of the relative molecular mass, starting at 40 V for *m/z* 100 and ending at 80 V for *m/z* 600, nebulizer pressure was 80 psi, and capillary voltage, 4000 V. The compounds were chemically ionized by proton transfer, the ions generated were introduced into the mass spectrometer and the abundance of selected *m/z* corresponding to $(M\text{-}H)^+/(M\text{-}H)^-$ ions of compounds was recorded.

2.5. Thiolysis conditions

Fractions F1 and F2 were dissolved in 2 ml of pure methanol and introduced in a glass vial with an equal volume of thiolytic reagent (toluene-α-thiol 5% in methanol containing 0.2 M HCl). After sealing, the mixture was shaken and heated at 90 °C for 2 min. The solutions were then analyzed by HPLC–DAD under the same conditions described above. Quantification of each terminal and extension unit was based on peak areas at 280 nm [24]. Each mixture was analyzed in triplicate.

2.6. CZE analysis

Capillary zone electrophoresis was performed using an Agilent CE instrument (Waldbronn, German) equipped with a standard cassette containing an uncoated fused-silica capillary and diode array detector. Sodium tetraborate buffer solutions (50 mM) of pH 9.4 with 10% methanol (v/v) content, and 56 cm (effective length) capillary were used to separate anthocyanins and polymeric anthocyanins. The remaining CZE conditions were those described by Sáenz López et al. [20].

Wine fractions were dissolved in 500 μl of synthetic wine (12% (v/v) ethanol in aqueous solution containing 6 g l^{-1} tartaric acid, pH 3.5). Thereafter, the samples were centrifuged (5000 rpm, 5 min, room temperature) using a 5804 Eppendorf centrifuge, and directly injected in the CZE system. Wine samples were also prepared in order to compare their response with that of the fractions. These wine samples were concentrated four times prior analysis in order to reach the same degree of concentration than wine fractions.

Electrophoregrams were recorded at 280, 420, 520 and 599 nm, and the spectrum from 200 to 600 nm was also collected for each peak. The polymeric anthocyanins were detected at 280 nm and 520 nm, and anthocyanins at 599 nm because they were present as a blue quinoidal base at pH 9.4. The identification of monomeric anthocyanin and pyranoanthocyanin peaks was based on the migration times of these compounds in the electrophoregrams and on [25,26]. All the analyses were performed in triplicate.

2.7. Determination of the total proanthocyanidin content by the vanillin assay

The vanillin assay was performed according to the method described by Sun et al. [27] but with few modifications. Fraction F2 was dissolved in a suitable quantity of methanol in order to have a final absorbance within the linear range of the standard curve; for the general case, 7.5 ml of methanol was added. One milliliter (1 ml) of this solution was placed in a vial and 2.5 ml of 1% (w/v) vanillin in methanol and 2.5 ml of sulphuric acid/methanol (10:90, v/v) were added. The absorbance of the coloured adducts formed between vanillin (4-hydroxy-3-methoxybenzaldehyde) and proanthocyanidins was measured at 500 nm in 1 cm-cuvettes. The reaction was performed at room temperature and left until the maximum absorbance value at 500 nm was reached, which occurred at around 15 min of reaction time.

The spectrophotometric measurements were performed on a Cary 300 Scan UV–vis spectrophotometer (Varian Inc., Madrid, Spain). First, a blank was made to eliminate the absorbance of residual pigments present in F2. The blank was prepared in the same way described for the sample, but adding methanol instead of vanillin. When measuring the samples, reference solutions (adding methanol instead of the sample) were used for each sample. Quantification of proanthocyanidins was performed by means of a standard curve prepared with different concentrations of catechin. In this case, the reactions were performed at 30 °C

and the maximum absorbance value at 500 nm was reached at 18 min. Samples were analyzed in triplicate.

2.8. Determination of the total astringent proanthocyanidin content

The method proposed for the quantification of astringent proanthocyanidins is a combination of two methods, the vanillin assay and the assay based on the ability of proanthocyanidins to precipitate with gelatin.

Ten milliliters of a solution of gelatin (35 g l^{-1}) was added to 50 ml of wine. The wines, with and without gelatin (control wine), were shaken and kept at 4 °C for 72 h. Thereafter, the samples were centrifuged and the supernatants were collected and fractionated by GPC. Two milliliters of the supernatants was directly applied onto the TSK Toyopearl gel HW-50F column (120 mm × 12 mm i.d.). The fractionation was carried out in the same conditions described in Section 2.2. Fractions F2 from both wines were then analyzed by the vanillin assay as described in Section 2.7. The quantity of astringent proanthocyanidins was calculated as the difference found between the control wine and that with gelatin addition.

2.9. Determination of the tannin content

In order to determine the gelatin index in wine samples, these were precipitated with gelatin as described above. Precipitated proanthocyanidins were determined and calculated by comparing the tannin content obtained before and after precipitation, as described in [28].

2.10. Study of validation: repeatability, reproducibility and recovery

Repeatability was checked by analyzing six samples of the same wine under normal operating conditions. Wines were fractionated by GPC and the resulting fractions submitted to HPLC–DAD and CZE (fractions F1) and to the vanillin assay (fractions F2). The amount of different families of monomeric phenolics was quantified in each sample by HPLC–DAD and the content of total PAs was determined by the vanillin assay. In CZE, the resulting peak areas were quantified. Moreover, the content of astringent PAs was also determined in the six wines as described in Section 2.8. Repeatability results were expressed as the coefficient of variation obtained for the six measurements.

Reproducibility was assessed using five different wines at different stages of vinification, except for the quantification of total PAs in fractions F2, for which 60 samples of wines and musts were analyzed (unpublished results). Reproducibility was expressed as the mean value of the coefficients of variation obtained for the different wines from three replicate measurements.

GPC recovery was studied using five aliquots of the same wine fractionated by GPC and monomeric phenolics were quantified by HPLC. The same aliquots were directly injected in HPLC, without previous fractionation, and the same phenolics were quantified. The recovery was calculated by comparing the amounts for each compound in the samples with and without fractionation. To study proanthocyanidin recovery in the proposed method (fractionation + vanillin reaction), 20 ml of wine was fractionated in a bigger Millipore Vantage L column (120 mm × 320 mm) to obtain a suitable quantity of proanthocyanidin extract. Using the vanillin assay, an aliquot was analyzed to estimate the PA richness in the extract. Two different quantities of the lyophilized extract were added to a wine (equivalent to 68 and 150 mg l^{-1} of PAs). The original wine and the two enriched samples were fractionated and the proanthocyanidin content was determined in fractions F2 by the vanillin assay. This protocol was repeated three times and the recovery expressed as the mean of the six recoveries calculated.

3. Results and discussion

3.1. Analysis of fractions F1 and F2 obtained by GPC

Red wine was submitted to GPC in order to separate the phenolic compounds and avoid interferences in further analysis. Fig. 1 shows the chromatogram recorded at 515 nm and obtained during the GPC fractionation of red wine. It can be observed that most wine pigments were eluted in fraction F1, although a small quantity was also collected in fraction F2. Several authors [24,13] employing the same chromatographic conditions as in the present study, report that fraction F1 is mainly composed of monomeric phenols (flavan-3-ols, anthocyanins, flavonols and phenolic acids) and dimeric flavan-3-ols, while fraction F2 contains the oligomeric and the polymeric material (proanthocyanidins). Remy et al. [24] observed that polymeric pigments and oligomeric proanthocyanidins are distributed between the two fractions F1 and F2.

In order to confirm the findings reported in the bibliography, both fractions obtained after GPC fractionation underwent different analysis. A thiolysis reaction was performed in order to obtain information about the mean degree of polymerization (mDP) of the compounds present in both fractions. The depolymerization of the possible polymeric pigments and tannins by the thiolysis assay enabled us to calculate the mDP. This value, calculated as the ratio between the total number of units and the number of terminal units, was estimated as 1.5 for fraction F1.

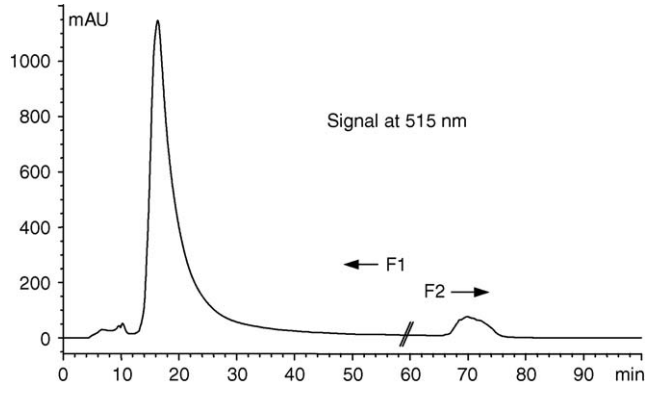

Fig. 1. Chromatogram of the GPC fractionation of a wine sample using a Toyopearl HW-50F column. See text for GPC conditions.

Table 1
Validation results[a] obtained for GPC analysis (see text for conditions and calculations)

	Monomeric anthocyanins	Phenolic acids	Flavonols	Flavan-3-ols
Repeatability (%)	4.9 ± 0.1	4.9 ± 0.1	4.7 ± 0.4	4.9 ± 0.1
Reproducibility (%)	4.9 ± 0.6	4.5 ± 0.2	4.8 ± 0.6	4.7 ± 0.5
Recovery (%)	101 ± 1	99 ± 4	96 ± 5	92 ± 7

[a] Expressed as a mean for each phenolic family (mean ± SD).

This suggested that most of the polyphenols present in F1 were monomers and dimers. Comparatively, the mDP of fraction F2 was estimated as 20, confirming the presence of proanthocyanidins.

Fraction F2 was also analyzed by HPLC–DAD and a minimum response (below the quantification limit) was observed for monomeric anthocyanins at 515 nm. This suggested that the low signal visualized at 515 nm when fractionating F2 (Fig. 1), may be due to the presence of this small amount of residual monomeric anthocyanins. However, the presence of polymeric pigments could not be ruled out. This possibility will be discussed later in Section 3.3., when analyzing polymeric pigments by CZE.

3.2. Analysis of pigments in fraction F1 by HPLC–DAD and HPLC–MS

Fraction F1 was submitted to HPLC–DAD and HPLC–MS analysis in order to identify and quantify the monomeric pigments present in it. Eleven monomeric anthocyanins were identified: delphinidin-3-*O*-glucoside; cyanidin-3-*O*-glucoside; petunidin-3-*O*-glucoside; peonidin-3-*O*-glucoside; malvidin-3-*O*-glucoside; delphinidin-3-glucosylacetate; petunidin-3-glucosylacetate; malvidin-3-glucosylacetate; delphinidin-3-glucosylcoumarate; petunidin-3-glucosylcoumarate; and malvidin-3-glucosylcoumarate. Different phenolic acids, flavan-3-ols, and flavonols were also identified and quantified in this fraction. Among the phenolic acids, gallic acid, *c*-caftaric acid, *t*-caftaric acid, *c*-coutaric acid, *t*-coutaric acid, caffeic acid and *p*-coumaric acid were identified; among the flavonols, rutin, myricetin-3-glucoside, kaempferol, myricetin, quercetin and isorhamnetin-3-glucoside; and among flavan-3-ols, catechin, epicatechin and epigallocatechin.

In order to validate the methods used, GPC fractionation and HPLC analysis, repeatability, reproducibility and recovery were assessed (see Section 2.10.) and expressed as a mean for each phenolic family (Table 1). All these results showed that the method proposed had good reliability and accuracy. These analytical characteristics are well established for the HPLC technique but not for fractionation by GPC on a Toyopearl column, which is widely used.

3.3. Analysis of pigments in fractions F1and F2 by CZE

Monomeric phenolics were successfully identified by HPLC–DAD and HPLC–MS; however, the polymeric pigments could not be visualized by these techniques. In order to be able to study this group of compounds, capillary zone electrophoresis was chosen. This technique allows the separation of many classes of compounds based on the electrophoretic migration of charged analytes. Recently, CZE has shown to be efficient enough to separate red wine polymeric pigments from monomeric anthocyanins and anthocyanin derivatives, and showed higher separation efficiency than HPLC and reduced analysis time and solvent consumption [20]. Moreover, pigmented polymers were properly separated in different peaks, whereas with other techniques these compounds eluted as diffuse humps or as a single peak [17,19,29].

Firstly, fractions F1 and F2 obtained after GPC fractionation were analyzed by CZE in order to determine where polymeric pigments were contained. Fig. 2A shows the electrophoregram recorded at 599, 520 and 280 nm obtained for fraction F1; Fig. 2B shows the electrophoregram recorded at 520, 420 and 280 nm obtained for fraction F2. In F1, two different zones of peaks were observed: a first group of peaks with migration times around 15 min (zone I, peaks 1–9); and a second one, with migration times around 24 min (zone II, peaks a–g). However, in F2 only a diffuse and big hump was observed, with a maximum migration time around 30 min (zone III), just after the second zone visualized in fraction F1.

Peaks 1–9 in zone I (Fig. 2A) were identified as reported in previous papers [25], corresponding to monomeric and dimeric anthocyanins. This finding demonstrated the presence of these compounds in F1, and was consistent with our observations in HPLC–DAD. Peaks a–g of zone II (Fig. 2A) corresponded to polymeric anthocyanins as described by Sáenz-López et al. [20], who observed that the global CZE response of these polymeric pigments was linearly correlated with the spectrophotometric determination of polymeric pigments (96%) and age index (92%). The migration times of these peaks were longer than those of peaks in zone I, confirming their higher charge/size ratios. Moreover, they absorbed at 520 nm, thus confirming their pigment nature. Finally, zone III in F2 (Fig. 2B) was attributed to the absorption of PAs. Compounds in this zone migrated more slowly than those of F1, which confirmed their higher charge/size ratio. These molecules, with an mDP of 20 (see Section 3.1.), are known to be in the F2 fraction [30]. Besides, its spectra, with a maximum absorbance at 200 and 280 nm and very low absorbance in the visible region, coincided with that of a mixture of monomeric and oligomeric apple PAs [31]. The low absorbance seen at 520 and 420 nm may be attributed to residual polymeric anthocyanins eluting in fraction F2.

After confirming the presence of monomeric anthocyanins in F1 and demonstrating that most of the polymeric pigments were

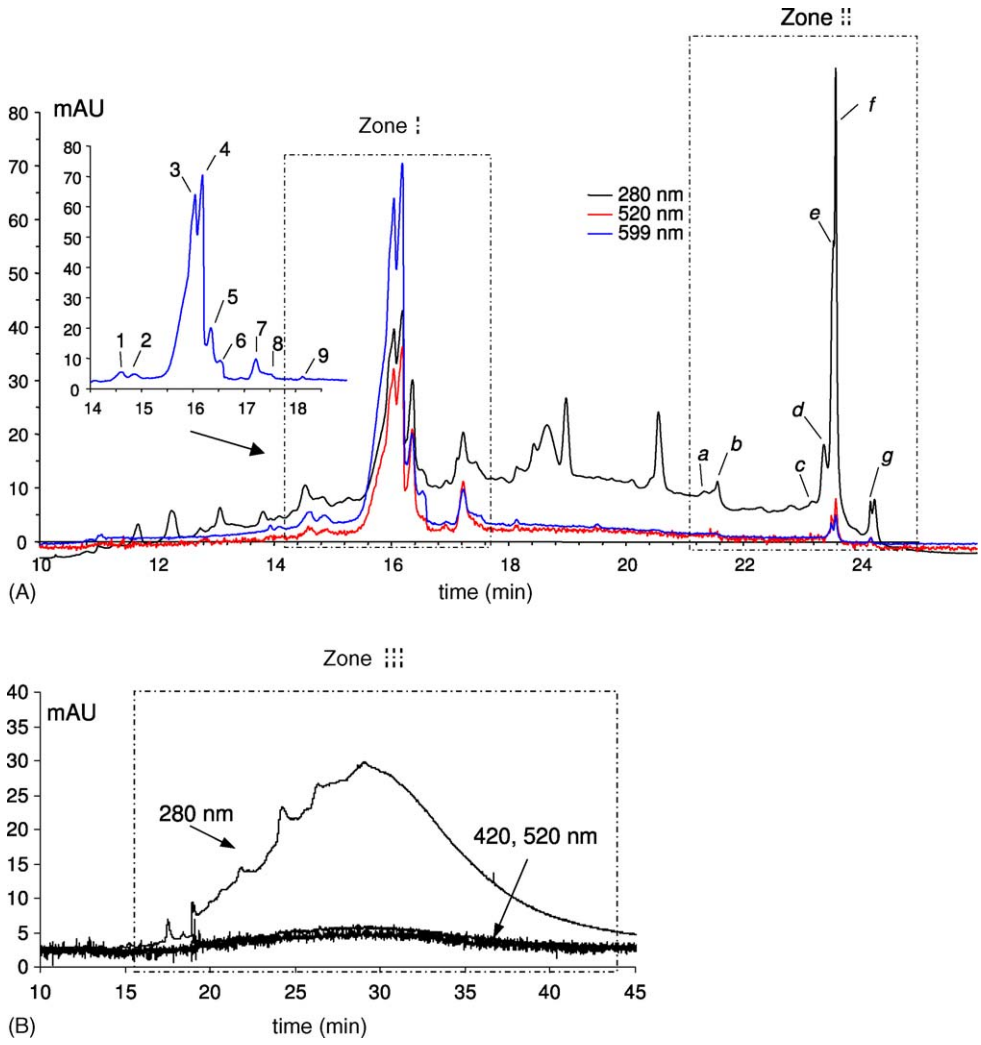

Fig. 2. Electrophoregrams of fraction F1 (A) and fraction F2 (B) from the same wine. See text for GPC and CZE conditions. Peaks: (1) malvidin-3-*O*-(6-coumaroyl)-glucoside; (2) malvidin-3-*O*-(6-acetyl)-glucoside; (3) malvidin-3-*O*-glucoside; (4) peonidin-3-*O*-glucoside; (5) malvidin-3-*O*-glucoside catechin dimer; (6) (4) malvidin-3-*O*-glucoside and pyruvic acid derivative; (7) petunidin-3-*O*-glucoside; (8) delphinidin-3-*O*-glucoside; (9) cyanidin-3-*O*-glucoside; (a–g) unidentified polymeric pigments.

contained in F1, the methods proposed, both GPC fractionation and CZE, were validated (see Section 2.10). The results obtained were also compared with those obtained from direct wine analysis in order to determine whether the fractionation step was worthwhile. Fig. 3 shows the electrophoregrams recorded at 520 and 280 nm for wine fraction F1 (Fig. 3A) and for the same wine without fractionation (Fig. 3B).

When analyzing the wines submitted to GPC fractionation, the repeatability values obtained were 2.99% for monomeric anthocyanins (zone I) and 2.68% for polymeric pigments

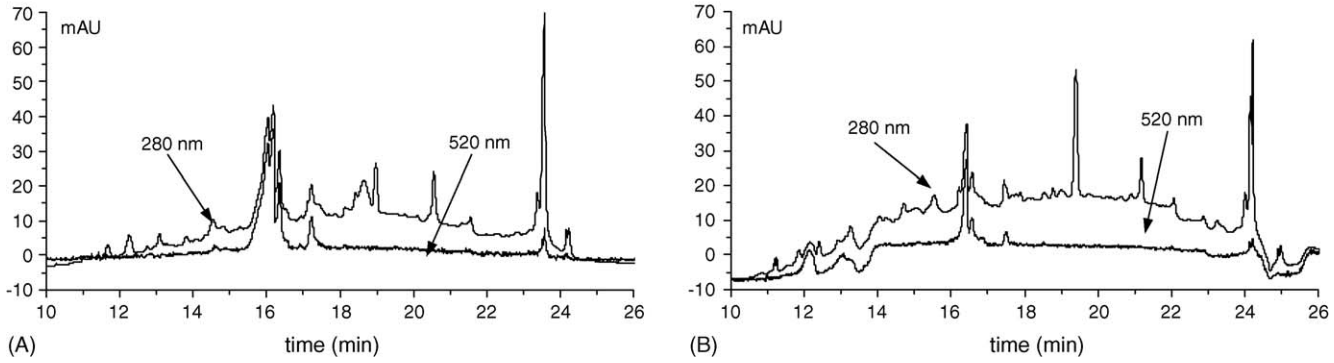

Fig. 3. Electrophoregrams for wine fraction F1 (A) and for the same wine without fractionation (B). See text for GPC and CZE conditions.

(zone II). The values of reproducibility were 2.2 ± 0.6% for monomeric anthocyanins and 2.7 ± 0.6% for polymeric pigments. These results showed a good reliability of the combined methods; however, the same parameters assessed in wine samples without previous fractionation revealed poorer analytical characteristics. In this case, the values obtained for repeatability and reproducibility were, respectively 16.2 and 14.6 ± 6.6% for monomeric anthocyanins, and 9.2 and 9.1 ± 10.8% for polymeric pigments. Additionally, the CZE response obtained was considerably higher (four to five times) in F1 fractions than in wines, and the baseline remained flatter and more stable. All these improvements could be explained by the fact that GPC fractionation eliminates interfering compounds, such as proanthocyanidins. Therefore, GPC fractionation is recommended prior to analysis by CZE.

3.4. Analysis of proanthocyanidins in fraction F2

As stated above, proanthocyanidins (polymers of flavan-3-ols) were contained in fraction F2 obtained after GPC fractionation. Several spectrophotometric methods have been developed for their quantification [21]. Of these, the vanillin reaction is an interesting procedure due to its simplicity and its specificity for flavan-3-ols, both monomers and polymers [32]. The formation of the coloured adduct is relatively slow, so the absorbance must be kinetically monitored and the value of the maximum absorbance taken as the signal value. Besides, autocondensation of vanillin in excess generates coloured compounds that cause interference. This was taken into account through subtraction of the absorption of a reference cuvette in which the sample was substituted by methanol.

When this procedure was performed directly in red wines without prior fractionation, several problems arose, giving poor values of reliability and accuracy. This was due to the interference of monomeric flavan-3-ols and the presence of large amounts of pigments absorbing at 500 nm. GPC fractionation allowed the elimination of all these interfering compounds, which remained in fraction F1. Thus, the vanillin assay was performed in fraction F2, which contains practically all the PAs.

According to Sun et al. [27], catechin can be used as a calibration standard for proanthocyanidins with a small error when the suitable concentration of acid (sulfuric acid 3.6 N) is selected. Hence, there is no need to isolate the proanthocyanidins to be used as standards, thus simplifying the analysis. Besides, the interferences of monomeric catechin itself, which is found in wine, monomeric flavan-3-ols, and wine pigments absorbing at 500 nm, are circumvented by the preliminary fractionation step. However, a blank had to be performed to eliminate the interference of the residual wine pigments present in F2, as discussed in Section 3.1.

The analytical parameters of the calibration curve are shown in Table 2. The values of the limit of detection (LD) and quantification (LQ) showed an acceptable sensitivity. A validation study of the proposed method (fractionation + vanillin reaction) was carried out as described in Section 2.10. The repeatability, reproducibility and recovery values obtained, 3.5, 1.90 ± 1.99, and 101 ± 6.4%, respectively, showed good reliability and accuracy.

Table 2
DL-Catechin standard calibration curve for vanillin assay (analytical parameters)

Equation[a]	$A = 0.002386 \times C - 0.0019$ (r^2[b] = 0.994)
SD_{slope} ($n = 7$)	0.0001
$SD_{intercept}$ ($n = 7$)	0.0079
LD (mg l^{-1})	1.66
LQ (mg l^{-1})	5.55
Repeatability[c] ($n = 6$)	2.5%
Recovery[d]	101.4 ± 0.2%

[a] A in absorbance units and C in mg l^{-1}.
[b] Linear correlation coefficient for the range 0–122 mg l^{-1} ($n = 7$).
[c] Expressed as the variation coefficient of 6 measurements of the same sample.
[d] Calculated by adding two different quantities of catechin (40 and 60 mg l^{-1}) to a previously analyzed fraction F2.

3.5. Gelatin precipitation of polymeric phenolics

Large polymeric pigments are said to precipitate in the presence of proteins like bovine serum albumin or gelatin, whereas smaller polymeric pigments remain in solution [33]. The addition of gelatin to wine samples before fractionation and further analysis by CZE of fraction F1 revealed a substantial decrease in polymeric anthocyanin content. An overall 65% decrease was measured by CZE for the seven main peaks (a–g) present in the electrophoregram (Fig. 2A). In contrast, the decrease in monomeric pigments was about five times lower (10% for malvidin-3-glucoside, according to HPLC quantification before and after gelatin precipitation). This suggested that most of the polymeric pigments present in wines were large macromolecules, confirming the findings reported in literature [33]. It also revealed a negative ionic nature in polymeric pigments, as gelatin itself is a positive-charged molecule. This supported the idea that large polymeric pigments, which should be the most important polymeric pigments in wines, are tannins containing a covalently bonded anthocyanidin moiety [33].

3.6. Analysis of astringent PAs. gelatin precipitation and vanillin assay

Through a combination of both proanthocyanidin analysis and precipitation with gelatin, a method was developed to measure the content of astringent PAs (the astringent PAs being defined by their ability for gelatin precipitation). This method permitted direct quantification of the precipitated PAs, not just an index in which the concentration of astringent PAs is only implicitly taken into account. Hence, the absolute value of astringent PA content should be a better estimate of wine astringency, if compared to the classical gelatin index.

Different types of wines from D.O.Ca.Rioja were chosen following a sensorial guidance and trying to cover a large range of astringent PA concentrations (Table 3). Wine astringency was assessed by six trained tasters and punctuated from 1 to 5. Wine 1 was a 2003 vintage young wine; Wine 2 was a 2000 vintage *Crianza* wine that was subjected to mannoprotein treatment. Wines

Table 3
Analysis of total PA concentration (TPA) and astringent PA concentration (APA) of different wine samples (comparison with the Gelatin Index)

Wine	TPA[a]	APA[a]	R[b]	GI[c]	SAP[d]
1	465 ± 15	437 ± 15	93.9	63 ± 2	3
2	238 ± 7	146 ± 14	61.1	35 ± 8	1
3	2411 ± 46	2377 ± 11	98.6	82 ± 1	5
4	428 ± 15	403 ± 19	94.4	41 ± 9	3
5	278 ± 12	271 ± 9	97.2	69 ± 8	2

[a] Expressed in mg l^{-1} (mean ± SD).
[b] Ratio APA/TPA expressed in % (mean ± SD).
[c] Gelatin Index (mean ± SD).
[d] Sensorial astringency punctuations (1–5) evaluated by six trained tasters.

3 and 4 were 2003 vintage young wines termed as "astringent" (3) and "non astringent" (4) by the tasters. Finally, Wine 5 was a very aged wine (1982 vintage), where PAs (and other compounds) had precipitated to a large extent during bottle storage.

From the results presented in Table 3, it can be observed that more than 90% of the PAs present in the wines analyzed had an "astringent" character. Wine 2 was an exception, probably due to the mannoprotein effect, which is supposed to diminish wine astringency. It was also observed that the absolute content of astringent PAs was well correlated with the sensorial astringency punctuations. However, there was a weak correlation between the content of astringent PAs and the gelatin index. This index is also based on the precipitation of PAs by gelatin. In this case, the amount of precipitated PAs is determined by the difference in tannin content before and after gelatin addition, and the index is expressed as a percentage of total tannin content. Tannin content measurement is based on the depolymerization of PAs in a hot acidic medium, and colorimetric determination of the anthocyanidins released. This assay has many drawbacks: the yield of this reaction is low due to the formation of polymeric byproducts, and depends on the structure of the PAs present in the sample [21]. For this reason, the gelatin index may be considered as a poor estimate of astringency. This index should be correlated to the ratio of astringent PAs against the total PA content. The poor correlation observed was an indication of the shortcomings of the gelatin index for measuring astringency.

To validate the method proposed, repeatability and reproducibility were assessed, being 3.4 and 4.5 ± 1.9%, respectively.

4. Conclusions

A multiple-step analytical method was developed to improve the analysis of polymeric phenolics. This method, based on the fractionation of wine phenolics by GPC on a TSK Toyopearl gel HW-50F column, was valid for analyzing different families of phenolic compounds. Monomeric flavonoids, dimeric anthocyanins and polymeric pigments were contained in fraction F1, while proanthocyanidins eluted in fraction F2. GPC fractionation enabled the elimination of interfering compounds, and thus the later analyses were substantially improved.

Monomeric flavonoids (anthocyanins, phenolic acids, flavonols and flavan-3-ols) were identified and quantified by HPLC–DAD and HPLC–MS. The methods used – both GPC fractionation and HPLC analysis – enabled high recovery of monomeric compounds in wine and yielded satisfactory repeatability and reproducibility values. The CZE technique was chosen for analyzing polymeric pigments. These compounds were properly separated in seven peaks, with longer migration times than the monomeric and dimeric anthocyanins. GPC fractionation offered a considerable improvement in response, repeatability and reproducibility in wine pigments analysis by CZE. Thus, GPC fractionation is recommended prior to analysis by CZE. GPC fractionation also improved the quantification of proanthocyanidins by the traditional vanillin assay.

Polymeric anthocyanidins were mostly precipitated with gelatin, confirming their overall negative charge. A combined method for quantifying astringent proanthocyanidins was proposed as an alternative to the general indexes available.

Acknowledgements

The authors would like to thank the University of La Rioja, INIA (Project VIN01-020) and the *Consejería de Educación del Gobierno de La Rioja* (Projects ANGI 2001/16 and ANGI 2004/10) for their financial support. Zenaida Guadalupe would like to thank the Spanish Ministry of Education and Sciences for her FPU grant.

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