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ScienceDirect
Food Chemistry 105 (2007) 248–259
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of phenolic compounds in wines: Influence of bottle storage of young red wines on their evolution

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Received 11 September 2006; received in revised form 1 November 2006; accepted 4 November 2006

Abstract

Various analytical methods based on the HPLC-DAD technique were used to determine 38 phenolic compounds in red wines. While anthocyanins and hydroxycinnamic acids were determined by direct injection of wine samples, hydroxybenzoic acids, catechins, procyanidins and flavonols required an analytical pretreatment involving liquid–liquid partitioning with ethyl ether followed by solid-phase extraction on C_{18} mini-columns. The proposed analytical methods were used to establish the phenol composition of Mencía and Brancellao, two varietal young red wines, and its influence on colour stability during storage in bottles for one year. At the end of malolactic fermentation, Mencía wine was found to contain much greater amounts of anthocyan pigments than was Brancellao wine. This resulted in a higher colour density but a weaker hue in Mencía wine than in Brancellao wine. Phenolic compounds evolved similarly in both wines during storage; changes in such compounds involved a decrease in the levels of monomeric anthocyanins, phenolic acids, epicatechin and flavonols, and an increase in those of procyanidins. The absence of a relationship between the changes in colour density and monomeric anthocyanins in both wines suggests that copigmentation and polymerization with other phenolic compounds (viz. phenolic acids, catechins and/or flavonols) prevail over degradation of the pigments.

Keywords: HPLC-DAD; Phenolic compounds; Young red wines; Bottle storage

1. Introduction

Colour is no doubt one of the most important attributes of quality red wines. Phenolic compounds in general, and anthocyanins, flavanol monomers and polymers, flavonols and phenolic acids in particular, play a major role in stabilizing colour in red wines during storage. The initial colour of red wine is due mainly to anthocyanins extracted from grape skin during crushing, pressing and fermentation. There is solid evidence that condensed pigments are formed in a gradual manner between free anthocyanins and colourless phenols present in grapes – particularly monomeric and polymeric flavanols (catechins and procyanidins), flavonols and phenolic acids – during storage and ageing of red wines. These compounds form in the presence and absence of oxygen, so the polymerization reactions occurring during wine storage in bottles are essentially anaerobic and more strongly influenced by temperature than by the dissolved oxygen concentration (Gómez Plaza, Gil Muñoz, López Roca, Martínez Cutillas, & Fernández Fernández, 2002). A number of mechanisms have been proposed to explain the formation of these pigments that involve copigmentation (Baranac, Petranovic, & Dimitric-Markovic, 1996; Baranac, Petranovic, & Dimitric-Markovic, 1997; Darias Martín et al., 2002), direct condensation between anthocyanins and flavanols, and acetaldehyde-mediated reactions between them (Dallas, Ricardo da Silva, & Laureano, 1996; Francia-Aricha, Guerra, Rivas-Gonzalo, &

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doi:10.1016/j.foodchem.2006.11.006

Santos-Buelga, 1997; Vivar-Quintana, Santos-Buelga, & Rivas-Gonzalo, 2002). These chemical changes cause colour changes from full red to orange-brown in wine during storage or ageing (Somers, 1971; Zamora, 2003).

Phenolic compounds in wines are usually identified by standard HPLC (Brú, Barroso, Cela, & Pérez Bustamante, 1996; Chilla, Guillén, Barroso, & Pérez Bustamante, 1996; Gómez Plaza, Gil-Muñoz, Lopez-Roca, & Martínez, 2000b; Gómez Plaza, Gil Muñoz, López Roca, Martínez Cutillas, & Fernández Fernández, 2001; Gómez Plaza et al., 2002; Ibern-Gómez, Andrés-Lacueva, Lamuela-Raventós, & Waterhouse, 2002; Mazza, Fukumoto, Delaquis, Girard, & Ewert, 1999; Salagoïty Auguste & Bertrand, 1984; Mayén, Mérida, & Medina, 1995; Pozo-Bayón, Martín-Álvarez, Hernández, & Polo, 2003; Revilla & González San José, 2003; Zafrilla et al., 2003; Rodriguez Delgado, González Hernández, Conde González, & Pérez Trujillo, 2002). By virtue of their high concentrations in red wines, anthocyanins can be determined simply by direct injection of samples into an HPLC-DAD instrument (Berente, De la Calle García, Reichenbächer, & Danzar, 2000; Gambelli, 2004; Zafrilla et al., 2003). On the other hand, phenolic acids, catechins, procyanidins and flavonols usually require a concentration and fractionation pretreatment. One of the most frequently used techniques for this purpose is liquid–liquid extraction with ethyl ether or ethyl acetate at pH 7 or 2, which allows polyphenols to be split into two groups, namely: phenolic acids and neutral phenolic compounds (catechins, procyanidins and flavonols) (Brú et al., 1996; Fernández de Simón, Pérez Izarbe, Hernández, Gómez Cordovés, & Estrella, 1990; Salagoïty Auguste & Bertrand, 1984). One more recent alternative is the use of solid phase extraction with various types of sorbents (e.g. C_{18}, polyamide, polystyrene-divinyl benzene) and subsequent fractionation by elution with appropriate solvents at a variable pH (Chilla et al., 1996; Oszmianski, Ramos, & Bourzeix, 1988; Ricardo da Silva, Rosec, Bourzeix, & Heredia, 1990).

The purpose of this study was to assess various existing methods for the fractionation of polyphenols in order to select the best choice. The proposed method involves liquid–liquid partitioning with ethyl ether and subsequent solid-phase extraction on C_{18} mini-columns for analysis by HPLC-DAD. Once optimized and validated, the method was used to determine the evolution of the polyphenolic composition of two monovarietal young red wines during storage in bottles for one year, and its influence on colour stability.

2. Materials and methods

2.1. Grape cultivars and vinification protocol

Grapes of the Mencía and Brancellao (*Vitis vinifera* L.) varieties grown in a test plot in the Viticultural and Enological Station of Galicia (EVEGA, Ourense, Spain) of the certification of origin Ribeiro were harvested at commercial maturity. The damaged grape clusters (broken or with visual microbial alterations) were separated in order to eliminate undesirable contamination and degradation compounds. Groups of intact clusters were processed. Following destalking and crushing, the grapes were placed in amounts of 15–45 kg in glass containers and supplied with 0.07 g kg^{-1} potassium metabisulphite, 30 g h L^{-1} commercial yeast (*Saccharomyces cerevisiae*) and 3 g h L^{-1} pectolytic enzymes (vinozym vintage). Alcoholic fermentation was conducted at temperatures below 25 °C for 10 days, after which the wine was strained off, grapes were pressed and the wine-must mixture transferred to other glass containers in order to allow alcoholic fermentation to complete and spontaneous malolactic fermentation to occur. At the end of malolactic fermentation (viz. in November–January 2005), the wine was transferred several times, supplied with 25 mg L^{-1} sulphite and treated with a 10 g h L^{-1} concentration of metatartaric acid. Following filtration and clearing with gum arabic (10 cm^2 L^{-1}), the wine was finally bottled (January 2005) and stored at 15 °C for 1 year. Both wines (Mencía and Brancellao) were analysed following the procedures described below at the end of malolactic fermentation, and also 3 and 12 months after bottling.

2.2. Chemicals and materials

Standards of anthocyanins (malvidin 3-*O*-glucoside, peonidin 3-*O*-glucoside chloride and cyanidin 3-*O*-glucoside chloride) were purchased from Extrasynthese; phenolic acids (gallic, protocatechuic, salicylic, *trans*-caffeic, *trans*-ferulic and *trans*-*p*-coumaric acids), catechins [(+)-catechin and (−)-epicatechin], flavonols (quercetin) and procyanidin B2 were obtained from Sigma–Aldrich; and procyanidins (B1, B3, B4 and trimer C1) were kindly supplied by Santos-Buelga's research group (University of Salamanca, Spain), what made possible their identification in the samples and the estimation of their recovery by spiking.

Methanol was HPLC grade and all other solvents (viz. ethanol, formic acid, hydrochloric acid, diethyl ether and acetone) were analytical grade; all were purchased from Merck. Purified water was obtained from a Milli-Q water purification system (Millipore). Tartaric acid (Riedel-de Haën) and NaOH (Panreac) were used to prepare synthetic wine and NaCl (also from Panreac) was used in the extraction procedure. Waters Sep-Pak C18 Plus (360 mg) cartridges were used as solid-phase extraction (SPE) minicolumns for purification and concentration. Cartridges were activated by passing through methanol (5 mL) followed by synthetic wine (10 mL; see below for preparation).

2.3. Preparation of solutions

2.3.1. Synthetic wine

The synthetic wine solution (1 L) was prepared from 120 mL of ethanol, 2.5 g of L-(1)-tartaric acid and water to the mark, and adjusted to pH 3.2 with 2 M NaOH.

2.3.2. Standard solutions for quantifying phenolic compounds

Stock solutions of malvidin 3-*O*-glucoside chloride (500 mg L^{-1}), gallic acid (500 mg L^{-1}), protocatechuic acid (500 mg L^{-1}), *p*-hydroxybenzoic acid (500 mg L^{-1}), vanillic acid (500 mg L^{-1}), syringic acid (500 mg L^{-1}), *trans*-caffeic acid (500 mg L^{-1}), *trans*-ferulic acid (500 mg L^{-1}), *trans*-*p*-coumaric acid (500 mg L^{-1}), (+)-catechin (2500 mg L^{-1}), (−)-epicatechin (2500 mg L^{-1}), procyanidin B2 (200 mg L^{-1}) and quercetin (100 mg L^{-1}) were prepared by dissolving the compounds separately in methanol. These solutions were used to make working-strength solutions in synthetic wine following evaporation of the methanol (see concentration ranges in Table 2) and stored in amber flasks at 4 °C, where they remained stable for at least 1 month. The concentrations of anthocyanins, procyanidins and flavonols in the wines were referred to those of malvidin 3-*O*-glucoside chloride, procyanidin B2 and quercetin, respectively.

2.3.3. Standard solutions for identifying phenolic compounds

Stock solutions of peonidin 3-*O*-glucoside chloride, cyanidin 3-*O*-glucoside chloride, procyanidins B1, B3 and B4, and trimer C1 were prepared by dissolving the individual compounds in methanol (procyanidins solutions were also used for recovery estimation purposes). Each standard was used to make working-strength solutions in synthetic wine following evaporation of methanol. *Cis*-*p*-coumaric and *cis*-caffeic acids were obtained from the *trans*-*p*-coumaric and *trans*-caffeic acids standard by exposure to UV light (340 nm) for 2 h. None of these solutions were used for quantitation purposes.

2.4. Determination of phenolic compounds

2.4.1. Chromatographic conditions

HPLC measurements were made by using a Thermo Separation Products (TSP) P2000 binary pump equipped with a TSP AS1000 autosampler, a TSP SCM1000 vacuum membrane degasser and a UV6000LP DAD detector. Chromatographic data were acquired and processed with Chrom-Quest software. Table 1 lists the optimum instrumental parameter values for the chromatographic determination of anthocyanins, phenolic acids, catechins, procyanidins and flavonols.

2.4.2. Determination of anthocyanins and hydroxycinnamic acids

These compounds were determined by direct transfer of untreated samples to HPLC vials for subsequent injection into the instrument. Sample filtration with syringe filters (0.45 μm; cellulose acetate or nylon membranes) or centrifugation (3000 rpm for 10 min) was abandoned since there were significant losses of the anthocyanins (losses ranging from about 30–40 to 60–70% were visible since anthocyanins are colored components). Losses could be explained by sorption of anthocyanins to the filter materials during filtration and to wine colloids during their separation by centrifugation. A guard column was efficient in protecting the analytical column from contamination build-up for at least one hundred analyses.

Table 1
Chromatographic conditions for the determination of phenolic compounds in wines

Chromatographic conditions	
Injected volume	50 μL
Guard column	Pelliguard LC-18 (5 × 4.6 mm i.d., 40 μm) (Supelco)
Analytical column	*Anthocyanins* Waters symmetry C18 (150 × 4.6 mm i.d., 5 μm) (Waters) *Phenolic acids, catechins, procyanidins and flavonol* ODS Hypersyl (250 × 4.6 mm i.d., 5 μm) (Thermo Separation)
Mobile phase	*Anthocyanins* A (formic acid in water, 5% v/v): B (methanol) 0–5 min: 90:10 (elution step) 15–20 min: 80:20 (elution step) 30–32 min: 50:50 (elution step) 33–40 min: 10:90 (flushing step) 41–47 min: 90:10 (conditioning step) *Phenolic acids, catechins and flavonols* A (formic acid in water, 2.5% v/v): B (methanol) 0–25 min: 100:0 (elution step) 60–75 min: 80:20 (elution step) 75–110 min: 40:60 (elution step) 111–116 min: 0:100 (flushing step) 117–127 min: 100:0 (conditioning step) *Procyanidins* A (formic acid in water, 2.5 % v/v): B (methanol) 0 min: 100:0 (elution step) 45–60 min: 91:9 (elution step) 61–66 min: 0:100 (washing step) 67–77 min: 100:0 (conditioning step)
Flow rate	1 mL min^{-1}
Temperature	35 °C
Detection conditions	
Scanning	200–600 nm
Detection wavelength	Anthocyanins: 520 nm Phenolic acids: 280 nm or 320 nm Catechins: 280 nm Procyanidins: 280 nm Flavonols: 360 nm
Scan rate	1 Hz
Step	2 nm
Bandwidth	3 nm

2.4.3. Determination of hydroxybenzoic acids, catechins, procyanidins and flavonols

The determination of these compounds entailed their fractionation into phenolic acids, catechins and flavonols (fraction 1) (Fig. 1) on the one hand and procyanidins (fraction 2) (Fig. 1) on the other. All analyses were carried out in triplicate.

Fraction 1 was obtained as follows: a volume of 5 mL of wine previously acidified to pH 1 by adding two drops of 98% formic acid was supplied with 2 g of NaCl, 5 mL of distilled water and 8 mL of diethyl ether, the mixture being

liquid–liquid extracted under magnetic stirring for 15 min. Separation of the ether layer was facilitated by centrifugation at 2500 rpm for 1 min. This extraction procedure was repeated twice and provided about 16 mL of ether extract that was evaporated to dryness on a rotary evaporator, the residue being dissolved in 1 mL of synthetic wine and poured into an HPLC vial for analysis.

Fraction 2 was obtained as follows: the previous diluted residual wine (*ca.* 10 mL), which contained no phenolic acids, catechins or flavonols, was passed through a pre-activated C_{18} mini-column which was then flushed with 10 mL of distilled water and dried with a nitrogen stream. Retained procyanidins were eluted by passing 8 mL of acetone, which was evaporated on a rotary evaporator, the extract being dissolved in 0.5 mL of synthetic wine and poured into an HPLC vial for subsequent analysis.

2.4.4. Identification and quantitation

Phenolic compounds in wine were identified from their UV-Vis spectra and, wherever possible, by chromatographic

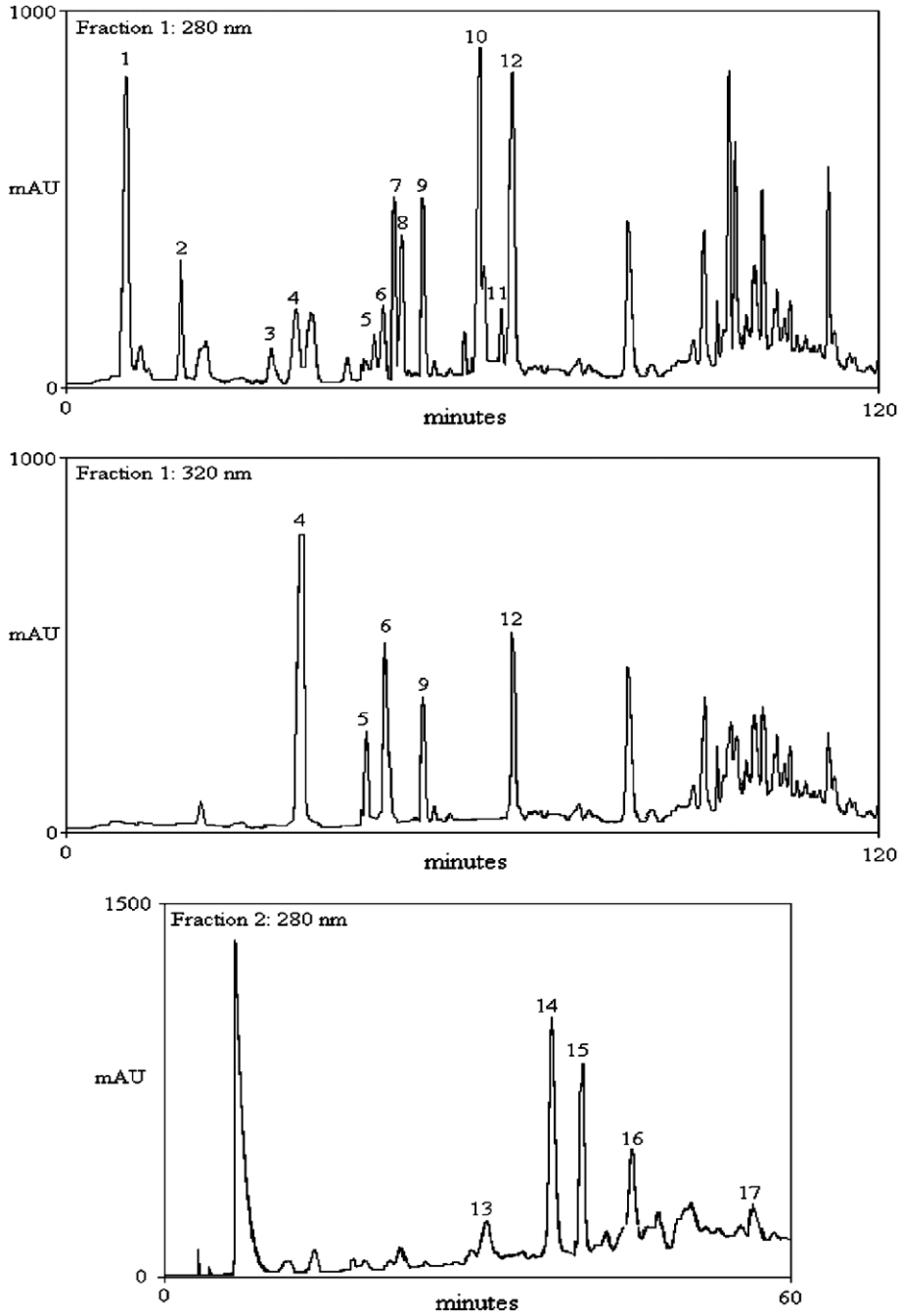

Fig. 1. Chromatograms of the fraction 1 (phenolic acids and catechins at 280 and 320 nm) and fraction 2 (procyanidins at 280 nm) of Mencia wine. Peaks identification: (1) gallic acid, (2) protocatechuic acid, (3) *p*-hydroxybenzoic acid, (4) *trans*-caftaric acid, (5) *cis*-coutaric acid, (6) *trans*-coutaric acid, (7) vanillic acid, (8) (+)-Catechin, (9) *trans*-caffeic acid, (10) syringic acid, (11) (−)-epicatechin, (12) *trans p*- coumaric acid, (13) procyanidin B3, (14) procyanidin B1, (15) trimer, (16) procyanidin B4 and (17) procyanidin B2.

comparison with authentic markers. Individual anthocyanins, flavonols, procyanidins, caffeic acid derivatives (viz. *cis*- and *trans*-caftaric acid) and coumaric acids (*cis*- and *trans*-coutaric acid) were quantified as malvidin 3-*O*-glucoside chloride, quercetin, procyanidin B2, caffeic acid and coumaric acid, respectively, all in mg L^{-1}.

2.4.5. Colour-related parameters

Colour density was determined from absorbance measurements at 420, 520 and 620 nm made in cells of 1 mm light path (Glories, 1984) on a Hitachi 2000 spectrophotometer. The absorbances were then multiplied by 10 to be referred a 1 cm path lengths and then added. Hue was quantified as the ratio between the absorbances at 420 and 520 nm (Glories, 1984).

3. Results and discussion

3.1. Performance of the phenol extraction procedure

All anthocyanins and hydroxycinnamic acids detected in the wines were found to be present at high enough concentrations to allow their determination by direct injection of samples. In addition, these compounds absorb at quite selective wavelengths and are thus scarcely prone to interference. All other phenolic compounds required purification, concentration and fractionation prior to their determination.

The feasibility of using solid-phase extraction (SPE) for the intended purpose was initially explored by using C_{18} mini-columns and procedures similar to others previously reported (Oszmianski et al., 1988). Tests were performed on a synthetic wine sample overloaded with phenolic acids, catechins, procyanidins and flavonols (see Table 2). Following adjustment of the pH to 7 with NaOH, the sample was passed through a mini-column that was previously activated with 5 mL of methanol and 10 mL of synthetic wine at pH 7. This pH allowed the neutral polyphenol fraction (viz. catechins, procyanidins and flavonols) to be retained by the sorbent, and phenolic acids to be eluted. The extract containing such acids was acidified to pH 2 with HCl and passed through another C_{18} mini-column pre-activated in the same manner as the previous one. Each analyte thus retained was subsequently eluted at an appropriate pH. The results obtained exposed the following restrictions:

(a) Recoveries were acceptable (85–95%) for all phenolic acids except those of low molecular weights (particularly gallic and protocatechuic acids). In fact, the latter two acids were not fully retained by the mini-column and provided very low recoveries (25% and 60%, respectively) as a result.
(b) Catechins and procyanidins were collected in the same eluate. The structural similarity of these two compound families precluded their chromatographic resolution as it resulted in overlap between some peaks for procyanidin B1 and catechin on the one hand, and some for procyanidin B2 and epicatechin on the other.

These shortcomings of SPE led us to explore liquid–liquid partitioning, using procedures similar to others previously reported (Brú et al., 1996). Most such procedures initially extract wine samples at pH 7 in order to separate the neutral fraction of polyphenols and then lower the pH to 1 to extract phenolic acids. In this work, however, we chose to operate in the opposite manner, i.e. we initially extracted the wine samples at pH 1. To this end, a volume of 5 mL of synthetic wine overloaded with all phenolic compounds was adjusted to pH 1 with formic acid for extraction in duplicate with 8 mL fractions of diethyl ether; this extracted both phenolic acids, catechins and flavonols into the ether layer and left procyanidins alone in the wine. This fraction scheme is compatible with the chromatographic methods used to resolve the analytes. Recoveries were quite acceptable (87–92%) for all phenolic acids except gallic, which was recovered by only 53%. Flavonols were extracted by 90% and catechins by only 50%. In order to improve these results, we examined the influence of ionic strength on the analyte recovery as previously done by some authors (Brú et al., 1996). Following saturation with NaCl, a sample of synthetic wine was extracted with diethyl ether using the above-described procedure. The recoveries thus obtained were much better for all compounds (particularly gallic acid and catechins, which were recovered by about 80%).

Finally, the extraction of procyanidins, which remained in the wine after phenolic acids, catechins and flavonols were removed, was addressed by using liquid–liquid partitioning with an organic solvent (ethyl acetate) and solid-phase extraction through a sep pak C_{18} column. The extraction with ethyl acetate required neutralizing the samples to pH 7 with NaOH. The recoveries obtained under these conditions ranged from 15% to 20% and, as with phenolic acids and catechins, increased to 20–50% when the ionic strength of the solution was raised by addition of NaCl. Even so, this procedure was discarded as it resulted in too high relative standard deviations. For SPE, samples were passed untreated (i.e. with no pH adjustment) through an activated C_{18} mini-column. This allowed procyanidins to be quantitatively retained on the sorbent. The most suitable solvent for their elution was chosen from among methanol, acetonitrile, tetrahydrofuran and acetone; a volume of 10 mL of the last was found to provide the highest recoveries (50–70%). Therefore, the pre-analytical procedure adopted involved extraction of phenolic acids, catechins and flavonols in a single fraction by liquid–liquid partitioning with diethyl ether and subsequent separation of procyanidins by SPE on a C_{18} mini-column.

3.2. Validation of the proposed methods

Anthocyanins and hydroxycinnamic acids required no estimation of their recoveries as samples were analysed

Table 2
Validation parameters for the determination of phenolic compounds in wines

	LOQ (mg/L wine)	Linearity		Added/wine concentration (mg/L)	Recovery (%) ($n = 4$)	RSD (%) ($n = 4$)
		Concentration range (mg/L sw)	r^2			
Phenolic acids determined following concentration (Concentration factor = 5)						
Gallic	0.4	2–60	0.999	5	79	3
Protocatechuic	1	5–100	0.998	5	91	5
p-Hydroxybenzoic	0.2	1–20	0.999	5	90	5
Vanillic	1	5–40	0.999	5	83	7
Syringic	0.4	2–60	0.998	5	95	3
Ferulic	0.2	1–40	0.998	5	91	7
Phenolic acids determined by direct injection (100% recovery)						
trans-Caffeic	1	1–40	0.997	7	100	2
cis-Caftaric[a]	1	–	–	1.5	100	5
trans-Caftaric[a]	1	–	–	30	100	2
trans-*p*-Coumaric	1	1–40	0.996	7	100	1.5
cis-Coutaric[b]	1	–	–	1.5	100	1.5
trans-Coutaric[b]				4	100	2
Catechins determined following concentration (Concentration factor = 5)						
(−)-Epicatechin	1	5–100	0.997	10	77	5
(+)-Catechin	1	5–100	0.997	10	82	2
Procyanidins determined following concentration (Concentration factor = 10)						
Procyanidin B1[c]	1	–	–	2	72	2
Procyanidin B2	1	1–200	0.999	2	61	3
Procyanidin B3[c]	1	–	–	2	72	2
Procyanidin B4[c]	1	–	–	2	70	6
Trimer C1[c]	1	–	–	2	51	3
Flavonols determined following concentration (Concentration factor = 5)						
Quercetin	0.2	1–60	0.999	5	92	1
Anthocyanins determined by direct injection (100% recovery)						
Delfinidin 3-glucoside[d]	0.5			1	100	8
Cyanidin 3-glucoside[d]	0.5			1	100	8
Petunidin 3-glucoside[d]	0.5			2	100	9
Peonidin 3-glucoside[d]	0.5			2	100	6
Malvidin 3-glucoside	0.5	1–150	0.999	24	100	2
Peonidin 3-(6-acetyl glucoside)[d]	0.5			2	100	1
Malvidin 3-(6-acetyl glucoside)[d]	0.5			2	100	2
Malvidin 3-(6-coumaryl glucoside)[d]	0.5			2	100	4

sw: synthetic wine, *LOQ:* limits of quantitation (limits of detection are three times lower), *RSD:* relative standard deviation.
[a] Result expressed as caffeic acid.
[b] Result expressed as coumaric acid.
[c] Result expressed as procyanidin B2.
[d] Result expressed as malvidin 3-glucoside.

directly (untreated). The precision of the method was thus determined by applying the same procedure to four identical samples of Mencía wine. The results are shown in Table 2.

The recoveries of all other phenolic compounds were determined by fractionating four samples of synthetic wine overloaded with phenolic acids, catechins, procyanidins and flavanols at the concentration levels listed in Table 2, which also shows the results thus obtained. It was not necessary to concentrate the red wines analysed to measure the levels of caffeic and coumaric acids; therefore no estimation of their recoveries is required as samples were analysed directly. Anyway, recoveries of caffeic and coumaric acids were established by overload of synthetic wine ($85 \pm 4\%$ for concentration factors of 5 and 10) just in case concentration might be necessary in other wine samples.

Limits of detection (LOD) and quantitation (LOQ) were determined from the amount of noise obtained in the analysis of synthetic wine ($n = 7$). LOD and LOQ were taken to be the concentrations of individual analytes resulting in a signal-to-noise ratio of 3 and 10 (ACS, 1980), respectively, and were also experimentally determined by spiking synthetic wine at the analyte levels shown in Table 2.

Phenolic compounds were quantified by using external standards and regressing the peak areas for the different analytes against their concentrations. Flavonol, procyanidin and anthocyanin concentrations were expressed in mg L^{-1} quercetin, procyanidin B2 and malvidin 3-*O*-glucoside, respectively. Hence, Table 2 only shows the linearity parameters for these compounds.

3.3. Phenolic compounds in the studied wines

The proposed method was used to analyse phenolic compounds (viz. anthocyanins, phenolic acids, catechins, procyanidins and flavonols) in the two Galician red wine varieties studied: Mencía and Brancellao (Table 3, Figs. 2 and 3). Samples were analysed immediately upon completion of malolactic fermentation, and also after 3 and 12 months of storage in bottles.

3.3.1. Phenolic acids

A total of five hydroxybenzoic acids and nine hydroxycinnamic acids were found at detectable levels in all wine samples (Table 3).

At the end of malolactic fermentation, Brancellao wine exhibited higher total phenolic acid contents than did the Mencía wine (viz. 201 mg L^{-1} vs 108 mg L^{-1}). The difference was due to the large contribution of hydroxycinnamic acids and, especially, their esters, in Brancellao wine; on the other hand, the levels of hydroxybenzoic acids were more similar in the two types of wine.

Gallic acid was the most abundant benzoic acid in both wine varieties, and accounted for 35–36% of all benzoates. Substantial amounts of vanillic and syringic acid were also found in both types of wine. Also, protocatechuic acid and, especially, hydroxybenzoic acid, were the two phenolic compounds found at the lowest levels in both wines.

Regarding hydroxycinnamates, *trans*-caftaric acid was by far the principal compound in both wines; in fact, it accounted for 66% of all hydroxycinnamates in Mencía wine and 65% in Brancellao wine. *Trans*-coutaric acid was also prominent in both. On the other hand, the *cis* forms of caftaric and coutaric acids accounted for less than 10% of all hydroxycinnamates in most wine samples. By contrast, neither ferulic acid nor the *cis* forms of caffeic and coumaric acids were found at detectable levels in any wine sample.

The total concentrations of hydroxybenzoic and hydroxycinnamic acids found in this work are higher than those previously determined by Mayén et al. (1995) at the end of malolactic fermentation in Cabernet Sauvignon wines (viz. 23 mg L^{-1} for hydroxybenzoic acids and 21 mg mL^{-1} for hydroxycinnamic acids) and much higher than those found by the same authors in Tempranillo wines (viz. <1 mg L^{-1} for both hydroxybenzoic and hydroxycinnamic acids). Mazza et al. (1999) also found differences – albeit not so marked – in tartaric ester contents in freshly fermented wines of the Cabernet Franc (201 mg L^{-1}), Merlot (162 mg L^{-1}) and Pinot Noir (117 mg L^{-1}) varieties. Arnous, Makris, and Kefalas (2001) reported levels of hydroxybenzoic acids (average content as gallic acid 340 mg L^{-1}) and hydroxycinnamic acids (average content as caffeic acid 500 mg L^{-1}) in Greek wines that were much higher than those in our wines; however, the relative proportion of each individual acid was very similar to that found in this work. As in our study, Zafrilla et al. (2003) found *trans*-caftaric and *trans*-coutaric acids to be the major hydroxycinnamic acids in Monastrell red wines, their levels in freshly fermented wines being in the region of 31 and 15 mg L^{-1}, respectively, as chlorogenic acid. Gómez-Plaza et al. (2000a) also studied freshly bottled Monastrell wines and found *trans*-caftaric and *trans*-coutaric acid levels over the ranges 171–187 and 117–124 mg L^{-1}, respectively. Unlike us, Revilla and González San José (2003) found large amounts of ferulic acid in young wines made from Tinto Fino grapes.

As previously found by other authors (Gómez-Plaza et al., 2000a; Gómez Plaza et al., 2001; Revilla and González San José, 2003), the total concentration of phenolic acids decreased during storage of the wines in bottles, particularly after the third month. Thus, the benzoic acid levels decreased by 16% in Mencía wine and 25% in Brancellao wine within one year of bottled storage. Hydroxycinnamic acid levels fell even more markedly: by 20% in Mencía wine and 34% in Brancellao wine (Figs. 2 and 3). The decrease was largely the result of those in the contents of *trans*-coutaric acid and, especially, *trans*-caftaric acid, in favour of caffeic and coumaric acids (Fig. 3). This phenomenon, which was previously observed by other authors (Wightman, Price, Watson, & Wrolstad, 1997; Zafrilla et al., 2003), has been ascribed to the presence of enzymes with estearase activity which can hydrolyse the tartaric esters of caftaric and coutaric acids to their free forms. The increased coumaric acid levels may also be associated to degradation of coumaryl anthocyanins during storage of the wines (see Table 3).

In addition, the decreased levels of phenolic acids (particularly those of caftaric acids) may have resulted from the formation of copolymers with anthocyanins (Darias Martín et al., 2002), the copolymers helping stabilize the colour of red wine during storage.

3.3.2. Anthocyanins

All wine samples were found to contain seven anthocyanins at the end of malolactic fermentation (Table 3). The lack of commercially available standards for determining the acyl derivatives entailed the use of the 290–340 nm spectral region, which allows one to confirm whether the acid esterifying the glucose molecule is of the aliphatic or aromatic type.

Based on the results, the total concentration of free anthocyans was higher in Mencía wine than in Brancellao wine. This is consistent with previous spectrophotometric results of Pérez Lamela, García Falcón, Simal Gándara, and Orriols Fernández (2007) for both types of wine. In both, malvidin 3-*O*-glucose was the major pigment,

Table 3
Mean concentrations (mg L^{-1}) of phenolic compounds in the selected wines ($n = 3$), together with their color parameters, along a year after the end of malolactic fermentation (EMF)

	Mencía			*Brancellao*		
	EMF	3 months	1 year	EFM	3 months	1 year
Hydroxybenzoic acids (RSD < 6%)						
Gallic	14	17	10	13	11	7
Protocatechuic	7	7	5	6	5	4
p-Hydroxybenzoic	2	2	1	1	1	1
Vanillic	5	6	6	10	10	10
Syringic	10	10	10	7	8	6
Total	38	42	32	37	35	28
Hydroxycinnamic acids (RSD < 2%)						
cis-Caftaric[a]	2	2	nq	3	3	nq
trans-Caftaric[a]	46	40	27	106	70	48
cis-Coutaric[b]	2	2	1	12	11	10
trans-Coutaric[b]	10	7	4	32	25	22
cis-Caffeic	nd	nd	nq	nd	nd	nq
trans-Caffeic	5	7	11	9	8	15
cis-p-Coumaric	nd	nd	nd	nd	nd	nd
trans-p-Coumaric	5	5	12	2	2	9
trans-Ferulic	nq	nq	nq	nd	nd	nd
Total	70	63	55	164	119	104
Catechins (RSD < 8%)						
(+)-Catechin	19	21*	37*	28	31*	31*
(−)-Epicatechin	5	4	2	20	19	16
Total	24	25	39	48	50	47
Procyanidins (RSD < 8%)						
Trimer[c]	9	9	14	20	19	11
Procyanidin B1[c]	20	21	36	21	22	42
Procyanidin B4[c]	2	2	3	7	8	15
Procyanidin B2	nq	nq	nq	nq	nq	nq
Total	31	32	53	48	49	68
Flavonols (RSD < 7%)						
Glycosylated flavonol?[d]	4	2	nq	nq	nq	nq
Glycosylated flavonol?[d]	3	2	nq	3	2	1
Glycosylated flavonol?[d]	6	1	nq	nq	nq	nq
Glycosylated flavonol?[d]	4	3	2	1	1	0.5
myricetin?[d]	nq	nq	5	nq	nq	1
kaempferol?[d]	nq	nq	3	nq	nq	nq
Quercetin	nq	nq	2	nq	nq	3
Total	17	8	12	4	3	5.5
Anthocyanins (RSD < 4%)						
Delfinidin 3-glucoside[e]	7	5	2	nq	nq	nq
Cyanidin 3-glucoside[e]	nq	nq	nq	nq	nq	nq
Petunidin 3-glucoside[e]	13	8	3	2	2	nq
Peonidin 3-glucoside[e]	10	6	2	10	6	nq
Malvidin 3-glucoside	126	79	33	48	40	5
Peonidin 3-(6-acetyl glucoside)[e]	3	2	4*	2	2	1
Malvidin 3-(6-acetyl glucoside)[e]	22	15	4	4	3	nq
Malvidin 3-(6-coumaryl glucoside)[e]	24	14	3	1	0.8	nq
Polymeric anthocyanin?[e]	nq	nq	3	nq	nq	2
Polymeric anthocyanin?[e]	nq	nq	4	nq	nq	2
Total	205	129	57	66	53	10
Colour density	12.6	11.5	11.2	5.0	5.0	5.2
Hue	67	82	89	101	102	120

nd: non detectable; nq: not quantified.
[a] Result expressed as caffeic acid.
[b] Result expressed as coumaric acid.
[c] Result expressed as procyanidin B2.
[d] Result expressed as quercetin.
[e] Result expressed as malvidin 3-glucoside.
* Coeluted with another compound.

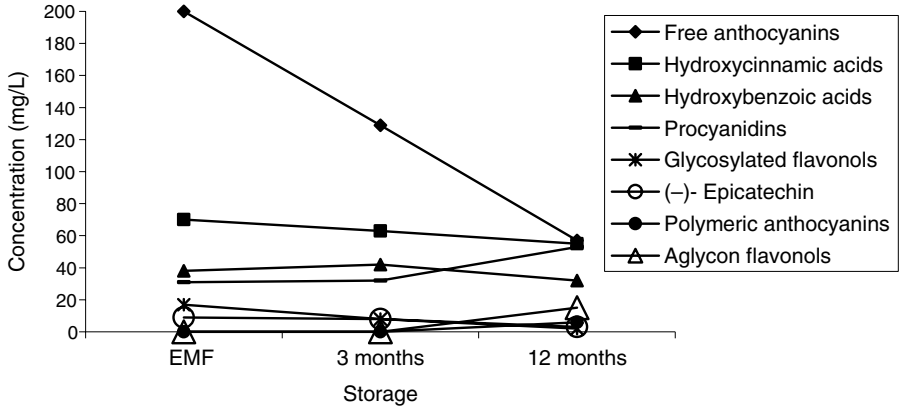

Fig. 2. Evolution of total polyphenol levels during bottle storage of Mencia wine along a year after the end of malolactic fermentation (EMF).

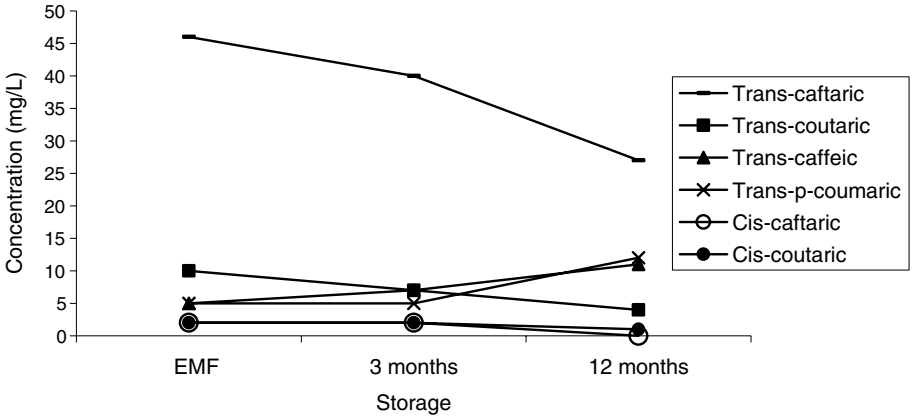

Fig. 3. Evolution of hydroxycinnamic acid levels during bottle storage of Mencia wine along a year after the end of malolactic fermentation (EMF).

followed by its acyl derivatives in Mencía wine and by peonidin 3-*O*-glucoside in Brancellao wine. All other anthocyanins were present in contents below 10%.

In addition to grape variety, anthocyan concentrations in freshly fermented wine depend on the particular vinification technique used (specifically, on factors such as the maceration temperature and time or whether any clearing agents are used, for example). In this respect, the anthocyan levels found in Mencía wine were similar to those previously reported by other authors for Monastrell (Gómez-Plaza, Gil-Muñoz, López-Roca, De la Hera-Orts, & Martínez Cultillas, 2000a; Gómez-Plaza et al., 2000b; Gómez Plaza et al., 2001; Zafrilla et al., 2003), Tinto Fino (Revilla & González San José, 2001) and Pinot Noir wines (Mazza et al., 1999), albeit lower than those found by Mazza et al. (1999) in Merlot and Cabernet Franc wines or Castillo-Sánchez, Mejuto, Garrido, and García Falcón (2006) in Vinhao wines. Brancellao grapes give wines with very low levels of pigments relative to the previous varieties.

The total content in monomeric anthocyanins decreased by 72% in Mencía wine and 85% in Brancellao wine over the 12 months of bottled storage. This may have been a result of degradation or combination with other compounds to give more stable polymeric pigments. Specifically, the degradation of the anthocyanin malvidin 3-(6-coumaryl glucoside) is known to raise the levels of coumaric acid during storage of wine. Even though our wines may have experienced some degradation of monomeric anthocyanins, this was not accompanied by a decrease in wine colour density (Table 3), so the synthesis of new polymers was the more likely origin of the decrease in anthocyanin levels. According to Gao, Girard, Mazza, and Reynolds (1997) and Mazza et al. (1999), polymeric anthocyanins are strongly hydrophobic, so they exhibit long retention times – in the region of acylated anthocyanins. After 12 months of storage, both types of wine were found to contain two new compounds exhibiting a typical anthocyan profile that eluted after acylated anthocyanins. Also, peonidin 3-(6-acetyl glucoside) in Mencía wine after 12 months of storage was found to coelute with another compound. The three new peaks were probably due to polymeric anthocyanins (Fig. 2).

3.3.3. Flavan-3-ol monomers and polymers

Flavan-3-ol derivatives were classified into two groups according to their degree of polymerization, namely: monomers (catechin and epicatechin) and polymers [dimers

(procyanidins B1, B2 and B4) and trimers (C1)]. In addition, both wines were found to contain other, minor compounds with a typical flavan-3-ol profile that could not be identified but were probably galloyl derivatives and tetramers, among others (Revilla & González San José, 2003). Brancellao wine exhibited higher concentrations of flavan-3-ol monomers (particularly catechin) than did Mencía wine. Also, as previously found by some authors (Gómez-Plaza et al., 2000a, 2000b; Gómez Plaza et al., 2001, 2002; Mayén et al., 1995; Rodriguez Delgado et al., 2002) in various wine varieties, catechin levels exceed epicatechin levels in both Mencía and Brancellao wine. The concentrations found in this work were similar to those reported by Gómez-Plaza et al. (2000a, 2000b), Gómez Plaza et al. (2001, 2002) in Monastrell, Mayén et al. (1995) in Cabernet Sauvignon and Revilla and González San José (2003) in Tinto Fino wines.

As regards polymers, procyanidin B1 was the major compound in both wine varieties, followed by trimer C1 and procyanidin B4. On the other hand, dimer B2 was found at detectable levels in neither wine. The total levels of these compounds were slightly higher in Brancellao wine than in Mencía wine. Even so, the combined contribution of unidentified compounds with a procyanidin profile was greater in the latter wine. The levels found in this work were similar to those reported by Gómez Plaza et al. (2002), Gómez-Plaza et al. (2000a, 2000b), Gómez Plaza et al. (2001) for Monastrell wine, Mayén et al. (1995) for Cabernet Sauvignon wine and Revilla and González San José (2003) for Tinto Fino wine.

As previously found by other authors (Gómez Plaza et al., 2002; Mayén et al., 1995; Gómez-Plaza et al., 2000a, 2000b; Gómez Plaza et al., 2001; Revilla & González San José, 2003), the epicatechin levels in our wines decreased slightly during storage (Fig. 2); contrary to previously reported values, however, those of catechin increased over the same period. This increase was unreal as the catechin peaks at 3 and 12 months were interfered with by the presence of another substance: *cis*-caffeic acid. This compound occurs at very low concentration in wines – particularly freshly fermented wines –, so it does not disturb flavan-3-ol measurements; however, it grew in concentration during storage (particularly in Mencía) wine, thereby posing an increasing disturbance on the quantitation of flavan-3-ol.

Consistent with previous results (Gómez-Plaza et al., 2000a, 2000b; Gómez Plaza et al., 2001, 2002; Mayén et al., 1995), procyanidin levels increased with time (Fig. 2). These oscillations in the levels of flavan-3-ol monomers and polymers was probably the result of complex mutual polymerization-depolymerization processes accompanied by combination with some anthocyanins to give more stable pigments.

3.3.4. Flavonols

None of the freshly fermented wines, nor those stored for 3 months were found to contain quercetin aglycon at detectable levels. However, Mencía wine was found to contain four compounds and Brancellao two with a typical flavonol profile, all with retention times shorter than that for quercetin; such compounds might thus be glycosyl derivatives of this flavonol or some other such as myricetin or kaempferol (Mayén et al., 1995; Wightman et al., 1997; Zafrilla et al., 2003). The total levels of these compounds were higher in Mencía wine and comparable to those previously found by Mayén et al. (1995) in freshly fermented Cabernet Sauvignon and Tempranillo wines – by exception, these authors also found detectable levels of quercetin aglycon. On the other hand, our levels were much lower than those reported by Mazza et al. (1999) and Zafrilla et al. (2003) at the end of malolactic fermentation in Cabernet Franc, Merlot, Pinot Noir and Monastrell wines.

Flavonol levels decreased considerably – simultaneously with an increase in those of quercetin aglycon and other, unknown compounds – during the 12 months of storage. Based on their retention times, such unknown compounds were probably aglycon forms of myricetin and kampherol (Zafrilla et al., 2003) resulting from the hydrolysis of conjugated flavonols during storage of the wines (Fig. 2).

3.3.5. Influence of the phenol composition on wine colour

As can be seen from Table 3, at the end of alcoholic fermentation Mencía wine exhibited a much stronger colour and a greater contribution of red to its hue than did Brancellao wine; this is consistent with the anthocyanin levels in the two wines. Colour density remained virtually constant but hue increased (particularly as regards yellow) throughout the storage period. The absence of a direct relationship between colour density and the levels of anthocyanins reveals that polymerization and copigmentation reactions prevail over degradation of these pigments. Since all phenolic compounds studied other than procyanidins decreased in concentration during storage of the wines, any could indeed be involved in the process.

4. Conclusions

Anthocyanins and hydroxycinnamic acids in the two varietal wines studied were successfully determined by direct injection into an HPLC-DAD instrument. All other phenolic compounds, however, required some pretreatment for analysis. Liquid–liquid partitioning with ethyl ether at an acid pH, followed by solid-phase extraction on C_{18} mini-columns, ensured optimum fractionation of the analytes for their subsequent determination by HPLC-DAD. Raising the ionic strength by addition of NaCl prior to partitioning with ethyl ether resulted in substantially improved recoveries of phenolic acids – particularly those with low molecular weights – catechins and flavonols.

At the end of malolactic fermentation, Mencía wine was found to have higher contents in anthocyan pigments, but lower levels of flavan-3-ol and hydroxycinnamic acids, than Brancellao wine. This distribution of polyphenols is the origin of the increased colour density and weaker hue of Mencía wine relative to Brancellao wine. Except for procy-

anidins, the levels all phenolic compounds decreased during storage of both types of wine. The fact that colour density remained constant, however, suggests the occurrence of polymerization and copigmentation reactions among the compounds leading to a decrease in their levels. The decreased contents of tartaric esters of hydroxycinnamic acids during storage was accompanied by a simultaneous increase in those of the free forms. Similarly, the decrease in the levels of glycosylated flavonols was accompanied by a simultaneous increase in those of aglycon forms. These phenomena were the likely result of enzymatic hydrolysis processes.

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