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Flavonoid and aromatic characterisation of cv. Albarín blanco (*Vitis vinifera* L.)

Antón Masa *, Mar Vilanova

Misión Biológica de Galicia (CSIC), P.O. Box 28, 36080 Pontevedra, Spain

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Abstract

In order to characterise the Albarín blanco, a white *Vitis vinifera* L. cultivar native of Asturias (North of Spain), the grape flavonoids and the volatile composition of their wines were studied during three consecutive years. A total of 16 flavonoids (five dihydroflavonols, seven quercetin derivatives and four kaempferol derivatives) and 34 aroma compounds were identified and quantified by HPLC and gas chromatography, respectively. The results are very significant, in that dihydroflavonols were found to be the most abundant flavonoids in Albarín blanco grapes because this class of phenolic compounds was always considered as very scarce in grape skins. On the other hand, higher alcohols and esters were quantitatively the largest group of volatile compounds in wines elaborated with this cultivar and made up more than 80% of the free volatiles. Linalool, β-ionone, isoamyl alcohols, ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate were determined to be an important contribution to the aroma of Albarín blanco wines, according to their perception threshold in the three vintages. We may conclude that flavonoid and aromatic compounds have been of great value for Albarín blanco description. In addition, with this paper we can contribute to improve the knowledge about flavonols and dihydroflavonols in grape.

Keywords: Albarín blanco; Aroma index; Flavonoid; HPLC; Volatile compounds; GC–MS

1. Introduction

The grapevine Albarín blanco (*Vitis vinifera* L.) is a white cultivar growing in Asturias (North of Spain), where it is very appreciated for the high quality of wine that it produces. As far as we known, this cultivar was only characterised by ampelographic, agronomic and molecular methods (Santiago, Boso, Vilanova, & Martínez, 2005). Phenolic compounds play an important role in grape and wine quality, and varietal and flavour characteristics, largely are due to the presence of these compounds. Moreover, the use of grape phenolic compounds as taxonomic markers in cultivar characterisation is well documented (Alonso Borbalán, Zorro, Guillén, & García Barroso, 2003; Downey, Harvey, & Robinson, 2003; Nuñez, Monagas, Gomez-Cordovés, & Bartolomé, 2004; Pomar, Novo, & Masa, 2005). In this sense, the pre-eminence of flavonoids as chemical markers among the plants metabolic compounds, which have frequently been used in chemotaxonomy, is evident. They have been widely used with this purpose, because their patterns tend to be specific, they are relatively stable and their biosynthesis/accumulation is largely independent from environmental influence (Markham, 1989). Actually, it is well-known that the composition of flavonoids is primarily determined by genetic factors and most of the references coincide with the fact that the non-genetic factors (environmental conditions, viticultural practices, etc.) have a greater effect on the concentration of flavonoids, rather than on their relative composition (Arozarena et al., 2002).

Three classes of flavonoids are commonly detected in white grapes: flavonols, flavanoids (also called dihydroflavonoids, since the carbon-2 and carbon-3 of their skeleton are hydrogenated) and tannins, polymeric flavan-3-ols found in the hypodermal layers of the skin and the soft

* Corresponding author.
E-mail address: amasa@mbg.cesga.es (A. Masa).

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parenchyma of the seed, between the cuticle and the hard seed coat (Adams, 2006). Different derivatives of the most commonly encountered flavonol aglycones, including quercetin, kaempferol, myricetin and isorhamnetin (3′-methylether of quercetin), have been found simultaneously in the skin of red varieties of *Vitis vinifera* (Makris, Kallithraka, & Kefalas, 2006) but depending on cultivar, isorhamnetin may be present as traces (Macheix, Fleuriet, & Billot, 1990). The presence of laricitrin and syringetin derivatives in red grapes was recently reported by Mattivi, Guzzon, Vrhovsek, Stefanini, and Velasco (2006). The main flavonol glycosides in the skins of white grapes were quercetin and kaempferol derivatives, but isorhamnetin glucoside was also found in very low concentrations (Mattivi et al., 2006; Rodriguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006). Within the three classes of dihydroflavonoids, (flavanones, dihydroflavonols and dihydrochalcones) there is a high variation in structure and they are widely distributed in the plant kingdom but, in any case, they are considered to be very rarely present in fruits except in *Citrus* (Macheix et al., 1990). Data on dihydroflavonoids in grapes are rather scarce and, as far as is known, only two dihydroflavonols were reported: dihydroquercetin-3-*O*-rhamnoside (astilbin) and dihydrokaempferol-3-*O*-rhamnoside (engeletin), isolated for the first time from white grape berry skins in 1983 (Trousdale & Singleton, 1983).

On the other hand, aroma constituents of several wines have been studied by different authors and more than 800 volatile compounds have been identified in grapes and wines. Monoterpenes, C_{13}-norisoprenoids, esters, acetates, alcohols, organic acids, phenols and thiols have been found in different wines (Aznar, Lopez, Cacho, & Ferreira, 2001; Ferreira, Lopez, Escudero, & Cacho, 1998; Rapp & Mandery, 1986). Monoterpenes and C_{13}-norisoprenoids are characteristic of the grape, forming the primary or varietal aroma (Peynaud & Blouin, 1996). Some volatile substances arise from components of the grapes and many of these compounds are modified during yeast fermentation (Lambrechts & Pretorius, 2000). Among the volatile compounds derived from yeast metabolism during fermentation, (named secondary aromas) ethyl esters and acetates are mainly known to be important contributors to wine flavour with fruity notes (Nykänen, 1986; Rapp & Mandery, 1986; Selli et al., 2004).

The contribution of each compound to the entire aroma can be estimated by its odour activity value (OAV). Compounds with OAV > 1 are considered to have active contribution. This does not take into account the depressive or synergic odour effects resulting from the interactions of different molecules present in the wines, it can serve as a first approximation to the potential contribution of each compound to the global aroma (Ferreira, Ortín, Escudero, López, & Cacho, 2002; Moyano, Zea, Moreno, & Medina, 2002).

This paper reports the results of the first study about flavonoid composition of grapes and volatile composition of the wine elaborated with cv. Albarín blanco, during three consecutive years. The main objective of this study was to identify and quantify the principal flavonoid and volatile compounds present in the grape and wine of Albarín blanco and contribute to the characterisation of this cultivar, according to the phenolic and aromatic profile.

2. Materials and methods

2.1. Determination of flavonoids in grape

2.1.1. Plant material

During three consecutive years, berries at technological ripening of cv. Albarín blanco, from the grape germplasm collection of the "Misión Biológica de Galicia" (Pontevedra, Northwest of Spain), were harvested and immediately frozen and stored at −20 °C, until extraction.

2.1.2. Chemicals

Acetonitrile, ethanol and methanol were HPLC-rade solvents from Merck (Darmstadt, Germany) and acetic acid was "for synthesis" grade from Merck. Phthalic acid was of chromatographic grade from Merck and all the other chemicals were of analytical grade from Merck, Scharlau (Barcelona, Spain) or Panreac (Barcelona, Spain). The phenolic standards were obtained from Extrasynthese (Genay, France), Sarsyntex (Merignac, France), Fluka (Buchs, Switzerland) and Sigma (St Louis, MO, USA), and sugar standards were obtained from Fluka. HPLC-grade water was obtained from a Milli-Q System from Millipore (Bedford, MA, USA).

2.1.3. Extraction

Peels (30 g fresh weight) were manually separated from pulp and seeds and homogenised in water (150 mL) in a T-25 Ultraturrax from IKA (Staufen, Germany), centrifuged for 15 min at 20,000*g* and 4 °C in a Centrikon H-401 from Kontron (Zurich, Switzerland) and filtered (Whatman 4). One hundred percent ethanol was added, double the volume, to the juice obtained and the resultant mixture was left in refrigerator for 1 h and then filtered (Whatman 4) to remove protein. Ethanol was removed in a vacuum evaporator (Büchi, Flawil, Switzerland), at 35 °C, and the aqueous extract obtained was deffated in a separatory funnel three times with *n*-hexane (v/v) and extracted five times with EtOAc (v/v), in separatory funnel. The extracts obtained were mixed, dried with Na_2SO_4, evaporated to dryness and resolubilised with 2 mL of methanol/water (v/v). These extracts were used for the chromatographic analysis.

2.1.4. Chromatographic analysis

The HPLC analyses were carried out on a Waters (Milford, MA, USA) high-performance liquid chromatograph, equipped with a 2690 Waters Separations Module and a 996 Waters photodiode array detector. The HPLC column was a C18 Kromasil (5 μm particle size, 300 mm × 4.6 mm

i.d.) from Scharlau and was protected with a C18 Nova Pack guard precolumn from Waters. The oven temperature was set at 30 °C. Extracts were passed through a 0.45 μm filter (Millex-HV, Millipore) and a volume of 20 μL of solution was injected through the RP-C18 column for analytical HPLC. The flow rate was 0.8 mL/min and the mobile phase consisted of acetonitrile/acetic acid/water (35:2:63) as solvent A and 2% acetic acid as solvent B. The gradient profile was 25% A at 0 min, 30% A at 10 min, 40% A at 25 min, 50% A at 30 min, 60% A at 40 min, 65% A at 50 min, 75% A at 60 min and 100% A at 65 min. The mobile phase was returned to its initial condition in 5 min. Data were recorded on a computer with the Millenium[32] software from Waters, chromatograms were acquired at 356 and 280 nm and photodiode array spectra were recorded between 240 and 460 nm.

In a parallel way at HPLC analyses, phenolic extracts were separated on successive descending paper chromatography (PC) on 3MM Whatman paper, with water as solvent. The different fractions obtained were eluted with methanol and rechromatographied in ascending preparative thin layer chromatography (TLC) (200 mm × 200 mm × 0.1 mm cellulose, Merck) with the upper phase of BAW (*n*-butanol/acetic acid/water, 4:1:5) and in descending preparative PC, with the same solvent. Fractions corresponding to flavonoids were repeatedly injected in HPLC and peaks were collected and studied by co-chromatography, with standards in analytical HPLC, TLC and PC (1M Whatman paper), by colours yielded with different reagents and according to their spectral properties (Grayer, 1989; Markham, 1989). The spectral measurements were performed with a DU-640 spectrophotometer from Beckman Coulter (Fullerton, CA, USA). Spectral analysis (between 240 and 460 nm) was achieved in methanol and shifts were recorded after addition of $AlCl_3$, $AlCl_3$/HCl, NaOMe and NaOAc. All the shift reagents were prepared according to Markham (1982a). Further evidence for the structure of flavonoids isolated was obtained by chromatographing the products of the acid an enzymatic hydrolysis, which were carried out following the methods described by Markham (1982b). Aglycones were identified by co-chromatography with standards (when it was possible) and by UV–vis spectral analysis. Sugars were identified by chromatographic comparison with authentic sugar markers on 3MM Whatman paper with BBPW (*n*-butanol/benzene/pyridine/water, 5:1:3:3) as solvent. Detection was achieved by dipping the chromatogram into a solution of aniline hydrogen phthalate (Partridge's reagent) that was prepared dissolving aniline (0.92 mL) and phtalic acid (1.6 g) in *n*-butanol/ether/water (49:49:2) (Markham, 1982b).

2.1.5. Oxidation of dihydrokaempferol to kaempferol

It was not possible to compare dihydrokaempferol with an authentic standard. Thus, the aglycone obtained by hydrolysis of dihydrokaempferol glycosides was dissolved in water with a few drops of ethanol, mixed (v/v) with 10% $Na_2S_2O_5$ and heated on a steam bath to 100 °C, for 45 min, cooled and evaporated to dryness (Markham, Webby, & Vilain, 1984). The product (extracted out with methanol) was identified as kaempferol by spectral and chromatographic methods and by means of colour tests.

2.2. Vinifications and wine analysis

The wines analysed in this study were elaborated from Albarín blanco grapes, harvested in three different vintages. Spontaneous fermentations were performed in 16 L glass vessels, containing 10 L of cv. Albarín blanco grape juice, at 18 °C for 15 days. Sugar density content was measured daily. At the end of fermentation the wines were centrifuged and sulphur dioxide (50 mg/L) was added. Finally, the wines were transferred to 0.75 L bottle. The bottles were stopped and stored at 4 °C until analysis, 2 months after fermentation.

The ethanol, total acidity, volatile acidity, tartaric acid, malic acid and reducing sugar content were measured according to OIV Official Methods (1990).

2.3. Determination of aromatic compounds

To determine the identities and amounts of aromatic compounds present, the extracts were analysed by gas chromatography. All gas chromatographic analyses were performed with a Hewlett–Packard 5890 Serie II chromatograph equipped with a flame ionisation detector (FID). Compounds were separated on a 50 m × 0.25 mm i.d. fused-silica capillary column coated with a 0.20 μm film of Chrompack CP-Wax57CB.

2.3.1. Free and bound volatile compounds

Free and bound volatile compounds were fractionated by selective retention on SepPak Vac C-18 (Waters, Milford, MA, USA), according to the procedure described by Di Stefano (1991), with some modifications (Cortés, 1997). The cartridge was sequentially conditioned with 5 mL of methanol and 10 mL of distilled water; 100 mL of sample (centrifuged wine diluted with distilled water, v:v) were mixed with 1 mL of internal standard (3-octanol at 10 mg/L in ethanol) and passed through the C-18 cartridge. Then the cartridge was washed with 25 mL of distilled water and finally the free fraction was eluted with 10 mL of pentane–dichloromethane (2:1). Before GC analysis, the elute was dried over anhydrous sodium sulphate and concentrated to 0.5 mL by evaporation with a stream of nitrogen. The bound fraction was eluted with 10 mL of methanol and concentrated to dryness using a vacuum, before dissolution in 5 mL of citrate–phosphate buffer (pH 5.0). AR-2000 (Gist Brocades, France) was added and the mixture was incubated at 40 °C for 18 h to accomplish enzymatic hydrolysis. Glycosides hydrolysed were extracted like free form fraction and the extract was concentrated by evaporation with a stream of nitrogen before GC analysis. Conditions used for chromatographic analysis were: injector temperature (250 °C), temperature program

(60 °C for 5 min, increased at 3 °C/min to 220 °C, 15 min isothermal), detector temperature (260 °C), injection type (splitless, 30 s) and injection size (1 μL). Carrier gas helium at 1.20 mL/min; make-up gas: nitrogen 15 mL/min. The detector gas flow rates were: hydrogen, 40 mL/min and air, 400 mL/min.

2.3.2. Fermentative compounds

In the determination of methanol and higher alcohols, 1 mL of an internal standard solution (1 g of 4-methyl-2-pentanol per 1 L of ethanol) was added to 10 mL of the sample prior to GC analysis. A 1 μL aliquot of sample was injected directly and split 1:1. The temperature program was as follows: held 15 min at 60 °C and raised at 3 °C/min to 200 °C.

Extraction of esters and acetates was carried out according to the method described by Bertrand (1981): 2 mL of 3-octanol (50 mg/L) as internal standard and 1 mL of sulphuric acid (1/3) were added to 50 mL of wine. Each sample was liquid–liquid extracted three times with 4, 2 and 2 mL of diethyl ether–hexane (1:1). One microlitre of the organic extract is injected into the chromatograph in splitless mode (30 s). Temperature program was as follows: held 15 min at 55 °C and raised at 3 °C/min to 200 °C.

Instrumental conditions for fermentative compounds were: injector temperature: 250 °C, detector temperature: 260 °C, carrier gas: helium at 1.07 mL/min; make-up gas: nitrogen 30 mL/min. The detector gas flow rates were: hydrogen, 40 mL/min and air, 400 mL/min.

2.3.3. Identification and quantification

Aromatic compounds were identified by comparing retention times with those of pure compounds and confirmed by GC–MS using a HP5890 Series II coupled to HP 5989A mass spectrometer, in the EI mode (ionisation energy, 70 eV, source temperature 250 °C). The acquisition was made in scanning mode from m/z 10 to 1000 at 5 scans/s.

Internal standards were used to quantify the concentrations of individual compounds.

3. Results and discussion

3.1. Flavonoid identification

The typical HPLC chromatogram of phenolic extracts of Albarín blanco grapes, captured at 280 nm, shows 16 peaks corresponding to flavonoids (Fig. 1). Attending to the retention time in HPLC and their UV–vis spectral properties, it was possible to identified totally or partly five dihydroflavonols compounds (DHF), seven quercetin derivatives (Q) and four kaempferol derivatives (K) (Table 1). Their chemical structure was confirmed by co-chromatographic analysis in PC and TLC, by the result of acid and enzymatic hydrolysis and by the colours yielded in presence of different agents (Table 2).

On preparative PC with water as solvent, our crude extracts gave a total of four dark UV-absorbing spots, two of them (R_{fs} 0.37 and 0.69) turn yellow with NH_3 vapour (in future, abs-y), one (R_f 0.74) turns green (in future, abs-g) and another one (R_f 0.47) that does not change in the presence of NH_3 vapours (in future, abs–abs). All these chromatographic fractions were eluted with methanol/water (v/v) and rechromatographied on preparative PC and TLC with BAW as solvent. A total of five abs–abs spots, four abs-y spots and three abs-g spots were produced and eluted with methanol/water, v/v.

Finally, these new phenolic fractions were repeatedly injected in HPLC and peaks manually collected for their identification. In this sense, co-chromatography with com-

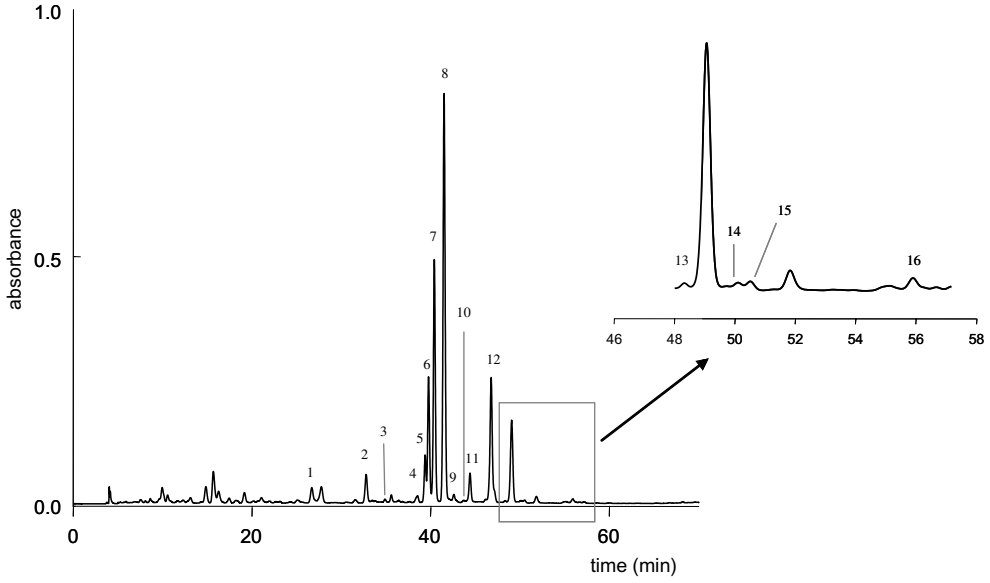

Fig. 1. Typical HPLC chromatogram of Albarín blanco phenolics captured at 280 nm.

Table 1
Flavonoids (in peak area %) for Albarín blanco grape from three consecutive vintages

Peak number	Class	Vintage			Mean	SD
		I	II	III		
1	DHF	1.12	1.49	1.43	1.35	0.20
2	DHF	1.74	2.23	1.78	1.92	0.27
3	DHF	0.45	0.19	0.54	0.39	0.18
4	Q	0.54	0.60	0.56	0.57	0.03
5	Q	1.94	2.81	2.17	2.31	0.45
6	Q	9.41	8.26	12.9	10.2	2.39
7	Q	9.39	16.0	12.7	12.7	3.29
8	DHF	23.9	29.0	25.2	26.0	2.63
9	Q	0.80	0.76	0.44	0.67	0.20
10	Q	0.33	0.14	0.13	0.20	0.11
11	K	1.51	2.04	1.86	1.80	0.27
12	K	4.67	9.65	9.22	7.85	2.76
13	Q	0.25	0.12	0.15	0.17	0.07
14	DHF	0.34	0.21	0.37	0.31	0.09
15	K	0.12	0.23	0.20	0.18	0.06
16	K	0.32	0.50	0.48	0.43	0.10
Sum-DHF		27.6	33.1	29.3	30.0	
Sum-Q		22.7	28.7	29.0	26.8	
Sum-K		6.62	12.4	11.8	10.3	
Total		56.9	74.2	70.0	67.0	

mercial standards (when it was possible) on HPLC and TLC, with several solvents (water, BAW and 5–15% acetic acid), was used. When a sufficient sample was obtained, the chemical structures of the collected peaks were confirmed by UV-spectroscopy and hydrolytic treatments.

The chromatographic behaviour (in reverse-HPLC, PC and TLC) confirms that all the compounds isolated have glycosilated structures.

3.1.1. Quercetin glycosides

All the abs-y compounds isolated (peaks 4, 5, 6, 7, 9, 10, and 13 in Fig. 1) present a methanol UV-spectra which exhibits one absorption band in the 348–355 nm range (Band I) and another (Band II) at 253–256 nm, which is characteristic of 3-OH substituted flavonols (Markham, 1989). The NaOAc induced bathocromic shift (7–14 nm) suggests the presence of an OH-7 group and the spectral behaviour with $AlCl_3$ (bathochromic shift around 70 nm in Band I in relation to the MeOH spectrum) and $AlCl_3$/HCl (hypsochromic shift around 35 nm in Band I respect to the $AlCl_3$ spectrum) points to the presence of a free OH-5 and two contiguous free OH's in ring B. The new absorption maximum in the 320–325 nm range, when NaOMe is added, confirms the existence of a free OH-7.

When we spray the PC and TLC chromatograms with "Naturstoff Reagenz" (NA) (1% solution in methanol of diphenyl–boric acid–ethanolamine), all these compounds appear as yellow-orange fluorescent spots, which suggests the presence of 3′,4′ or 3′,4′,5′-hydroxylated compounds. Acid and enzymatic hydrolysis confirm that the aglycone is, in all cases, quercetin and the conditions of acid hydrolysis using 2 N HCl/MeOH (1:1, v/v), that occur in 5–10 min in all cases, confirm glycosylation at position 3. The nature of the liberated sugars was confirmed using PC chromatography as in Section 2. Thus, a total of seven quercetin glycosides (Q) were isolated and partly or totally identified.

3.1.2. Kaempferol glycosides

Methanol UV-spectra of the abs-g compounds isolated (peaks 11, 12, 15, and 16 in Fig. 1) exhibit, in all cases, one absorption band in the 345–350 nm range (Band I)

Table 2
Retention times, spectral characteristics and colours with different reagents of the chromatographic peaks isolated

Peak number	R_t (min)	λ_{max} (nm)[a]	Colours						Identification
			UV	UV + NH_3	NA	Zn/HCl	$NaBH_4$/HCl	$NaBH_4$/DDQ	
1	25.7	291, 340sh	Abs	Abs	Y	Purple	Neg	Neg	Dihydroquercetin-3-*O*-glycoside
2	31.8	290, 340sh	Abs	Abs	Y	Purple	Neg	Neg	Dihydroquercetin-3-*O*-glycoside
3	34.0	291, 340sh	Abs	Abs	Y	Purple	Neg	Neg	Dihydroquercetin-3-*O*-galactoside?
4	37.6	256, 354	Abs	OY	Y	–	–	–	Quercetin-3-*O*-rutinoside
5	38.4	256, 354	Abs	OY	Y	–	–	–	Quercetin-3-*O*-glucuronide?
6	38.8	256, 354	Abs	OY	Y	–	–	–	Quercetin-3-*O*-galactoside
7	39.4	256, 354	Abs	OY	Y	–	–	–	Quercetin-3-*O*-glucoside
8	40.5	290, 340sh	Abs	Abs	Y	Purple	Neg	Neg	Dihydroquercetin-3-*O*-rhamnoside
9	41.5	256, 353	Abs	OY	Y	–	–	–	Quercetin-3-*O*-rhamnnoside
10	42.6	255, 358	Abs	OY	Y	–	–	–	Quercetin-3-*O*-glycoside
11	43.3	265, 347	Abs	G	G	–	–	–	Kaempferol-3-*O*-rutinoside
12	45.6	265, 347	Abs	G	G	–	–	–	Kaempferol-3-*O*-glucuronide
13	47.0	255, 354	Abs	OY	Y	–	–	–	Quercetin-3-*O*-glycoside
14	48.7	291, 340sh	Abs	Abs	G	Red	Neg	Neg	Dihydrokaempferol-3-*O*-rhamnoside
15	49.0	266, 351	Abs	G	G	–	–	–	Kaempferol-3-*O*-glycoside
16	54.1	264, 346	Abs	G	G	–	–	–	Kaempferol-3-*O*-glycoside

Abs, absorbent (dark spot); OY, orange-yellow; Y, yellow; G, green and Neg, colourless.
? = Tentative identification.
[a] Absorption spectra in photodiode array; sh, shoulder.

and another in the 264–266 nm range (Band II), which indicates the presence of 3-OH substituted flavonols. The NaOAc induced bathochromic shift suggests the presence of an OH-7 group and the spectral behaviour with $AlCl_3$ induces a bathochromic shift (around 45-50 nm), which did not disappear when HCl was added. This points to the presence of a free OH-5 and only one OH in the B ring. As in quercetin glycosides, the new absorption maximum in the 320–325 nm range, when NaOMe was added, confirms the existence of a free OH-7.

All these compounds gave green-yellowish fluorescent spots when PC and TLC chromatograms were sprayed with NA, which suggests the presence of 4′-hydroxylated compounds. Acid and enzymatic hydrolysis confirm kaempferol as the only aglycone and glycosylation at position 3. The nature of the liberated sugars was confirmed using PC chromatography (see 2). In this case, it was possible to isolate a total of four kaempferol glycosides (K) that were totally or partly identified.

3.1.3. Dihydroflavonols

All the abs–abs compounds isolated (peaks 1, 2, 3, 8 and 14 in Fig. 1) showed the characteristic methanol UV-spectra of flavanoids (flavanones, dihydroflavonols and dihydrochalcones) (Grayer, 1989), with a very strong maximum between 280 and 294 nm (Band II) and an inflection around 340 nm. Their UV-spectra, with standard spectral shifts and the characteristic dark UV-absorbing unaffected by ammonia vapours, suggests structures lacking free 3-hydroxyl groups. The NaOAc induced shift of Band II (around 37 nm) suggests the presence of a free OH-7 group and the spectral behaviour with $AlCl_3$ (20–37 nm) and $AlCl_3$/HCl (22–25 nm) points to the presence of a free OH-5. The absence of change in colour to blue-green using NH_3 vapour confirms this (Markham et al., 1984). All these compounds yielded red or purple colours with the Zn/HCl reagent, specific for the detection of dihydroflavonols, and did not give any colour, either with $NaBH_4$/HCl (specific for flavanones) or with $NaBH_4$/DDQ, specific for dihydrochalcones detection. Besides, four of those compounds (peaks 1, 2, 3 and 8) gave a yellow colour with NA reagent while the peak 14 produces a green colour with this reagent. Conditions of acid hydrolysis confirm in all cases glycosylation at position 3. Acid and enzymatic hydrolysis yielded two different aglycones, both UV-absorbing that do not change when fumed with ammonia and that show the typical methanol UV-spectra for dihydroflavonols. One of these aglycones, that also gave a yellow colour with NA reagent, was identified as dihydroquercetin (also known as taxifolin) by direct comparison with an authentic standard, while the other aglycone, that gives a green colour with the NA reagent, seems to be a derivate of kaempferol. Actually, since colour with NA reagent depends on the B-ring structure, it is logical to think that dihydrokaempferol is present (also known as aromadendrin). Besides, oxidation of this aglycone with sodium metabisulfite (see Section 2) yielded kaempferol. A total of five dihydroflavonols (DHF) were isolated and partly or totally identified.

From the chromatograms obtained for the three consecutive years studied, the contents (in peak area %) of the 16 flavonoids isolated were calculated. From Table 2, we can deduce that DHF are quantitatively the largest group of the flavonoid compounds in this cultivar (made up more than 29%), followed by quercetin glycosides (26.2%) and kaempferol glycosides (9.9%). Dihydroquercetin-3-*O*-rhamnoside (peak 8), named astilbin, was the most abundant flavonoid in Albarín blanco and represents 25.2% of the total phenolic content, whereas dihydrokaempferol-3-*O*-rhamnoside (peak 14), named engeletin, represents only 0.30% of the total phenolic content. The other three dihydroflavonols (peaks 1, 2 and 3) were tentatively identified as dihydroquercetin glycosides and in all cases were found for the first time in grape cultivars by Masa, Vilanova, and Pomar (2007). The main flavonol, by far, was quercetin-3-*O*-glucoside (peak 7) followed by quercetin-3-*O*-galactoside (peak 6) and kaempferol-3-*O*-glucuronide (peak 12). Traditionally it was accepted that only quercetin and kaempferol derivatives were present in white grape cultivars, but recently, several research studies have described numerous white grape cultivars that contain isorhamnetin derivatives (Mattivi et al., 2006; Rodriguez Montealegre et al., 2006). In this sense, the results of Mattivi et al. (2006) found isorhamnetin derivatives in 21 of the 27 white grape cultivars studied. Thereby, it is very significant that isorhamnetin derivatives were absent in Albarín blanco cultivar and we can consider it as a varietal characteristic of this cultivar.

3.2. General wine composition

The general composition of wines elaborated with Albarín blanco is shown in Table 3. All wines were fermented to dryness. The ethanol of the wines was higher in the first and second vintages than the third one. Moreover, the first and third vintage showed important levels of total acidity and tartaric acid. Probably the climatic conditions are the principal factor that explain the existence of differences, among years, in the general composition of Albarín blanco wines in the three years studied. This indicates that the degree of maturation was possibly incomplete in the third vintage.

Table 3
General composition of cv. Albarín blanco wine

Parameter	Vintages			Mean	SD
	I	II	III		
Ethanol (vol%)	12.5	11.2	10.7	11.5	0.9
Total acidity (g tartaric acid L)	7.8	5.3	7.1	6.7	1.2
Volatile acidity (g acetic acid L)	0.4	0.3	0.3	0.3	0.1
Reducing sugar (g/L)	1.6	1.0	1.3	1.3	0.3
Tartaric acid (g/L)	3.5	1.5	3.6	2.9	1.1
Malic acid (g/L)	2.0	1.7	2.1	1.9	0.2
Lactic acid (g/L)	0.2	0.3	0.3	0.3	0.1

3.3. Bound volatile compounds

To assess the aromatic potential of Albarín blanco wines, the concentration of free and bound aroma compounds was determined. The bound compounds, do not contribute directly to the aroma, but are a reservoir of odourless precursors of flavour. Bound compounds can be released during vinification process by glycosidase enzymes produced by the grapes themselves, or by the microorganisms taking part in the process and hence increase the aroma of the wine (Cabaroglu, Canbas, Lepoudre, & Gunata, 2002; Strauss, Wilson, Gooley, & Williams, 1986; Ugliano, Bartowsky, McCarthy, Moio, & Henschke, 2006).

A total of 9 bound volatile compounds were identified in Albarín blanco wines, including monoterpenes, alcohols, C_{13}-norisoprenoids and others (Table 4). Quantitatively bound aroma released by enzyme hydrolysis was lower than the direct glycoside composition. The levels of bound compounds in the Albarín blanco wines, analysed in the three vintages, were 1569, 1012 and 123 μg/L, respectively, dominated by 2-phenylethanol and terpinen-4-ol. Vilanova and Sieiro (2007) reported that 2-phenylethanol was the most important bound compound of Albariño wines. The alcohols, when glycoconjugated, were present at lower concentrations than free compounds. These results were similar to a previous study (Carro, López, Gunata, Baumes, & Bayonove, 1996).

In the third vintage, low bound compound levels were detected. These results for a given vintage may be influenced by weather factors during grape ripening. Generally, 90% of terpenes present as non-volatile glycosides can be hydrolysed (enzymatically or chemically) to the free form during fermentation and aging (Ebeler, 2001). The alcoholic fermentation, with adequate *Saccharomyces cerevisiae*, actively contribute to the liberation of glycosidically bound volatiles of different origins (Ugliano et al., 2006).

It can be seen that the total concentration of free forms is higher than that of bound forms, in the vintages studied. In this case, only eugenol was detected in the bound form. Most compounds found in the free aroma were also present in the bound fraction. In contrast citronellol and theaspirane-b were detected only in the free fraction.

Table 4
Bound and free compound levels (μg/L) in Albarín Blanco wine over three vintages, mean and standard deviation

Compound	Vintage			Mean	SD
	I	II	III		
Bound compounds					
Linalool	ND	46	59	35	31.0
Terminen-4-ol	121	146	ND	89	78.1
α-Terpineol	50	34	31	38.3	10.2
Nerol	42	20	5	22.3	18.6
Geraniol	17	25	18	20	4.36
Benzyl alcohol	51	51	ND	34	29.4
2-Phenylethanol	1222	640	ND	621	611
β-Ionone	21	16	10	15.7	5.51
Eugenol	45	34	ND	26.3	23.5
Free compounds					
Terpenes					
Linalool	81	236	122	146	80.3
Terpinen-4-ol	87	273	ND	120	139
α-Terpineol	305	208	19	177	145
Citronellol	ND	12	5	5.67	6.03
Nerol	ND	17	5	7.33	8.74
Geraniol	62	78	7	49	37.2
C_{13}-norisoprenoids					
Theaspirane-b	254	72	ND	163	129
β-Ionone	24	18	12	18	6.00
Alcohols					
Methanol	7300	22920	22080	17433	8786
1-Propanol	17890	24390	69180	37153	27926
1-Butanol	450	ND	1200	550	606
2-Methyl-1-propanol	68390	81950	63380	71240	9607
Isoamyl alcohol	267350	328590	199950	265297	64345
Benzyl alcohol	106	70	ND	58.7	53.9
2-Phenylethanol	95420	58140	28740	60767	33418
Acetates					
Ethyl acetate	60250	61110	54410	58590	3645
Isoamyl acetate	170	140	270	193	68.1
Hexyl acetate	270	250	ND	173	150
Ethyl esters					
Ethyl lactate	12440	11900	17250	13863	2945
Ethyl butyrate	4820	170	2100	2363	2336
Ethyl hexanoate	280	290	1130	567	488
Ethyl octanoate	330	300	1840	823	881
Ethyl decanoate	90	90	2210	797	1224
Diethyl succinate	7570	1790	ND	3120	3956
Ethyl mirystate	190	50	ND	80	98.5

ND, not detected.

3.4. Free volatile compounds

The free volatile composition of the Albarín blanco white wines is shown in Table 4. A total of 25 compounds were identified and quantified in the three vintages, six terpenes, two C_{13}-norisoprenoids, seven alcohols, three acetates and seven ethyl esters. The total concentration of free volatile compounds was very similar in the three vintages (544,664 μg/l, 593,888 μg/l, 464,068 μg/l, respectively).

Higher alcohols and esters were quantitatively the largest group of the volatile compounds in Albarín blanco wines and made up more than 80% of the free volatiles. Both are produced by yeast metabolism during alcoholic fermentation and play an important role in the flavour of the wines (Swiegers & Pretorius, 2005; Valero, Moyano, Millan, Medina, & Ortega, 2002). In all vintages, the major alcohols in Albarín blanco were isoamyl alcohols, 2-methyl-1-propanol and 2-phenylethanol. In the first vintage, the level of 2-phenylethanol was higher than that in the other two vintages. This compound is characterised by a floral (rose) odour and its level in wine is reportedly related to the grape variety and to the yeast metabolism during fermentation (Gomez-Plaza, Gil-Muñoz, Carrero-Espin, Fernández-Lopez, & Martinez-Cutillas, 1999).

The wines from the three vintages contained 86,410, 76,090 and 79,210 μg/L, respectively, of acetates and ethyl esters. These compounds are important in young wine

aroma and they contribute to the fruity flavour of wines (Nykänen, 1986; Rapp & Mandery, 1986; Selli et al., 2004). High levels were observed for ethyl acetate in all of the vintages. Fresh, fruity aromas in wines derive to a great extent from the presence of the mixture of esters produced by the yeast during fermentation, but esters significant to a specific grape cultivar have also been identified (Cabaroglu et al., 2002).

Terpenoid compounds are an important contribution to varietal aroma of wines because of their low perception threshold and they are related with a floral odour. The concentration of monoterpenes in Albarín blanco wines from second vintage (824 μg/L) was higher than in the first (535 μg/L) and third vintage (158 μg/L). Monoterpenes are particularly abundant in aromatic grape varieties, such as Muscat, Riesling and Gewürztraminer (Valero et al., 2002). In this study, among monoterpenes α-terpineol was the most abundant in the first vintage and linalool and terpinen-4-ol were the most abundant in the second vintage. Linalool is, together with citronellol, one of the most important terpenic compounds, because it also presents a low perception threshold (López, Ferreira, Hernández, & Cacho, 1999), with a citrus aroma. C13-norisoprenoids are also considered to be important to the aroma of wine (Swiegers & Pretorius, 2005). High levels of theaspirene-b and β-ionone were detected in the first vintage.

4. Conclusion

From the results obtained, we can conclude that Albarín blanco grape skins have a specific flavonoid profile with a high concentration of quercetin derivatives, especially dihydroflavonols. Moreover, only quercetin and kaempferol derivatives are present in this cultivar. The presence of three quercetin dihydroflavonols (tentatively identified) was reported for the first time in *Vitis* sp. In volatile compounds, linalool, β-ionone, isoamyl alcohols, ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate were determined as important contributors to the aroma of Albarín blanco wines, according to their perception threshold in the three vintages. We have found only quantitative but not qualitative differences among the three vintages. As can be expected, the composition of the different vintages is clearly influenced by the climatic conditions.

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