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Phenolic characterisation of red grapes autochthonous to Andalusia

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ABSTRACT

Twenty six phenolic compounds in wine grapes were identified and quantified in five winegrape varieties using the complementary information from high-performance liquid chromatography coupled to diode array and fluorescence detectors, and mass spectrometry in both positive and negative mode. Fourteen different anthocyanins were identified in these grapes. In all varieties, malvidin-3-glucoside and its derivatives, mainly *p*-coumaroyl derivatives, were the major compounds. Seven flavonols were detected, most as quercetin and myricetin derivatives, and few qualitative differences were found among varieties. Total hydroxycinnamic content was rather low in all varieties. Lastly, catechin and epicatechin were detected in both skin and seed; differences in respect of the content in the seeds can be attributed to differences in the number and weight of seed per berry in each variety. The results of the characterisation can be used to select winemaking techniques aimed at improving the quality of the final wine.

1. Introduction

Content in phenolic compounds is one of the main factors in the quality of grapes and wine. The phenolic composition of a wine depends primarily on the phenolic content of the grapes and secondarily on the winemaking techniques employed. The grape anthocyanins are monoglucosides of five anthocyanidins, namely delphinidin, cyanidin, petunidin, peonidin and malvidin. The acylated anthocyanins are esters of the glucose moiety of the free anthocyanins with acetic, *p*-coumaric or caffeic acids. Flavonols are found in grape skins as glycosides of myricetin, quercetin, kaempferol, isorhamnetin, syringetin and laricitrin. Flavan-3-ols (monomeric catechins and polymeric proanthocyanidins) are another large family of polyphenolic compounds comprising mainly catechin, epicatechin, gallocatechin, epigallocatechin and their corresponding polymers, which are found in skin and seed. Hydroxycinnamic esters are the third most abundant group of phenolic compounds in grapes, comprising mainly caftaric, coutaric, fertaric, and tartrate esters (Rodriguez, Aguilar, & Gomez, 2006).

Anthocyanins are directly responsible for colour. Flavonols and hydroxycinnamic acid derivatives are involved in the stabilization of anthocyanins in young red wines through copigmentation (Boulton, 2001). Flavan-3-ols are mainly responsible for wine astringency, bitterness and the "structure" of wines (Kennedy, Saucier, & Glories, 2006). Moreover, recent studies have shown that they can play a very important role in the stabilization of red colour in wines (Sun, Santos, Leandro, De Freitas, & Spranger, 2007).

The interest of winemakers in the polyphenol content of grapes is increasing, as it offers ways of influencing the colour, bitterness, astringency, "mouth-feel" and "age-ability" of wines. This interest is bound to increase further, since phenolics have been reported to have multiple biological properties such as antioxidant, anti-inflammatory, anti-atherosclerosis, cardioprotective and cancer protective effects (Fresco, Borges, Diniz, & Marques, 2006; Soleas, Grass, Josephy, Goldberg, & Diamandis, 2006).

In grape berries, phenolic compounds are mainly found in skin and seed. Anthocyanins are the most abundant phenolics in red grape skins, while seeds are rich in flavan-3-ols. The amount of phenolics in grapes depends on the variety of grapevine and is highly influenced by viticultural and environmental factors such

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as light, temperature, altitude, soil type, water, nutritional status, pathogenesis, and various developmental processes (Downey, Dokoozlian, & Krstic, 2006). Temperature has a great influence on anthocyanin biosynthesis. Anthocyanin levels in Cabernet Sauvignon grapes are higher when day temperatures are constant at 20 °C than at 30 °C; therefore, increasing anthocyanin content is associated with grapes grown at higher altitudes. However this relationship is complicated by the effect of diurnal differences in temperature: lower night temperature result in greater accumulation of anthocyanins (Mori, Sugaya, & Gemma, 2005). In very hot seasons, reduction in grape berry colour has been observed. Whether this decrease occurs through degradation of existing anthocyanins or reduced anthocyanin biosynthesis is not known.

An additional climate impact on phenolic compounds is the positive relationship between sunlight exposure and increased flavonol accumulation. (Cortell & Kennedy, 2006; Downey, Harvey, & Robison, 2004). In contrast, bunch exposure to sunlight has little effect on flavan-3-ols, as these compounds occur mainly in the seed. The effect of sunlight on the concentrations of hydroxycinnamics is still under study (Kolb, Kopecky, Riederer, & Pfundel, 2003).

Information is available on the identification and quantification of phenolic compounds in grapes by families (Castillo-Muñoz, Gomez-Alonso, Garcia-Romero, & Hermosin-Gutierrez, 2007; Pomar, Novo, & Masa, 2005). However information on particular phenolic compounds in grape varieties is rather scarce, and to the best of our knowledge, does not exist for varieties of red grape for winemaking autochthonous to Andalusia. Andalusian red grape varieties for winemaking have been cultivated since the 19th century. They almost disappeared during the 20th century but recently these varieties have shown a steady increase in area cultivated and wine produced, associated with the introduction of red wines as part of the diversification plan for the Andalusian wines sector (MAPA: Ministerio de Agricultura y Pesca. Annual data of Andalusian vineyards, 2007).

The objective of the present study is to identify and quantify phenolic compounds in five red winegrape varieties cultivated under particularly warm conditions. Grapes of three varieties autochthonous to Andalusia (Jaén tinto, Palomino negro and Tintilla de Rota) and two others most commonly used in Spain, and all over the world (Cabernet Sauvignon and Tempranillo) were analysed and compared, with a view to advising on the most appropriate winemaking techniques, and to promoting the varietal character of the respective young red wines.

2. Materials and methods

2.1. Reagents

Malvidin-glucoside was purchased from Polyphenols A.S. (Sandnes, Norway); quercetin-3-rutinoside was purchased from Merck (Darmstadt, Germany); catechin and caffeic acid were purchased from Sigma (Madrid, Spain). Analytical grades of acetic acid, formic acid and methanol (MeOH) were supplied by Panreac (Barcelona, Spain). Mili-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout in this research.

2.2. Winegrapes

Three autochthonous red grape varieties: Jaen tinto (JT), Palomino negro (PNO), Tintilla de Rota (TR) and two varieties used in many countries: Cabernet Sauvignon (CS) and Tempranillo (TEM) were harvested at their stage of optimum maturity in the summer of 2006. They were grown in Jerez de la Frontera (IFAPA, Rancho de la Merced).

2.3. Extraction of anthocyanins and flavonols

Grapes were stored at −20 °C until analysed. Analyses were carried out according to the protocol of Cantos, Espín, and Tomas-Barberan (2002) with some modifications. Briefly, grapes were peeled using a sharp knife, and the skins were homogenised in Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Deutschland, Germany) at 24,000 rpm for 1 min after addition of 2 ml of a solution MeOH–formic acid (95:5, v/v) per gram of grape skin. Then the extracts were subjected to extraction for 5 h in conditions of darkness and cold. The extract was centrifuged at 5000 rpm for 5 min in a Centromix centrifuge (Selecta, Barcelona, Spain), filtered through a 0.22 μm membrane filter Olim Peak (Tecknocroma, Barcelona, Spain) and analysed by HPLC. All experiments were performed in triplicate.

2.4. HPLC analysis of anthocyanins and flavonols

The filtered skin extracts (20 μl) were analysed using a Waters HPLC system with a model 1525 pump and a Waters 996 diode array detector. Separations were performed on a Mediterranea Sea_{18} column (Tecknokroma, Barcelona, Spain) (RP-18, 250 × 4.6 cm; 5 μm particle size) and a guard column of the same material, at 30 °C. The mobile phase consisted of water with 5% formic acid (v/v) (solvent A) and HPLC grade MeOH (solvent B) at a flow rate of 1 ml/min. The elution program involved gradient elution from 20% B to reach 35% at 30 min, 40% B at 45 min, 50% B at 60 min, 60% B at 70 min, and 95% at 75 min; and isocratic elution, 95% B from 75–80 min. Anthocyanins were quantified at 520 nm as Malvidin-3-glucoside (LOD = 0.074 ppm, LQD = 0.240 ppm) and flavonols at 360 nm as quercetin-3-rutinoside (LOD = 0.123 ppm, LQD = 0.409 ppm).

2.5. Extraction of flavan-3-ols and hydroxycinnamic derivatives

An ASE-200 extractor (Dionex, Sunnyvale, CA, USA) was used for the pressurised liquid extractions of both flavan-3-ols and hydroxycinnamic derivatives (Piñeiro, Palma, & Barroso, 2004). The extraction cell volume was 11 ml and the collection vial volume was 40 ml. Sea sand (Panreac, Barcelona, Spain) has been used as supporting material in the extraction chamber. The extraction cell was filled with the extraction solvent (MeOH), pressurised up to 100 atm and then heated up to 100 °C. The sample (grape skins for hydroxycinnamics and flavan-3-ols, and grape seed for flavan-3-ols) was then extracted by three static extraction cycles of 10 min. After the extraction, the cell was rinsed with fresh solvent (100% of the extraction cell volume) and purged with a flow of nitrogen for 300 s. The extract was collected into a 60 ml chamber glass vial. The process consumed approximately 22 ml of the solvent. The sample volume was brought up to 25 ml, and then filtered through a 0.45 μm nylon syringe filter (Millex-HN, Ireland) before chromatographic analysis. All experiments were performed in triplicate.

2.6. HPLC analysis flavan-3-ols and hydroxycinnamic derivatives

The analyses of the extracts were performed by HPLC in a Waters system consisting of an autosampler (717 plus), pump controller (600S), pump (616), a photodiode array detector (996) and a fluorescence detector (474), using a RP-18 column (LiChrospher 100, 250 mm × 3 mm, 5 μm particle size, Merck, Germany) and a gradient of acidified water (2% acetic acid, v/v) (solvent A) and methanol–water–acetic acid (90:8:2, v/v/v) (solvent B) at a flow rate of 0.3 ml/min. The gradient was as follows: 0 min, 20% B; 10 min, 25% B; 20 min, 50% B; 21 min, 100% B. The UV absorbance was monitored from 200 to 400 nm. The identification of com-

pounds was made by comparison of retention times with pure standards, as well as by UV–visible spectra and MS spectra. Hydroxycinnamic acid derivatives were quantified at 320 nm as caffeic acid (LOD = 0.064 ppm, LQD = 0.212 ppm) and flavan-3-ols as catechin using the fluorescence signal (excitation wavelength 290 nm, emission wavelength 320 nm) (LOD = 0.024 ppm, LQD = 0.074 ppm).

2.7. HPLC–MS–MS

Chromatographic separation was carried out for each phenolic class as detailed above (Sections 2.4 and 2.6). An Agilent Technologies series 1100 system, equipped with vacuum degasser, a binary pump, an autosampler, a thermostated column compartment, a DAD and an on-line ion-trap mass spectrometer, connected to Agilent ChemStation software (Waldbronn, Germany). MS interface was an electrospray ionisation system (ESI). Capillary temperature and capillary voltage were maintained at 350 °C and 4 kV, respectively. Mass scans (MS) and MS/MS spectra were recorded from *m/z* 100 up to *m/z* 1500. Collision-induced fragmentation experiments were performed in the ion-trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. Maximum accumulation time of the ion-trap and the number of MS repetitions to obtain the MS average spectra was set at 300 ms and 5, respectively. Mass spectrometry data were acquired in both positive and negative ion mode.

3. Results and discussion

3.1. General

Polyphenol analyses were carried out on the grape skin since that is the part of the grape that contributes the greater part of these compounds to wine. In addition, the content of flavan-3-ol monomers was also determined in seeds, because they are very abundant in seeds and can be extracted during the winemaking process. The anthocyanins have maximum sensitivity in positive mode due to their inherent positive charge. In contrast, flavonols, hydroxycinnamics and flavan-3-ols provide highest sensitivity in negative ionisation mode (Cuyckens & Claeys, 2004).

3.2. Anthocyanins

A total of 14 phenolic compounds were identified as anthocyanins thanks to the information provided by their UV–vis spectra (Fig. 1). This primary information differentiated 5 glycosylated anthocyanins (λ maximum at 277–280, and 516–527 and shoulder at 328–349 nm): delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside; and 9 acylglycosylated anthocyanins (λ maximum at 278–283, and 522–535 and shoulder at 311–347 nm): delphinidin-3-acetylglucoside, petunidin-3-acetylglucoside, peonidin-3-acetylglucoside, malvidin-3-acetylglucoside, malvidin-3-*cis*-*p*-coumaroylglucoside, malvidin-3-caffeoylglucoside, cyanidin-3-*p*-coumaroylglucoside, petunidin-3-*p*-coumaroylglucoside and malvidin-3-*trans*-*p*-coumaroylglucoside. Among the non-acylated anthocyanins and after MS^2 events, 5 fragments ions were identified at *m/z* 303, 287, 317, 301 and 331 corresponding to the aglycones delphinidin, cyanidin, petunidin and malvidin, respectively (**1**–**5**) (Table 1). The analyses of the molecular ions identified these anthocyanins as monoglucosilated thanks to the common neutral loss at 162 amu (Table 1). The position of the glucosylation was placed at 3 on the aglycone ring due to their UV–vis spectra (Boido, Alcalde-Eon, Carrau, Dellacassa, & Rivas-Gonzalo, 2006; Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006). Regarding the acylated anthocyanins, compounds **6**–**9** showed the molecular ions at *m/z* 507, 521, 505 and 535 and corresponding fragment ions at *m/z* 303, 317, 301, and 331, respectively. The difference of amu between the molecular ions and the fragment ions provided the identification the deacetylglucosylated anthocyanins: delphinidin-3-acetylglucoside, petunidin-3-acetylglucoside, peonidin-3-acetylglucoside and malvidin-3-acetylglucoside (Boido et al., 2006). Compounds **10**, **12**, **13**, and **14** presented a shoulder in the UV–vis spectra within the range of 309–313 nm and fragmentation patterns characteristic of compounds acylated with *p*-coumaric acid, thus allowing their identification as *p*-coumaroyl derivatives of the monoglucosylated malvidin (*cis* and *trans*), cyanidin, and petunidin (Table 1). Only an acylated anthocyanin showed UV–vis spectra of caffeoyl derivative of anthocyanin (282, 326, 436 and 532 nm). This information plus the fragmentation pattern of its molecular ion led to the identification of the malvidin-3-caffeoylglucoside (compound **11**, Table 1).

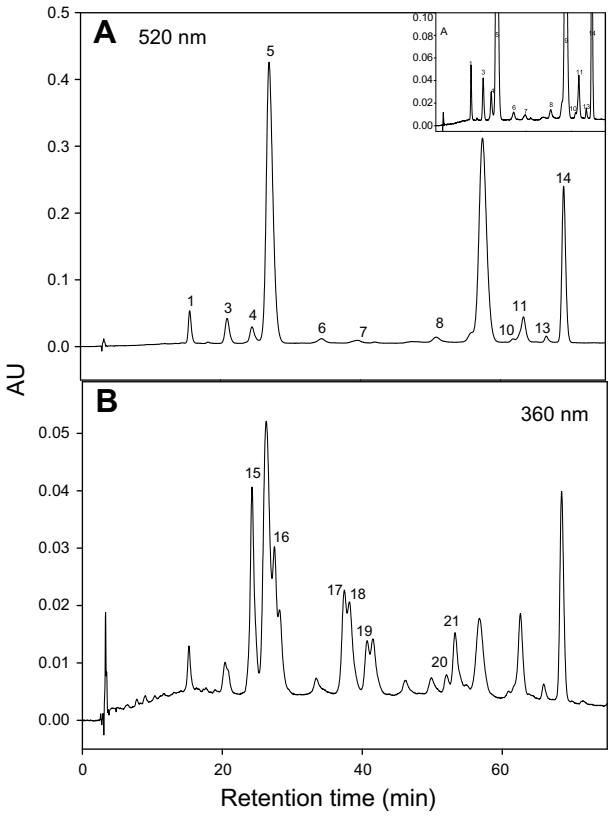

Fig. 1. Anthocyanin (A) and flavonol (B) chromatographic pattern of grape skin. (A) Delphinidin-3-glucoside (4), cyanidin-3-glucoside (2), petunidin-3-glucoside (3), peonidin-3-glucoside (4) and malvidin-3-glucoside (5), delphinidin-3-acetylglucoside (6), petunidin-3-acetylglucoside (7), peonidin-3-acetylglucoside (8), malvidin-3-acetylglucoside (9), malvidin-3-*cis*-*p*-coumaroylglucoside (10), malvidin-3-caffeoylglucoside (11), cyanidin-3-*p*-coumaroylglucoside (12), petunidin-3-*p*-coumaroylglucoside (13) and malvidin-3-*trans*-*p*-coumaroylglucoside (14). (B) myricetin-3-glucuronide (15), myricetin-3-glucoside (16), quercetin-3-glucuronide (17), quercetin-3-rutinoside (18), kaempferol-3-glucoside (19), isorhamnetin-3-glucoside (20) and syringetin-3-glucoside (21).

Quantitative differences in anthocyanins from red winegrape varieties were also found (Table 1). Total anthocyanin content ranged from 906 mg/kg of fresh weight of grapes (fw) for JT (the poorest variety) to 2640 mg/kg of fw for TR (the richest source) (Table 1). These ranges are in agreement with those previously described for TEM and CS (Ryan & Revilla, 2003; Hebrero, Santos-Buelga, & Rivas-Gonzalo, 1988). As in previous characterisations (Kallithraka, Mohdaly, Makris, & Kefalas, 2005; Mazza, 1995; Revilla, Garcia-Beneytez, Cabello, Martín-Ortega, & Ryan, 2001), malvidin-3-glucoside was the main anthocyanin in all the varieties (from 30% of total anthocyanin in TEM to 50% in JT). For autochthonous

Table 1
Anthocyanins in red-skinned grape varieties[a]

		t_R (min)	M^+, MS^2 fragments	JT	PNO	TR	CS	TEM
1	Delphinidin-3-glucoside	15.63	465, 303	28 (2)	197 (30)	98 (12)	46 (2)	107 (35)
2	Cyanidin-3-glucoside	18.40	449, 287	7 (0.3)	37 (7)	36 (1)	tr	15 (3)
3	Petunidin-3-glucoside	21.06	479, 317	42 (2)	179 (19)	119 (11)	51 (1)	91 (27)
4	Peonidin-3-glucoside	24.72	463, 301	43 (2)	96 (7)	437 (11)	43 (3)	30 (8)
5	Malvidin-3-glucoside	27.29	493, 331	425 (17)	508 (27)	1207 (67)	665 (6)	292 (76)
6	Delphinidin-3-acetylglucoside	39.64	507, 303	tr	18 (1)	tr	21 (1)	10 (2)
7	Petunidin-3-acetylglucoside	51.53	521, 317	tr	27 (1)	tr	26 (0)	15 (7)
8	Peonidin-3-acetylglucoside	56.64	505, 301	15 (1)	16 (0)	43 (5)	29 (2)	tr
9	Malvidin-3-acetylglucoside	58.43	535, 331	137 (4)	67 (2)	153 (14)	552 (14)	49 (11)
10	Malvidin-3-*p*-coumaroylglucoside (*cis*)	61.61	639, 331	11 (0)	78 (5)	21 (2)	18 (0)	59 (12)
11	Malvidin-3-caffeoylglucoside	63.43	655, 331	38 (3)	42 (5)	56 (6)	62 (1)	tr
12	Cyanidin-3-*p*-coumaroylglucoside	65.07	595, 287	tr	19 (1)	14 (0)	tr	tr
13	Petunidin-3-*p*-coumaroylglucoside	66.38	625, 317	16 (1)	67 (2)	29 (1)	21 (1)	56 (10)
14	Malvidin-3-*p*-coumaroylglucoside (*trans*)	68.62	639, 331	144 (4)	222 (7)	427 (23)	220 (2)	220 (35)
	Total anthocyanins			906 (18)	1573 (46)	2640 (75)	1754 (16)	944 (97)

[a] Values are expressed as mg/kg fw of grape berry. Standard deviation between brackets ($n = 3$). t_R, retention time; JT, Jaen tinto; PNO, Palomino negro; TR, Tintilla de Rota; CS, Cabernet Sauvignon; TEM, Tempranillo; tr, detected but not quantified.

Andalusian varieties, this is the first time that these data are reported. CS grapes did not contain cyanidin-3-glucoside, a finding reported by other authors (Revilla et al., 2001; Mattivi et al., 2006). Among the grape varieties studied, malvidin derivatives (acylated and non-acylated) are the main anthocyanins present in the skin. However, not all acylated derivatives were detected in all varieties. Malvidin-3-caffeoylglucoside was detected in all winegrape varieties except TEM, and accounted for 2–4% of total anthocyanin. In two of the three autochthonous varieties studied (JT and TR) delphinidin-3-acetylglucoside or petunidin-3-acetylglucoside were not detected. In the TEM variety peonidin-3-acetylglucoside, malvidin-3-caffeoylglucoside and cyanidin-3-*p*-coumaroylglucoside were not detected. In CS all the anthocyanin derivatives were present except derivatives from cyanidin. PNO was the only variety with detectable amounts of all the anthocyanins described.

Malvidin-3-*p*-coumaroylglucoside (*cis* and *trans* together) was the most abundant derivative in all the varieties (accounting for around 20% of total anthocyanin) (Table 1). In contrast with other varieties, CS showed a high content of malvidin-3-acetylglucoside (around 34% of total anthocyanin) (Table 1) as in previous studies (Revilla et al., 2001).

Other minor *p*-coumaroyl derivatives were also detected in grapes, some of them only in trace amounts. Peonidin-3-*p*-coumaroylglucoside was detected in TR and JT but not in quantities comparable with those previously reported in different winegrape varieties (Boido et al., 2006). Results showed higher contents of *p*-coumaroyl-anthocyanin derivatives, mainly from malvidin and petunidin, a finding which has previously been associated with warm climates (Downey et al., 2006; Cortell & Kennedy, 2006; Ryan & Revilla, 2003).

Although the anthocyanin profile may be complex and quite different for each variety studied, it is notable that the TR variety showed the highest anthocyanin concentration in grapes, largely due to the small berry size, among other factors. On the other hand, PNO presented the widest variety of anthocyanin types. In the non-autochthonous varieties, the total anthocyanin content of CS was nearly double that of TEM.

From the description presented above, anthocyanins could be considered useful markers for distinguishing grape varieties, although this characteristic should be used with care since anthocyanin content is heavily influenced not only by agronomic factors such as soil composition, irrigation, light intensity, etc., but also by the year's climatic conditions (Conde et al., 2007; Adams, 2006).

3.3. Flavonols

The UV–vis spectra of flavonols were similar, giving only information about the glycosylation at the 3 position on the main ring. Therefore, the LC–MS/MS was the only tool to identify the flavonols detected in red winegrape varieties. Eight main flavonols were found in the red winegrape varieties studied (**15**–**21**): myricetin-3-glucuronide, myricetin-3-glucoside, quercetin-3-glucuronide, quercetin-3-rutinoside, kaempferol-3-glucoside, isorhamnetin-3-glucoside and syringetin-3-glucoside (Fig. 1B; Table 2). Compounds **15** and **16** showed different deprotonated ions, but a common ion fragment at *m/z* 317 which corresponded to myricetin. The difference about the neutral loss, 176 and 162 identified these compounds as myricetin-3-glucuronide and myricetin-3-glucoside, respectively. For compounds **17** and **18**, the ion after MS^2 experiments as 301, quercetin. The first parent ion at *m/z* 477 was quercetin-3-glucuronide and the deprotonated ion at *m/z* 609 provided the fragment ion at *m/z* 301 that corresponded to a rhamnoglucosilated quercetin. The other three compounds, **19**, **20** and **21** were described as kaempferol, isorhamnetin and syringetin according to the corresponding fragment ions after

Table 2
Flavonols in red-skinned grape varieties[a]

		t_R (min)	$[M-H]^-$, MS^2 fragments	JT	PNO	TR	CS	TEM
15	Myricetin-3-glucuronide	25.08	493, 317	52 (3)	60 (4)	131 (7)	50 (8.95)	32 (0)
16	Myricetin-3-glucoside	28.55	479, 317	56 (6)	59 (2)	79 (6)	98 (10.98)	178 (39)
17	Quercetin-3-glucuronide	38.84	477, 301	32 (4)	41 (3)	103 (4)	70 (1.48)	115 (8)
18	Quercetin-3-rutinoside	39.55	609, 463, 301	35 (3)	43 (2)	100 (3)	69 (12.79)	133 (1)
19	Kaempferol-3-glucoside	40.60	447, 285	21 (1)	18 (1)	32 (4)	36 (1.70)	29 (1)
20	Isorhamnetin-3-glucoside	53.72	477, 315	13 (1)	tr	31 (3.89)	27 (3.18)	tr
21	Syringetin-3-glucoside	54.06	507, 345	23 (3)	tr	62 (5)	67 (6.48)	tr
	Total flavonols			232 (9)	221 (6)	538 (11)	417 (20)	487 (39)

[a] See footnote Table 1.

MS^2 events. In these three cases, the sugar moiety was glucose (162, neutral loss). Therefore, these compounds were identified as kaempferol-3-glucoside (**19**), isorhamnetin-3-glucoside (**20**) and syringetin-3-glucoside (**21**). These results were in agreement with previous studies (Mattivi et al., 2006).

Other authors have described the presence of laricitrin derivatives in TEM and CS (Castillo-Muñoz et al., 2007; Mattivi et al., 2006). However, the flavonol laricitrin was not found in any of the five grape varieties studied here. This discrepancy could be explained by agronomic factors and/or extraction protocols.

Total flavonols content ranged from 221 mg/kg fw to 538 mg/kg fw (Table 2). The main flavonols found were quercetin and myricetin derivatives, both in similar proportions (Mattivi et al., 2006), and differences found between varieties were small. Two of the autochthonous varieties, JT and PNO, showed lower levels of flavonols in comparison with the non-autochthonous. Kaempferol-3-glucoside accounted for 6–12% of total flavonol content, in agreement with previous results for CS and TEM (Mattivi et al., 2006). Isorhamnetin-3-glucoside and syringetin-3-glucoside were detected in CS, TR and JT, although in the latter variety, in relative low amounts.

It should be noted that total flavonol content was higher than previously described (Mattivi et al., 2006). This could be explained by higher exposure to UV in warm climates (Cortell & Kennedy, 2006). This fact is important because of the anti-carcinogenic activity of flavonols shown in clinical trials with humans (Williamson & Manach, 2005).

3.4. Hydroxycinnamic acid derivatives

The UV spectra of the compound **22** gave as result a maxima at 326 nm and a shoulder at 296 nm, characteristic of a caffeoyl derivative. The deprotonated molecular ion at *m/z* 311 and the fragment ion at *m/z* 179 confirmed the caffeic acid nature of the compound. Therefore, the additional information about the neutral loss of 132 amu identified the compound as caftaric acid (caffeoyltartaric acid) (Table 3). Compound **23** showed a deprotonated molecular ion at *m/z* 325 and main fragment ion at *m/z* 193, and so was identified by mass spectrometry as fertaric acid (feruloyltartaric acid). Compound **24** showed UV maxima at 306 nm and a shoulder at 226 nm characteristic of a coumaroyl derivative. The fragment ion after MS^2 event at m/z 163 (coumaric acid residue) and the neutral loss respect to the parent ion (m/z 295) identified the compound as coutaric acid (coumaroyltartaric acid) (Table 3). The *trans* nature of these compounds as confirmed by comparison with authentic markers of them. The fragmentation pattern of these compounds was in agreement with that found for the esters of caffeic acid, like caffeoylquinic acids which are fragmented at the ester bond (Gil-Izquierdo & Mellenthin, 2001). These compounds have been previously reported in grapes (Palma, Piñeiro, & Barroso, 2001) (Fig. 2).

The total amount of hydroxycinnamic acid derivatives ranged from 1.5 mg/kg for CS up to 3.1 for TR (Table 3). However, ferouyltartaric acid could not be quantified as it was found at less than the LQ (0.05 mg/kg fw) in all varieties. Levels previously described for these hydroxycinnamic derivatives are rather scarce. For CS and TEM some authors have previously described higher contents than those found in our study (Rodriguez et al., 2006).

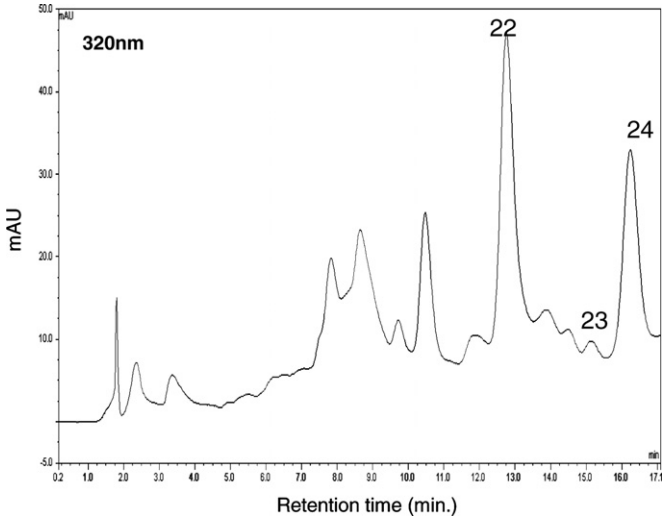

Fig. 2. Hydroxycinnamic acid derivative chromatographic pattern of grape skin. (22) *trans*-Caftaric acid, (23) *trans*-fertaric acid and (24) *trans*-coutaric acid.

TR showed the highest levels in hydroxycinnamic derivatives, as occurred with anthocyanins and flavonols. In contrast, for the hydroxycinnamic derivatives, JT did not show lower levels than the other varieties; it showed similar values as PNO and higher levels than the non-autochthonous varieties.

Table 3 shows for each variety the individual concentrations of both main hydroxycinnamic derivatives. The ratio between them was almost constant, ranging from 1.4 to 1.6 for the five varieties studied. This suggests that both compounds are being synthesized and/or degraded in the same way, regardless of grape variety.

These data show a clear difference between autochthonous and non-autochthonous varieties. Levels found for both coutaric and caftaric acids in the autochthonous varieties were from 1.5 to 1.8 times higher than in the non-autochthonous varieties. As stated before, the effect of sunlight on the levels of these compounds is not yet well established.

3.5. Flavan-3-ols

Seeds are the main source of these monomers in grapes, their presence in grape pulp and grape skins usually being negligible (Cortell & Kennedy, 2006; Cheynier, Fulcrand, Brossaud, Asselin, & Moutounet, 1998). These compounds are relevant to wine since flavan-3-ols are extracted from seeds during winemaking (Gambuti, Strollo, Ugliano, Lecce, & Moio, 2004).

Catechin and epicatechin were identified by fluorescence in grape skins and seeds (Fig. 3). As expected, the content of flavan-3-ol monomers in skin was low, ranging from 1.8 mg/kg of fw for TR, to 7.6 mg/kg of fw for TEM (Table 4). With respect to content in seed, these compounds ranged from 47 mg/kg of fw for JT to 179 mg/kg of fw for CS (Table 5). Despite the fact that a warm climate has been reported to produce a high content of flavan-3-ol

Table 3
Hydroxycinnamic derivatives in red-skinned grape varieties[a]

		t_R (min)	$[M-H]^-$, MS^2 fragments	JT	PNO	TR	CS	TEM
22	*trans*-Caftaric acid	12.95	311, 179	1.4 (0.1)	1.4 (0.1)	1.9 (0.1)	0.9 (0.0)	1.0 (0.0)
24	*trans*-Coutaric acid	16.21	295, 163	1.0 (0.0)	0.9 (0.0)	1.2 (0.0)	0.6 (0.0)	0.7 (0.0)
	Total hydroxycinnamics			2.4 (0.1)	2.3 (0.1)	3.1 (0.1)	1.5 (0.0)	1.7 (0.0)

Fertaric acid (peak **23** Fig. 2) was detected in all varieties but it could not be quantified (LQ 0.05 mg/kg fw).
[a] See footnote Table 1.

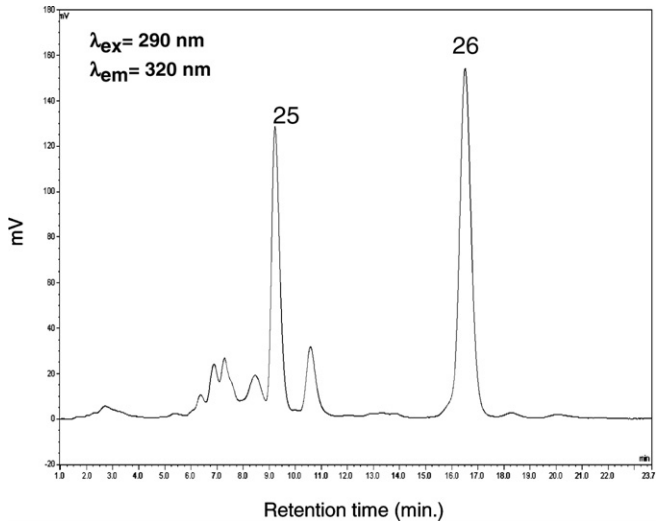

Fig. 3. Flavan-3-ol monomers chromatographic pattern grape skin. (25) Catechin and (26) epicatechin.

monomers (Rodriguez et al., 2006; Fernandez, Kennedy, & Agosin, 2007), the levels found in the varieties studied were lower than those previously described (Piñeiro et al., 2004). As previous stated (Conde et al., 2007), apart from sunlight, there are many factors that influence phenolic content including flavan-3-ols.

Catechin was more abundant than epicatechin in four of the varieties analysed, as reported by others (Piñeiro et al., 2004) but in JT the content of both compounds was similar, and relatively low.

As flavan-3-ols can be found mainly in grape seeds, two different factors may determine the final amount found in grapes, i.e. the concentration of these compounds in the grape seeds, and the total weight of the seeds in the grape. The proportion of seed to berry (seed/berry, w/w) in percentage together with the concentration of flavan-3-ols in seeds were the highest for CS (Table 5). Regarding the final concentration of flavan-3-ols in wines, the total number of seeds in grapes can play another important role: since seeds are not usually crushed during winemaking process, the efficiency of extraction of flavan-3-ols depends on the surface area of seeds. Therefore grapes containing more seeds per berry (Table 5) could contribute higher contents of these compounds in the final wine. TEM is the variety with the largest number of seeds; therefore the low concentration of flavan-3-ols in grape seeds could be overcome by a more efficient extraction process in this variety.

There is additional interest in flavan-3-ol monomers due to their wide range of beneficial effects for human health (Thomasset et al., 2007).

3.6. Total phenolic compounds

Total phenolics content was determined as the sum of anthocyanins, flavonols, hydroxycinnamic acid derivatives and flavan-3-ol monomers (together in skin and the seeds for each grape variety).

The TR variety presented considerably higher total phenolic content than the rest of the varieties; and the JT variety showed the lowest content. PNO, TEM and CS contained average concentrations related to the other two varieties (Table 6).

It is notable that all varieties showed similar proportions of the various types of phenolics, although TEM stood out because of its relatively low percentage of anthocyanins and high percentage of flavonols. Similarly, CS stood out for its high flavan-3-ol monomer contribution to total phenolics (Table 6).

From the data presented in this paper, several specific winemaking techniques could be suggested depending on the variety. For example, for TEM grapes cold maceration or soaking could be considered for a better extraction of anthocyanins and therefore for enhanced colour. In contrast, for CS a short period of maceration after alcoholic fermentation would be recommended to avoid

Table 4
Flavan-3-ol monomers in red-skinned grape varieties[a]

		t_R (min)	JT	PNO	TR	CS	TEM
25	Catechin	9.48	3.2 (0.1)	2.7 (0.1)	1.5 (0.0)	4.4 (0.1)	6.1 (0.2)
26	Epicatechin	16.75	0.6 (0.0)	0.1 (0.0)	0.3 (0.0)	0.4 (0.0)	1.5 (0.0)

[a] See footnote Table 1.

Table 5
Seed content and flavan-3-ol monomers in red grape varieties[a]

	JT	PNO	TR	CS	TEM
No. of seeds/berry	1.8 (0.2)	1.8 (0.5)	1.1 (0.1)	1.7 (0.3)	2.9 (0.4)
Individual seed weight	0.023 (0.003)	0.034 (0.003)	0.045 (0.005)	0.028 (0.001)	0.029 (0.001)
Seed/berry %, by wt.	3.1 (0.6)	4.6 (1.9)	4.0 (0.6)	5.5 (1.0)	4.1 (1.1)
Catechin (mg/kg fw)	24 (1)	45 (1)	98 (3)	111 (4)	37 (1)
Epicatechin (mg/kg fw)	23 (1)	24 (1)	79 (2)	68 (2)	11 (0)

[a] See footnote Table 1.

Table 6
Total phenolics content (mg/kg fw) and percentages of each phenolic group in red grape varieties[a]

	JT	PNO	TR	CS	TEM
Total anthocyanins (%)	906 (76%)	1573 (84%)	2640 (79%)	1754 (74%)	944 (63%)
Total flavonols (%)	232 (19%)	221 (12%)	538 (16%)	417 (18%)	487 (33%)
Total hydroxycinnamics (%)	2.4 (0.2%)	2.3 (0.1%)	3.1 (0.09%)	1.5 (0.06%)	1.7 (0.11%)
Total flavan-3-ol monomers (%)	51 (4.3%)	72 (3.8%)	180 (5.5%)	184 (7.8%)	56 (3.8%)
Total polyphenols (mg/kg fw)	1191	1868	3361	2356	1489

Percentage respect total phenolics content between brackets.
[a] See footnote Table 1.

high astringency in the wine (Sacchi, Bisson, & Adams, 2005). TR presents high potential according to its polyphenolic profile. A standard winemaking process is suggested to obtain wines which express the varietal flavours of this variety; meanwhile a proof extraction process, such as an increase in the frequency of pumping over, can produce a full-body wine optimum for aged in oak.

4. Conclusions

In summary, 26 phenolic compounds belonging to four phenolic classes (anthocyanins, flavonols, hydroxycinnamic acids and flavan-3-ols) have been characterised in the skin and seeds of five red wine grape varieties by liquid chromatography coupled with DAD, fluorescence detector and ESI–MS in negative and positive modes. As far as we are aware, such a complete study has not previously been carried out, nor has any similar data related to varieties autochthonous to Andalusia been published.

TR was the variety that presented the highest content of anthocyanins, flavonols and hydroxycinnamic derivatives, but not of flavan-3-ol monomers. In this last case, the CS variety stood out for its relatively high content of flavan-3-ol monomers, probably due to the high seed/berry proportion in this variety. Thus, the phenolic content of grapes does not depend on whether the variety is autochthonous or non-autochthonous; it appears to be mainly a question of the characteristics of the particular variety, as well as the agroclimatic factors.

The findings of this and similar studies is useful for optimising winemaking processes to produce wines "tailor-made" to predetermined requirements, depending largely on their detailed phenolic content. This approach is especially relevant in an area such as Andalusia (Southern Spain) which has such a warm climate, yet does not have a tradition of making red wines, but where interest and ability in this sector is currently increasing.

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