Concentration of flavanols in red and white winemaking wastes (grape skins, seeds and bunch stems), musts, and final wines

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Running title: Flavanols in winemaking by-products

Abstract

The winemaking industry produces huge quantities of different types of waste, such as bunch stems, grape skins and grape seeds. Knowledge of the composition of these wastes is essential if their disposal is to be appropriate. However, they can contain compounds such as flavanols that are beneficial to human health and of interest to the pharmaceutical and cosmetics industries.

The aim of this work was to establish whether flavanols are present, and in what concentration, in the above-mentioned wastes, as well as in the musts and final wines derived from the internationally known Albariño (white) and Mencía (red) grapevine varieties. Extractions were performed using appropriate solvents, and the compounds obtained identified by HPLC-MS QTOF. All three Albariño wastes had higher concentrations of flavanols than did the Mencía wastes. Flavanols were in very low concentration in the Albariño must, but virtually absent from the Mencía must. The Mencía wine, however, had much higher concentrations of these compounds than did the Albariño wine. This is explained in that these compounds are passed to the final wine in Mencía (and likely in other red wines). The present results suggest that red winemaking wastes are poorer in these compounds, while white winemaking wastes - certainly Albariño wastes - provide a potential source useful to industry.

Keywords: Albariño, Mencía, flavanols, white wine, red wine, steeping, winemaking by-products

INTRODUCTION

Viticulture is of great importance to many countries. According to the International Organisation of Vine and Wine (OIV 2017), some 7586 million ha of land around the world were given over to the growing of grapevines in 2016, enough to produce some 241 million hL of wine. Spain has the largest area under grapevines with 975 million ha, or 14% of the world's total viticultural land. It was the world's largest wine producer in 2013, and the third largest in 2016 after France and Italy. In Galicia

(northwestern Spain), winemaking is a major agroindustrial activity. The region possesses five Denomination of Origin (DO) areas, and in 2012/2013 produced 307,728 hL of wine (<http://www.magrama.gob.es/es/alimentacion/temas/calidadagroalimentaria/calidaddiferenciada/dop/htm/cifrasydato.aspx#> accessed 16.01.15). The Albariño (white) and Mencía (red) grapevine varieties are those most commonly grown. The former is native to Galicia but is of growing interest in other parts of Spain and abroad. Its cultivation has been authorised in France, and it is being planted in Australia and other countries.

The vitiviniculture industry produces both vineyard wastes, such as pruned wood and green material, and winemaking wastes, such as bunch stems left over after the grapes are removed from the clusters, and grape skins and seeds (together known as grape pomace) left over after pressing. Grape pomace has traditionally been used to make spirits, but this is ever less common, and new ways of making use of this waste are being sought.

Polyphenols are some of the most numerous secondary metabolites of plants (Aubert et al. 2018; El Gharras 2009; Liu and White 2012; Shi et al. 2005), and are commonly found in grape pomace. These compounds are divided into several groups, one of which is the flavonoids. Flavonoids can themselves be classified into six major groups: anthocyanins, flavones, isoflavones, flavanones, flavonols, and flavanols (Galanakis 2018). The maximum concentration of these compound is highly dependent on the vintage, the grape variety, fruit developmental stage, and fruit part (De la Cerda et al. 2015; Jordão et al. 1998; Marjan et al. 2016). Most authors report procyanidin B1 (an oligomeric procyanidin) to be the major oligomer in the bunch stems and skins, and procyanidin B2 to be the major oligomer in the seeds (De la Cerda et al. 2015; Jordão et al. 1998, 2001; Marjan et al. 2016).

Flavanoids have antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties. They may protect from oxidative stress and therefore help prevent the appearance of a number of diseases (De la Cerda et al. 2015; El Gharras 2009; Galanakis 2018; Liu and White 2012; Jordão et al. 1998, 2001; Quideau et al. 2010; Marjan et al. 2016). Consequently, they are of interest to the health and cosmetics industries. Flavanols from grape seeds have attracted considerable attention

given their apparent potential to prevent cancer (Chen et al. 2014; Fontana et al, 2013; Kampa et al. 2011; Katiyar and Athar 2013; Lachman et al. 2013), to reduce the risk of developing cardiovascular, cerebrovascular and neurodegenerative diseases, to reduce cholesterol levels, and to prevent different immune disorders (Liu and White 2012). Many studies have examined the flavanol content of musts, wines, grape seeds and grape skins, but only a few have examined their contents in bunch stems (Marjan et al. 2016; Gonzalez-Centeno et al. 2012; Jara-Palacios et al. 2016). The present work examines the flavanol concentration of Mencía (red) and Albariño (white) winemaking wastes (i.e., grape skins, seeds and bunch stems), as well as in the musts and final wines made from these varieties. It was hypothesised that the passage of flavanols from the different waste fractions to the must and final wines might differ. The results also provide preliminary indications regarding the potential of winemaking wastes as a source of flavanols for the pharmaceutical and cosmetics industries.

MATERIALS AND METHODS

Plant material and growing conditions

The Albariño and Mencía plants (*Vitis vinifera* L) used in this work were grown in an experimental plot at the *Misión Biológica de Galicia-CSIC* research station in Pontevedra (Galicia, Spain) (42° 25' N, 8° 38' W, altitude 20 m). All vines were grown *en espalier* and pruned according to the Sylvoz system. Rows were set 2.5 m apart; the distance between plants was 2 m. All plants were the same age, were cultivated in the same way, and received the same plant protection treatments.

Sample processing

Randomized portions of clusters from around the experimental vineyard, weighing a total of approximately 1 kg, were collected for each variety during the period of grape ripening (on the 17th October for Albariño, and the 24th for Mencía). The berries were separated from the clusters and finger-pressed to remove the pulp. The leftover grape stems, skins and seeds were then stored at -80°C until processing to extract their flavanols. Other collected berries were crushed in a glass mortar and the juice obtained was statically racked at 4°C for 24 h. The juice was then decanted to fill 50 mL

Falcon tubes (two per genotype) and immediately frozen at -40°C. Wines were made as shown in Figure 1. All waste, must and wine analyses were made in duplicate.

HPLC-MS and MS/MS analyses for flavanols

The grape skins, seeds and bunch stems were ground to a powder. Their flavanols were then extracted according to the method of Perez-Jimenez et al. (2009) with slight modifications, using 1 g of each powdered sample (performed in duplicate). The first extraction was performed with 20 mL methanol/water/formic acid (50:49:1 v/v/v) in an ultrasound bath for 1 h, followed by centrifugation at 2500 g for 10 min, keeping the supernatant. A second extraction of the residue left over from the first extraction was then performed using 20 mL acetone/water (70:30 v/v) in an ultrasound bath for 1 h, and then centrifuging again at 2500 g for 10 min. The supernatant was mixed with that from the first extraction to obtain a final sample and an aliquot was then again centrifuged at 2500 g for 10 min, decanted into vials, and analysed in a high performance liquid chromatograph coupled to a quadrupole time-of-flight mass spectrometer (HPLC-MS QTOF).

The musts and wines were diluted 50% with deionised water, decanted into vials, and analysed using the same HPLC-MS QTOF system.

The HPLC-MS QTOF system involved an Agilent 1200 series HPLC system equipped with an Agilent ZORBAX Eclipse XDB-C18 column (United States) (4.6 mm × 150 mm × 5 µm) at 40°C.

The mobile phase consisted of water containing 1% formic acid (A), and acetonitrile with 1% formic acid (B). The elution gradient was 5% B at 0 min, 15% B at 20 min, 25% B at 30 min, 30% B at 40 min and 5% at 32 min to 35 min. The flow rate was 1 ml/min. Flavanol identification/quantification was performed by MS and MS/MS (Q-TOF acquisition: 2GHz, low mass range [1700 m/z], negative polarity, drying gas 10 l 350°C, sheath gas 11 l 350°C, nebulizer 45 psi, cap voltage 4000 V, fragmentor voltage 150 V). A collision energy of 20V was used for all MS/MS experiments. Data capture and analysis were performed using Data Analysis v.B.02.01 and Qualitative Analysis v.B.04.00 or MassHunter Workstation software (Agilent Technologies, Waldbroon, Germany). Flavanols were identified as monomers, dimers, trimers and tetramers. All monomers were quantified

using the (+)-catechin pattern from Cymit Quimica (Barcelona, Spain); dimers, trimers and tetramers were quantified using the procyanidin B1 pattern from Extrasynthese (Genay, France). Results were expressed as $\mu\text{g/g}$ for the wastes and $\mu\text{g/mL}$ for the musts and wines.

Technical summary of flavanols detected

The flavanol monomers identified were catechin, epicatechin, galocatechin, epigallocatechin, epigallocatechin gallate, and epicatechin-3-gallate.

Six compounds were found with an exact mass and fragmentation pattern compatible with a dimer structure (Table 1, Figure 2): these were named dimers 1-6 according to their elution order. Dimers 1 and 4 were identified by their retention times as procyanidin B1 and procyanidin B2. All the dimers showed the same MS/MS fragmentation pattern with a fragment at m/z 289 compatible with a monomer.

Eight compounds were found with an exact mass and fragmentation pattern compatible with a trimer structure (Table 1, Figure 3): these were named trimers 1-8 according to their elution order. None could be identified according to retention time agreement with available information. However, all had the same MS/MS fragmentation pattern, with a fragment at m/z 577 compatible with a dimer.

Two compounds were found with an exact mass and fragmentation pattern compatible with a tetramer structure (Table 1, Figure 4): these were named tetramers 1 and 2 according to their elution order. None could be identified according to retention time agreement with available information. However, both had the same MS/MS fragmentation pattern, with a fragment at m/z 865 compatible with a trimer.

RESULTS AND DISCUSSION

The Albariño variety, had the greatest flavanol content in the bunch stems, followed by the seeds, the grape skins, must and finally wine. The must contained over twice the proanthocyanidin content of

the wine, but both showed little variability in terms of the types of flavanol present (Table 1, Figure 5). In the bunch stems, the major flavanol was catechin. Procyanidin B1 was also abundant, as were some of its derivatives, along with types of procyanidin trimer (Table 1). The Albariño seeds also contained large amounts of catechin (again, the majority compound), as well as epicatechin and procyanidins B1 and B2 and some of their derivatives. The grape skins mainly possessed procyanidin B1, along with catechin and different types of procyanidin trimer, although in much lesser quantity than in the bunch stems and seeds. Only very small quantities of other compounds were detected (or not detected at all). The Albariño must had a very low concentrations of the compounds analysed - indeed they were quite insignificant compared to those of the bunch stems and seeds. The Albariño wine had even lower concentrations.

The concentrations of flavanols detected in the Albariño seeds, grape skins and musts were smaller than those reported for the same variety by Lecce et al. (2014), although these authors used different extraction and analytical techniques. However, both sets of results agree in that, among these wastes, the seeds had the highest concentrations, followed by the grape skins and then the must. The literature contains no reports of proanthocyanidin measurements for bunch stems or wine for these varieties.

The Mencía variety, had the greatest proanthocyanidin content in the seeds, followed by the bunch stems, grape skins, wine and finally the must (Table 1). The most abundant flavanols in the seeds was catechin, followed by epicatechin, procyanidin B1 and its derivatives, and procyanidin B2. In the bunch stems, catechin was the most common, followed by procyanidin B1 and its derivatives, and procyanidin trimers. The grape skins contained mainly procyanidin B1, followed by catechin and some procyanidin trimer and tetramers (Figure 6). No other compounds were detected, or detected only in very small quantities. The must contained very low concentrations of these compounds, even lower than those found for the Albariño must (Table 1, Figure 7). The Mencía wine had a lower concentration of these compounds than did the bunch stems and seeds, but close to that recorded for the skins, and much greater than that recorded for the Albariño wine.

The literature contains few reports on the concentration of flavanols in Mencía material, and those that do exist involved solvents and analytical methods different to those employed in the present work (Letaief et al., 2007; Pazo et al., 2004; Segade et al., 2009). The present results do, however, agree with those of Segade et al. (2009) for this variety, at least in terms of the types of proanthocyanidin present. The content of the seeds was also reported to be higher than in the grape skins, although only about three times higher rather than the 37 times seen in the present work.

The difference between the flavanol contents of the Mencía and Albariño wines (e.g., the proanthocyanidin content of the former was 16 times that of the latter) is undoubtedly a consequence of the production methods used. Mencía wine is made by fermenting the must with the skins and seeds, and flavanols must be released from them, along with other polyphenols (Flanzy 2003; Fontana et al. 2013). This also explains why the Albariño skin and seed wastes were much richer in these compounds. Indeed, in its seeds, bunch stems and grape skins, the Albariño variety had respectively 1.3, 4.8 and 23 times the concentration recorded for the corresponding Mencía wastes (Table 1). In the Albariño must, the concentration was some 60.8 times higher.

In summary, the present results show the concentration of flavanols to be much greater in the bunch stem, seed and skin wastes of the Albariño variety compared to those of the Mencía variety. The Albariño must also had a higher concentration than the Mencía must, although both concentrations were very low. In contrast, the wine of the Mencía had a much higher flavanol content than the Albariño wine. In agreement, Pussa et al. (2006) indicated white grape stem bunch waste to contain significantly lower concentrations of total polyphenols than those of red grapes. These authors also reported the bunch stem waste of red varieties to be richer in anthocyanins than those of white varieties; however, anthocyanins are only one component of flavanols. Other authors (González-Centeno et al. 2012) have reported a lack of significant differences in the flavanol concentrations of the bunch stem wastes of red (Cabernet Sauvignon, Callet, Manto Negro, Shyrah, Tempranillo) and white (Chardonnay, Macabeu, Parellada and Prebsal Blanc) varieties. The flavanol content of different parts of grape clusters may therefore be a varietal trait, quite independent of whether the

variety is red or white. This requires further study if the best use is to be made of the varieties grown in different places. Nonetheless, the present work indicates that the winemaking wastes of the Albariño variety could be important sources of flavanols.

CONCLUSIONS

The present results provide preliminary data regarding the content of flavanols in the bunch stems, grape skins and grape seeds from the Albariño and Mencía varieties, the most cultivated varieties in the northwest of Spain. The concentration of flavanols was much greater in all three waste fractions of the white variety than the red variety, and was particularly high in the Albariño bunch stems. Surprisingly, the Mencía wine had a higher flavanol concentration than did the Albariño wine. This is almost certainly due to the fact that the Mencía wine is produced from must fermented in the presence of the wastes (from which the flavanols leach out), whereas the Albariño wine is made in their absence. Naturally, this leaves the Albariño wastes with higher flavanol concentrations than the Mencía wastes, and therefore of greater interest as a source of these compounds.

Acknowledgments: The authors also thank Adrian Burton for editing and linguistic assistance and Elena Zubiaurre, Iván González, Ana Martín for technical assistance. We are grateful to the Analysis Service Unit of the ICTAN for performing the chromatography and mass spectrometry analyses.

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269

270 **Table 1. Mean flavanol contents of the examined materials**

COMPOUNDS				Bunch stems				Grape seeds				Grape skin				Must				Wine			
				Albariño		Mencia		Albariño		Mencia		Albariño		Mencia		Albariño		Mencia		Albariño		Mencia	
FLAVONOLS (ug/g wastes) (ug/mL Must-Wine)	FORMULA	M-H	TRmin	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Monomer 1 Catechin	C ₁₅ H ₁₄ O ₆	289.1	8.7	1201.00	13.38	157.57	16.84	823.90	107.71	457.66	30.11	19.65	5.51	4.83	0.93	1.5	0.25	ND		0.47	0.03	2.48	0.38
Monomer 2 Epicatechin	C ₁₅ H ₁₄ O ₆	289.0718	13.3	59.85	3.30	7.04	0.23	445.20	58.20	429.86	34.99	4.00	1.40	1.26	0.19	ND		ND		0.68	0.17	5.23	0.55
Monomer 3 Gallocatechin	C ₁₅ H ₁₄ O ₇	305.0667	4.2	34.08	0.62	9.72	2.13	ND		ND		0.70	0.17	0.95	0.12	ND		ND		ND		1.16	0.26
Monomer 4 Epigallocatechin	C ₁₅ H ₁₄ O ₇	305.0667	7.7	2.96	0.36	0.81	0.07	ND		ND		ND		0.16	0.02	ND		ND		ND		0.76	0.15
Monomer 5 EpiGalocatechin gallate	C ₂₂ H ₁₈ O ₁₁	457.0776	13.3	1.63	0.15	1.94	0.19	ND		ND		ND		ND		ND		ND		ND		ND	
Monomer 6 Epicatechin-3-gallate	C ₂₂ H ₁₈ O ₁₀	441.0827	21.0	21.61	1.67	14.41	0.93	25.72	4.38	61.50	5.29	0.67	0.19	0.28	0.03	ND		ND		ND		ND	
TOTAL MONOMERS (Catechin standard)				1321.13	19.48	191.50	20.39	1294.83	170.29	949.02	70.39	25.01	7.27	7.49	1.28	1.5	7.27	0.00	1.28	1.15	7.27	9.63	1.28
Dimer 1 Procyanidin B1	C ₃₀ H ₂₆ O ₁₂	577.1351	7.1	925.54	34.71	213.15	10.29	106.27	14.16	97.75	13.90	39.41	3.32	19.84	2.76	2.5	0.5	ND		0.69	0.17	8.85	0.7
Dimer 2	C ₃₀ H ₂₆ O ₁₂	577.1351	7.7	350.00	12.22	60.49	4.18	177.62	23.70	97.24	16.82	6.33	0.86	1.60	0.04	0.3	0.17	ND		ND		3.15	0.59
Dimer 3	C ₃₀ H ₂₆ O ₁₂	577.1351	10.2	19.85	0.93	4.86	0.15	205.57	18.82	97.96	17.32	2.62	0.45	0.73	0.13	ND		ND		ND		2.53	0.51
Dimer 4 Procyanidin B2	C ₃₀ H ₂₆ O ₁₂	577.1351	11.34	13.27	0.46	3.53	0.00	141.92	12.53	149.00	26.39	1.67	0.52	0.95	0.08	ND		ND		0.14	0.04	6.59	0.63
Dimer 5	C ₃₀ H ₂₆ O ₁₂	577.1351	12.8	20.20	0.38	4.41	0.64	20.76	3.64	14.59	4.62	ND		ND		ND		ND		ND		0.16	0.03
Dimer 6	C ₃₀ H ₂₆ O ₁₂	577.1351	16.4	64.73	2.35	18.35	1.06	22.17	1.85	23.85	4.51	2.50	0.34	0.83	0.13	ND		ND		0.01		0.81	0.2
TOTAL DIMERS (Procyanidin B1 standar)				1393.60	51.05	304.79	16.01	674.31	74.70	480.39	83.55	52.54	5.49	23.96	2.97	2.8	5.49	0.00	2.97	0.84	5.49	22.09	2.97
Trimer 1	C ₄₅ H ₃₈ O ₁₈	865.1923	3.0	170.56	1.02	46.40	1.62	41.06	5.11	45.07	7.19	5.09	0.36	1.15	0.23	0.38	0.02	ND		ND		1.69	0.03
Trimer 2	C ₄₅ H ₃₈ O ₁₈	865.1923	8.3	36.03	1.90	6.22	0.50	48.72	6.17	29.92	5.20	0.87	0.10	ND		ND		ND		ND		ND	
Trimer 3	C ₄₅ H ₃₈ O ₁₈	865.1923	9.1	191.22	3.36	55.02	2.36	52.64	4.79	58.49	8.66	14.63	0.85	8.31	0.40	0.41	0.01	ND		ND		2.76	0.55
Trimer 4	C ₄₅ H ₃₈ O ₁₈	865.1923	9.6	42.41	0.82	12.41	0.96	12.39	0.93	13.30	1.98	1.98	0.01	1.23	0.09	ND		ND		ND		0.65	0.1
Trimer 5	C ₄₅ H ₃₈ O ₁₈	865.1923	11.0	ND		ND		18.42	2.07	15.11	3.79	ND		ND		ND		ND		ND		0.88	0.07
Trimer 6	C ₄₅ H ₃₈ O ₁₈	865.1923	11.5	8.13	1.30	ND		36.70	3.46	17.56	2.55	ND		ND		ND		ND		ND		0.24	0.01
Trimer 7	C ₄₅ H ₃₈ O ₁₈	865.1923	14.5	107.19	7.64	25.65	1.50	55.23	5.28	40.26	4.05	5.39	0.31	1.98	0.22	ND		ND		ND		0.83	0.02
Trimer 8	C ₄₅ H ₃₈ O ₁₈	865.1923	15.4	8.04	1.51	1.66	0.11	67.07	4.77	67.19	10.73	1.00	0.24	0.52	0.00	ND		ND		ND		1.9	0.03
TOTAL TRIMERS (Procyanidin B1 standard)				563.57	17.55	147.36	6.83	332.23	32.59	286.88	44.14	28.97	1.88	13.19	0.94	0.79	1.88	0.00	0.94	0.00	1.88	8.95	0.94
Tetramero 1	C ₆₀ H ₅₀ O ₂₄	1153.262	9.2	41.64	2.72	14.97	0.50	12.05	0.73	14.69	2.14	3.47	0.42	2.99	0.22	0.14	0.01	ND		ND		0.56	0.01
Tetramero 2	C ₆₀ H ₅₀ O ₂₄	1153.262	16.6	8.62	1.04			26.75	1.49	21.73	2.74	0.61	0.14	0.08	0.01	ND		ND		ND		0.37	0.01
TOTAL TETRAMERS (Procyanidin B1 standard)				50.26	3.76	14.97	0.50	38.80	2.22	36.42	4.88	4.08	0.57	3.07	0.22	0.14	0.57	0.00	0.22	0.00	0.57	0.93	0.22
TOTAL FLAVANOLS				3328.56	88.08	658.62	43.24	2340.17	277.58	1752.71	198.09	110.60	14.64	47.70	5.19	5.23	14.64	0.00	5.19	1.99	14.64	41.60	5.19

272 ND: not

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FIGURE LEGENDS

Figure 1. Differences in the white and red winemaking process

Figure 2. Dimer MS/MS fragmentation

Figure 3. Trimer MS/MS fragmentation

Figure 4: Tetramer MS/MS fragmentation

Figure 5. Total flavanols in wastes ($\mu\text{g/g}$), musts and wines ($\mu\text{g/mL}$)

Figure 6. Flavanols in wastes ($\mu\text{g/g}$): A = monomers, B dimers, C trimers, and D tetramers

Figure 7: Flavanols in musts and wines ($\mu\text{g/mL}$): A = monomers, B dimers, C trimers, and D tetramers

Figure 1

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Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7