Analytical Methods

Phenolic profiling of the skin, pulp and seeds of Albariño grapes using hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry

Giuseppe Di Lecce [a], Sara Arranz [b,d], Olga Jáuregui [c], Anna Tresserra-Rimbau [a,d], Paola Quifer-Rada [a,d], Rosa M. Lamuela-Raventós [a,d,*]

[a] Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain
[b] Department of Internal Medicine, Hospital Clinic, Institut d'Investigacions Biomédiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain
[c] Unitat de Tècniques Separatives, Centres Cientifics i Tecnologics (CCiTUB), Universitat de Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain
[d] CIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain

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ABSTRACT

This paper describes for the first time a complete characterisation of the phenolic compounds in different anatomical parts of the Albariño grape. The application of high-performance liquid chromatography coupled with two complementary techniques, hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry, allowed the phenolic composition of the Albariño grape to be unambiguously identified and quantified. A more complete phenolic profile was obtained by product ion and precursor ion scans, while a neutral loss scan at 152 u enabled a fast screening of procyanidin dimers, trimers and their galloylated derivatives. The compounds were confirmed by accurate mass measurements in QqToF-MS and QqToF-MS/MS modes at high resolution, and good fits were obtained for all investigated ions, with errors ranging from 0.2 to 4.5 mDa. To the best of our knowledge, two flavanol monomer hexosides were detected in the grape berry for the first time.

1. Introduction

Albariño (*Vitis vinifera* L.) is the most important white grape variety grown in the northwest of Spain (Galicia), notably in the Rías Baixas Denomination of Origin. Although there are five different varieties of grape cultivated in this region, Albariño grape provide 95% of the annual harvest obtaining a total of 40 million kg of grapes to produce 286,000 hectoliters of wine annually. Albariño wine is characterised by an appreciated aromatic profile and organoleptic properties (Diéguez, Lois, Gómez, & de la Peña, 2003; Vilanova, Genisheva, Masa, & Oliveira, 2010). Masa, Vilanova, and Pomar (2007) and Rodríguez-Bernaldo de Quirós, Lage-Yusty, and López-Hernández (2009) determined the flavonoid profile of Albariño grape skin and the antioxidant activity of Albariño wines by high performance liquid chromatography (HPLC) (Masa et al., 2007; Rodríguez-Bernaldo de Quirós et al., 2009).

Phenolic compounds are responsible for the colour, astringency and bitterness of wines and it has been demonstrated that the sensory perception of coarseness increases with the degree of galloylation of proantocyanidins (Vidal et al., 2003).

Grape phenols consist of a wide range of structures diversely distributed in every part of the berry (Adam, 2006), but they are present mainly in the skin and seed (Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006; Dietmar, Achim, Reinhold, & Schieber, 2004).

The most abundant phenolic compounds in white grape skin are flavonols, while flavan-3-ol monomers such as (+)-catechin and (−)-epicatechin, as well as dimers, trimers and polymeric forms, also called procyanidins (2–10 units), are present mainly in grape seed. These compounds may contain subunits of gallic acid, epigallocatechin or epicatechin gallate linked by an interflavan bond (Hayasaka, Waters, Cheynier, Herderich, & Vidal, 2003).

In last two decades liquid chromatography mass spectrometry has been widely employed for the characterisation of several food matrices (Justesen, Knuthsen, & Leth, 1998; Zhou, Xu, & Choi, 2009). Electrospray ionisation has proven to be a powerful tool that facilitates the analysis of non-volatile, thermally labile compounds. Different mass analysers, triple-quadrupole instruments (Sánchez-Rabaneda et al., 2004; Cavaliere et al., 2008), ion-trap mass analysers, and high-resolution instruments such as time-of-flight (or the hybrid configuration quadrupole-time-of-flight, Vallverdú-Queralt,

* Corresponding author. Address: Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain. Tel.: +34 934034843; fax: +34 934035931.
E-mail address: lamuela@ub.edu (R.M. Lamuela-Raventós).

Jáuregui, Di Lecce, Andrés-Lacueva & Lamuela-Raventós, 2011b) and Fourier trasformation mass spectrometry (FTMS, Vallverdu-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010) have been used for chemical characterisation of food matrices.

Specifically, polyphenol composition in food has been analysed using triple quadrupole instruments, applying MS/MS techniques such as product ion scan (PIS), precursor ion scan (PrI), and neutral loss scan (NL) (Sánchez-Rabaneda et al., 2004; Vallverdu-Queralt et al., 2010). A quadrupole instrument in full scan mode shows a poor signal-to-noise-ratio (if compared with the ratio of a high resolution instrument) but MS/MS techniques such as PrI or NL allow polyphenol families to be screened. In addition, hybrid high-resolution instruments such as Qq-ToF and IT-FTMS can produce high-quality MS/MS spectra, including high-resolution data for the determination of molecular formulae. Both MS and MS/MS experiments can be performed for high accuracy and high resolution analysis.

As far as we know, recent studies of the phenolic profile of the Albariño *cultivar* have only described the flavonoid composition. The aim of this paper is to report the first study on the qualitative and quantitative characterisation of phenolics in the different anatomical parts of the Albariño grape using two complementary QqToF and QqQ instruments to determine structures based on fragmentation patterns.

2. Materials and methods

2.1. Chemicals

The standards were handled without exposure to light. Vanillic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, *trans*-caffeic acid, *trans*-ferulic acid, protocatechuic acid, *m*-, *o*- and *p*-coumaric acids, gallic acid, homovanillic acid, (+)-catechin, (−)-epicatechin, *trans*-resveratrol, *trans*-piceid, kaempferol, myricetin, apigenin, (−)-epigallocatechin, (+)-catechin-gallate, quercetin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside and L-tryptophan were purchased from Sigma–Aldrich (St Louis, MO, USA). Kaempferol-3-*O*-glucoside, procyanidin dimers A2, B1 and B2, trimer C1, (−)-epicatechin-gallate, ethyl gallate, catechin-gallate and tyrosol were purchased from Extrasynthèese (Genay, France). *cis*-Resveratrol and *cis*-piceid were obtained after exposure of the *trans*-isomer standards to UV light (Romero-Pérez, Ibern-Gómes, Lamuela-Raventós, & de la Torre-Boronat, 1999), whereas *trans*-caftaric and *trans*-coutaric acids were isolated from grapes (Vrhovšek, 1998). HPLC-grade acetonitrile and acetic acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain), while ultrapure water was obtained from a Millipore system (Millipore, Bedford, MA, USA).

2.2. Extraction procedure

Albariño grape berries were harvested at the Miguel Torres winery in Vilafranca del Penedès (Barcelona, Spain). The samples were collected at 22 ± 0.6 Brix and immediately frozen ($-20\ °C$) until analysis. Sample extraction was performed in a dark room with a red safety light to prevent oxidation of the analytes during the process. Frozen grapes were manually separated into skin (0.5 g), pulp (5 g) and seeds (0.5 g). The extraction procedure, for the three fractions, was carried out under low temperature, as previously described by our research group (Vallverdú-Queralt et al., 2011a), using 5 mL of ethanol/water 80:20 (v/v) at pH 3.5 (acetic acid). The homogenates, obtained by an Ultra Turrax (IKA, Staufen, Germany), were centrifuged (2500g, 20 min at 4 °C), and the supernatants were collected; the extraction procedure was repeated two

times. Both fractions were combined and the ethanolic portion was evaporated with a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas. After filtration of the aqueous extracts with 0.45 μm PTFE syringe filters (Waters Corporation, Massachusetts, USA), the samples were stored at $-20\ °C$ and then injected into the HPLC-UV-QqToF and QqQ systems.

2.3. HPLC-UV and mass spectrometry conditions

The chromatography was performed with an HPLC Agilent 1200 RRLC (Santa Clara, CA, USA), using a Nucleosil 120 C18 column (250 mm × 4 mm, 5 μm particle size, Teknokroma, Barcelona, Spain). A constant flow rate of 0.8 mL min^{-1} was used with two solvents: solvent A consisted of water with 0.8% acetic acid (pH 2.65), and solvent B was 20% solvent A mixed with 80% acetonitrile; injection volume was 20 μL. The column was kept at 40 °C and the separation of phenolic compounds was carried out in 45 min under the following conditions: 0 min, 100% A; 5 min, 98% A; 10 min, 96% A; 15 min, 90% A; 30 min, 80% A; 35 min, 70% A; 40 min 0% A and 45 min, 100% A (Betés-Saura, Andrés-Lacueva, & Lamuela-Raventós, 1996). The column was equilibrated for 5 min prior to each analysis. The chromatograms were monitored, with UV detector Agilent SL Plus, at three wavelengths: 280, 320, and 365 nm. Each wavelength was suitable for each group of compounds: 280 nm was used for hydroxybenzoic acids, flavan-3-ols and the oligomeric procyanidins, 320 nm for hydroxycinnamic acids and their tartaric esters, and 365 nm for flavonols.

Individual compounds were quantified using a calibration curve of the corresponding standard compound. When reference compounds were not available, the calibration of structurally related substances was used. All analysis were performed in triplicate.

2.3.1. QqToF analysis

The HPLC system was coupled on-line to a hybrid quadrupole time-of-flight QSTAR Elite (ABSciex, Concord, Ontario, Canada). The MS acquisition was performed using negative ionisation between *m/z* 100 and 1050 with the Turbo Ionspray source. In addition, QqToF was used to obtain product ion information. The MS parameters were: ion spray voltage, −4200; declustering potential (DP), −60; focusing potential (FP), 190; declustering potential two (DP2), 15; ion release delay (IRD), 6 V; ion release width (IRW), 5 ms; nebulizer gas, 50 (arbitrary units), curtain gas, 60 (arbitrary units), and auxiliary gas N$_2$, 6000 cm^3 min^{-1} heated at 400 °C. The QqToF-MS instrument was calibrated after every three samples injected using two external reference compounds at *m/z* 112.9854 (CF$_3$COO$^-$) and *m/z* 1033.9880 (P$_3$N$_3$(OCH$_2$(CF$_2$)CF$_2$H)$_6$-CF$_3$COO$^-$), respectively (1 pmol μL^{-1}, ESI Tuning Mix Agilent solution). The MS/MS acquisition was also performed using information-dependent acquisition (IDA) between *m/z* 100 and 1050. IDA experiments were done at a fixed collision energy of 30 V and modified if no-fragmentation (or excessive) was produced. Acquisition and analysis of data were performed with Analyst QS 2.0 software (ABSciex, Concord, Ontario, Canada).

2.3.2. QqQ analysis

An API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) equipped with a Turbo Ionspray source in negative-ion mode was used to obtain product ion and neutral loss information. Turbo Ionspray source settings were as follows: ion spray voltage, −3500 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 4 (arbitrary units); focusing potential, −200 V; entrance potential, 10 V; drying gas (N$_2$), heated to 400 °C and introduced at a flow rate of 8000 mL min^{-1}. The DP and collision energy (CE) were optimised for (+)-catechin (DP −50 and CE −25 V), procyanidin B1 (DP −50

and CE −35 V), *trans*-caffeic acid (DP −40 and CE −20 V), and quercetin-3-O-glucoside (DP −60 and CE −30 V) in infusion experiments. Individual standard solutions (10 μg mL^{-1}) dissolved in 50:50 (v/v) mobile phase were infused at a constant flow rate of 5 μl min^{-1}, using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Data acquisition was performed scanning from *m/z* 100 to 1050 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In NL experiment, loss of 162 u corresponds to the loss of a glucose or galactose, while loss of 152 u, derived from the product of Retro-Diels–Alder rearrangement, correspond to dimer and trimer procyanidins as well as flavanol galloyl derivatives. Neutral loss experiments at 162 u and 152 u, were performed by scanning within the range of 300–600 u and from 250–900 u, respectively.

3. Results and discussion

3.1. HPLC–ESI-QqToF-MS and HPLC–ESI-QqQ-MS for the determination of phenolic compounds in grape skin, pulp and seed extracts

Phenolic extracts of skin, pulp and seed of Albariño grapes were analysed with two complementary QqToF and QqQ instruments to determine structures based on fragmentation patterns, using QqToF in PIS mode and QqQ in PrI and NL mode. In addition, information-dependent acquisition (IDA) by QqToF was used to generate a peak list of ions present in the spectrum at the time of analysis; this peak list was subjected to a series of user-defined criteria to select precursor ions of interest based on filters such as intensity threshold, charge state, isotope pattern and others. In general, we observed the deprotonated molecule [M-H]$^-$ and its characteristic product ions by MS/MS experiments. The 43 compounds are depicted in Fig. 1 and listed in Table 1 along with their retention time, molecular formulae and mDa of error between the experimental mass and the theoretical mass of each phenol investigated.

Thus, bearing in mind the importance of phenolic compounds as taxonomical markers (Vallverdu-Queralt et al., 2010), a precise characterisation of Albariño grape was obtained.

3.1.1. Hydroxybenzoic acid and its derivatives

Gallic acid (*m/z* 169) was the first compound to elute in skin and seed extract chromatograms. The product ion scan of the deprotonated molecule [M-H]$^-$ showed the typical loss of CO_2, giving an ion at *m/z* 125 [M-H-44]$^-$ as the characteristic fragment. This compound was confirmed by comparison with the calculated mass error (0.9 mDa) and reference compound.

LC-QqToF-MS analysis of seeds showed ions at *m/z* 331 and 315 (peaks 2, 14 and 4, 10, respectively), which were tentatively identified as deprotonated molecules of isomers of gallic acid hexose and protocatechuic acid hexose, respectively. Product ion scan of both ions showed the loss of hexose [M-H-162]$^-$, followed by the loss of CO_2 [M-H-162-44]$^-$. It was not possible to differentiate between the isomers on the basis of fragments and relative intensities in MS/MS spectra in PIS mode (Table 1).

Gallic acid dihexose (*m/z* 493) was also tentatively identified in seed and skin extracts: the PIS of the deprotonated molecule (*m/z* 493) showed two ions at *m/z* 331 and 169 derived from the loss of one and two hexose units, respectively. Furthermore, a very low mass error (0.3 mDa) was obtained with the QqToF instrument. The identification hypothesis was strengthened by the information obtained with the QqQ instrument, through neutral loss scan of 162 u and precursor ion scan of *m/z* 169.

3.1.2. Hydroxycinnamic acid and its derivatives

The skin extract revealed the presence of *p*-coumaric acid (*m/z* 163), which was corroborated by product ion scan experiment showing a predominant ion at *m/z* 119 (loss of CO_2). The presence of coumaric acid hexose (*m/z* 325) was also detected in skin and pulp. The PIS of this ion showed a characteristic fragmentation involving cleavage of the intact sugar [M-H-162]$^-$ (*m/z* 163), and an ion corresponding to the loss of a methyl (*m/z* 148) and CO_2 from aglycone (*m/z* 119). Peak identification was accomplished by comparing MS/MS fragmentation with reported data obtained by LC-ESI-MS in negative mode (Vallverdu-Queralt et al., 2010). NL of 162 u and PrI of *m/z* 163 by QqQ were useful for providing an unequivocal identification of hydroxycinnamic hexose.

3.1.3. Hydroxycinnamic tartaric esters

The skin and pulp extract chromatograms showed two ions at *m/z* 311 (peaks 7 and 8), two ions at *m/z* 295 (peaks 15 and 17), and one ion at *m/z* 325 (peak 21). These deprotonated molecules [M-H]$^-$ were tentatively identified as hydroxycinnamic acid tartaric esters. The ions at *m/z* 311 were identified as *cis* and *trans*-caffeoyl tartaric acid (caftaric acid); their PIS revealed a fragment at *m/z* 179 corresponding to caffeic acid, after the cleavage of the ester bond, and a low intensity signal at *m/z* 135 was ascribed to decarboxylated caffeic acid. MS/MS data were corroborated by comparison with reference compounds isolated from grape pomace (Vrhovšek, 1998). The ions at *m/z* 295 showed a fragmentation pattern similar to caftaric acid and were identified as *cis* and *trans*-coumaroil tartaric acid (coutaric acid) after comparison with the reference compound. The PIS produced only one ion fragment at *m/z* 163, which was ascribed to coumaric acid. In contrast, the deprotonated molecule at *m/z* 325 showed a fragment at *m/z* 193 attributed to ferulic acid, and an ion at *m/z* 149 that indicated a loss of CO_2 from the free ferulic acid. It was not possible identify the isomeric configuration of feruloyl tartaric acid, otherwise known as fertaric acid. The PrI at *m/z* 179, 163 and 193 confirmed the presence of the described peaks. Only *cis* and *trans*-caftaric acid and fertaric acid were found in the pulp extract. Among the aforementioned compounds, *trans*-caftaric acid is considered a major substance for coupled oxidation and enzymatic browning reactions in grape processing (Kroon & Williamson, 1999).

3.1.4. Flavan-3-ols

Reverse phase HPLC procedures provided a good baseline resolution for the flavan-3-ols, which consisted of (+)-catechin, (−)-epicatechin, their condensed product and corresponding galloylated derivatives that exhibited monomeric, dimeric and trimeric degrees of polymerisation. When the degree of polymerisation increased, the procyanidins were eluted as a single peak at the end of the chromatogram (Fig. 1). The resolved procyanidins present in Albariño seed, skin and pulp are mainly dimers (*m/z* 577) and trimers (*m/z* 865) in which the elemental units are linked by C4-C8 interflavan bonds (B-type). Structural variations in procyanidin oligomers may also occur with the formation of a second interflavanoid bond by C–O oxidative coupling to form A-type oligomers. Due to the complexity of this conversion, A-type procyanidins are not as frequently encountered in nature as B-type oligomers. Procyanidin A-type linkage shows a different fragmentation pathway than B-type linkage (Flamini, 2003), and in Albariño grapes no procyanidin A-type linkages were observed.

Peaks 23 and 28 (*m/z* 289) were identified as (+)-catechin and (−)-epicatechin, respectively, after comparison with the authentic standard. Both flavan-3-ols were identified in the three different fractions, with mass errors below 0.7 mDa. Up to four procyanidin dimers (*m/z* 577, peaks 19, 20, 26 and 27) were identified in the seed extract. The PIS at *m/z* 577 showed a Retro-Diels–Alder

(RDA) product with a neutral loss of 152 u [M-H-152]⁻ followed by loss of a water molecule [M-H-152-H₂O]⁻. Other fragments at m/z 289 and 245, derived from the interflavanic bond cleavage, were also observed (Table 1). Procyanidins B1 and B2 were corroborated by reference compounds, while the elution order of dimers B3 and B4 were assigned referring to the study by Monagas, Suárez, Gómez-Cordovés, and Bartolomé (2005).

LC-QqToF-MS analysis of the seed extract revealed four peaks (6, 24, 25 and 30) at m/z 865. Product ion scan showed a base peak at m/z 289 and two minor ions at m/z 577 and 425, which were also observed for the reference procyanidin C1. Additionally, an ion at m/z 695 was registered due to the RDA and successive loss of water [M-H-152-H₂O]⁻. Peaks 24, 25 and 30 were tentatively identified as procyanidin trimer isomers, but complete identification was not possible without standards.

Thus, on the basis of information obtained by PIS, when a NL experiment of 152 u was conducted by QqQ, the total ion current showed deprotonated molecules belonging to dimer (m/z 577) and trimer procyanidins (m/z 865, Fig. 3). Various studies of oligomeric and polymeric procyanidins in grape seed extracts have proposed a fragmentation scheme of ions derived from B-type procyanidins (Zhao, Pang, & Dixon, 2010; Sun & Miller, 2003; Gu et al., 2003).

Waterhouse, Ignelzi, and Shirley (2000) demonstrated that in grape seeds, the single unresolved peak, at the end of the chromatogram, corresponds to a mixture of high molecular mass procyanidin polymers.
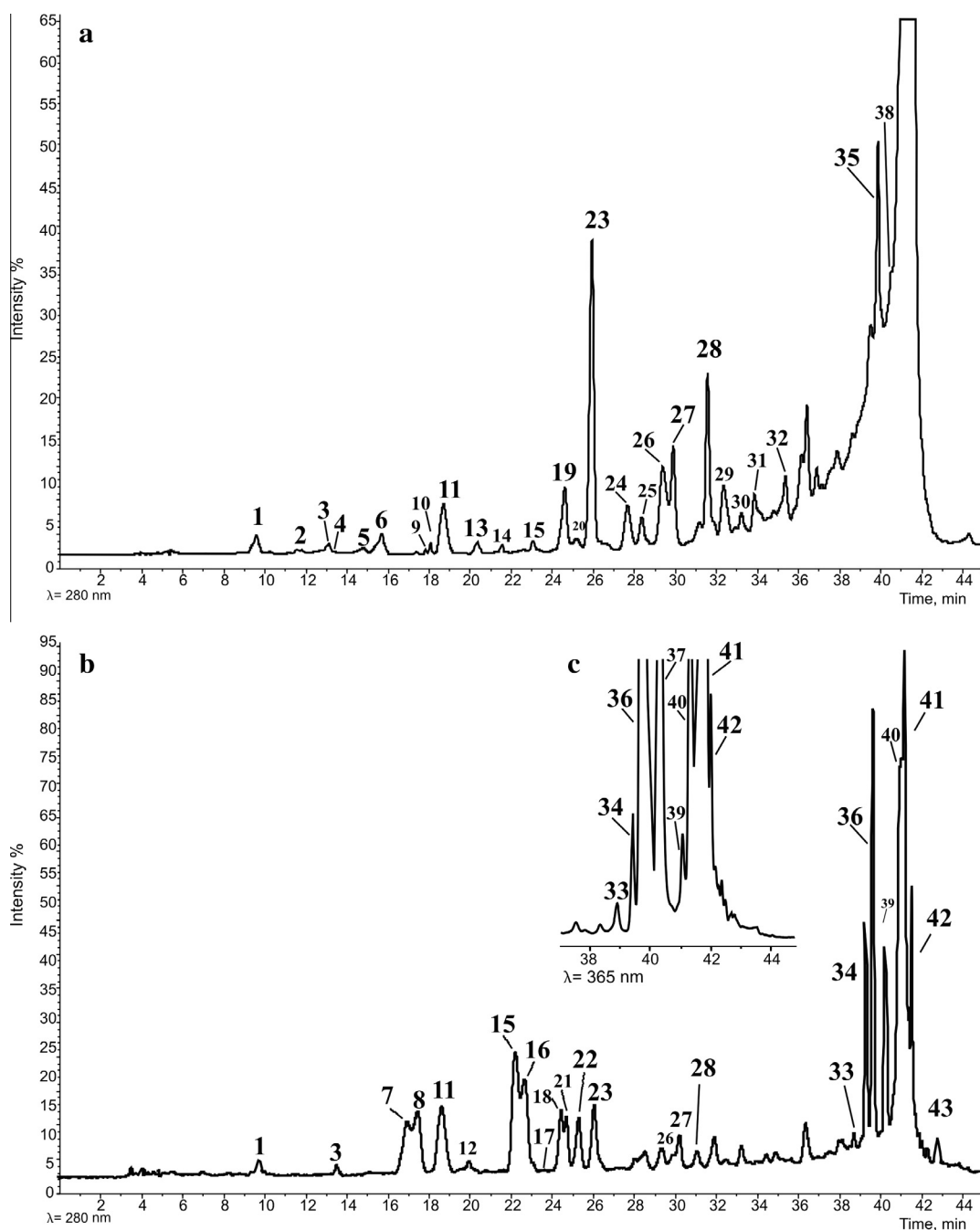

Fig. 1. HPLC-DAD chromatograms at λ = 280 nm of phenolic compounds identified in seed (a) and skin (b) of Albariño grape. (c) Flavonol profile (λ = 365 nm). Peak identification is shown in Table 1.

Table 1
List of compounds identified in three fractions of Albariño grape.

Peaks	Retention time	Compounds	Fractions	[M-H]⁻	Fragments m/z (% intensities)	PIS (Qq-ToF)	NL	PrI (QqQ)	Theoretical mass	Mass difference (mDa)[a]	Formula
1	9.35	Gallic acid*	se, sk	169.0151	169 (100), 125 (25)	169			169.0142	0.9	$C_7H_6O_5$
2	11.82	Gallic acid hexose I	se	331.0700	331 (5), 169 (70), 125 (100)	331	162	169	331.0670	3.0	$C_{13}H_{16}O_{10}$
3	13.08	Gallic acid dihexose	se, sk	493.1195	493 (9), 331 (100), 169 (100)	493		169	493.1198	−0.3	$C_{19}H_{26}O_{15}$
4	13.21	Protocatechuic acid-O-hexoside	se	315.0723	153 (100), 109 (40)	315	162	153	315.0721	0.2	$C_{13}H_{16}O_9$
5	14.76	(epi)gallocatechin-(epi)catechin	se	593.1332	423 (78), 305 (100), 289 (28)	593			593.1300	3.2	$C_{30}H_{26}O_{13}$
6	15.72	Procyanidin trimer C1	se	865.1950	865 (37), 695 (100), 577 (1), 407 (64), 289 (42)	865	152	289	865.1985	−3.5	$C_{45}H_{38}O_{18}$
7	16.90	cis-caftaric acid	sk, pu	311.0413	179 (100), 135 (54)	311		179	311.0418	0.5	$C_{13}H_{12}O_9$
8	17.49	trans-caftaric acid*	sk, pu	311.0411	179 (100), 135 (40)	311		179	311.0414	0.3	$C_{13}H_{12}O_9$
9	17.84	(epi)gallocatechin-3-gallate	se	457.0783	305 (100), 169 (65)	457			457.0790	0.7	$C_{22}H_{18}O_{11}$
10	18.01	Protocatechuic acid-O-hexoside	se	315.0751	153 (100), 109 (40)	315	162	153	315.0781	3.0	$C_{13}H_{16}O_9$
11	18.64	L-tryptophan*	se, sk, pu	203.0847	142 (9), 116 (100)	203			203.0868	2.1	$C_{11}H_{12}N_2O_2$
12	19.82	Epigallocatechin*	sk	305.0699	261 (100), 221 (25), 179 (34)	305			305.0732	3.3	$C_{15}H_{14}O_7$
13	20.23	(epi)catechin-hexose	se	451.1266	289 (100), 245 (40)	451	162	289	451.1287	2.1	$C_{21}H_{24}O_{11}$
14	21.56	Gallic acid hexose II	se	331.0700	331 (12), 169 (100), 125 (84)	331	162	169	331.0670	3.0	$C_{13}H_{16}O_{10}$
15	22.35	cis-coutaric acid	sk	295.0469	163 (32), 119 (100)	295		163	295.0479	1.0	$C_{13}H_{12}O_8$
16	22.83	(epi)catechin-hexose	se	451.1266	289 (100), 245 (54)	451	162	289	451.1287	2.1	$C_{21}H_{24}O_{11}$
17	22.91	trans-coutaric acid	sk	295.0460	163 (53), 119 (100)	295		163	295.0461	0.1	$C_{13}H_{12}O_8$
18	23.34	Coumaric acid-O-hexoside	sk, pu	325.0919	163 (50), 148 (30), 119 (100)	325	162	163	325.0928	0.9	$C_{15}H_{18}O_8$
19	24.51	Procyanidin B3	se, pu	577.1331	407 (75), 289 (81), 245 (67)	577	152	289	577.1311	−2.0	$C_{30}H_{26}O_{12}$
20	24.75	Procyanidin B1*	se	577.1334	407 (75), 289 (70), 245 (45)	577	152	289	577.1317	−1.7	$C_{30}H_{26}O_{12}$
21	24.45	Fertaric acid	sk, pu	325.0585	193 (100), 149 (30)	325		193	325.0605	2.0	$C_{14}H_{14}O_9$
22	24.82	p-Coumaric acid*	sk	163.0418	163 (20), 119 (100)	163			163.0436	1.8	$C_9H_8O_3$
23	25.93	(+)-Catechin*	se, sk, pu	289.0710	245(100), 205 (65)	289			289.0703	−0.7	$C_{15}H_{14}O_6$
24	27.72	Procyanidin trimer I	se	865.1954	865 (55), 695 (80), 577 (68), 425 (88), 289 (81)	865	152	289	865.1923	−3.1	$C_{45}H_{38}O_{18}$
25	28.30	Procyanidin trimer II	se	865.1971	865 (28), 695 (19), 577 (33) 575 (21), 289 (100)	865	152	289	865.1957	−1.4	$C_{45}H_{38}O_{18}$
26	29.24	Procyanidin B4	se, sk	577.1332	407 (93), 289 (73), 245 (59)	577	152	289	577.1313	−1.9	$C_{30}H_{26}O_{12}$
27	29.85	Procyanidin B2*	se, sk	577.1331	407 (93), 289 (73), 245 (59)	577	152	289	577.1311	−2.0	$C_{30}H_{26}O_{12}$
28	31.58	(−)-Epicatechin*	se, sk, pu	289.0712	245 (100), 205 (60)	289			289.0707	−0.5	$C_{15}H_{14}O_6$
29	32.42	(epi)catechin-(epi)catechingallate I	se	729.1476	577 (26), 451 (34), 407 (96), 289 (100)	729		289	729.1491	1.5	$C_{37}H_{30}O_{16}$
30	33.26	Procyanidin trimer III	se	865.1959	865 (42), 695 (49), 577 (52), 407 (70), 289 (100)	865	152	289	865.1933	−2.6	$C_{45}H_{38}O_{18}$
31	33.81	(epi)catechin-(epi)catechingallate II	se	729.1472	577 (37), 407 (100), 289 (70)	729	152	289	729.1483	1.1	$C_{37}H_{30}O_{16}$
32	35.42	(epi)catechin-(epi)catechingallate III	se	729.1473	577 (43), 407 (100), 289 (94)	729	152		729.1485	1.2	$C_{37}H_{30}O_{16}$
33	38.76	Quercetin-3-O-rutinoside*	sk	609.1466	609 (67), 301 (100)	609	308	301	609.1471	0.5	$C_{27}H_{30}O_{16}$
34	39.30	Quercetin-3-O-glucuronide	sk	477.0636	301 (72), 151 (100),	477	176	301	477.0598	−3.8	$C_{21}H_{18}O_{13}$
35	39.44	(−)-Epicatechin-3-O-gallate	se	441.0866	289 (100), 271 (47), 169 (85), 125 (6)	441			441.0905	3.9	$C_{22}H_{18}O_{10}$
36	39.65	Quercetin-3-O-glucoside*	sk	463.0857	301 (67), 151 (100)	463	162	301	463.0833	−2.4	$C_{21}H_{20}O_{12}$
37	40.27	Dihydroquercetin-3-O-rhamnoside	sk	449.1109	303 (100), 151 (75)	449		303	449.1129	2.0	$C_{21}H_{22}O_{11}$
38	40.52	Dimer digallate	se	881.1975	881 (25), 729 (93), 407 (100)	881	152		881.2016	4.1	$C_{45}H_{38}O_{19}$
39	40.95	Quercetin-3-O-pentoside	sk	433.0731	301 (100), 151 (77)	433			433.0692	−3.9	$C_{20}H_{18}O_{11}$
40	41.21	Kaempferol-3-O-glucuronide	sk	461.0770	285 (100), 257 (18), 229 (25) 135 (8)	461	176		461.0815	4.5	$C_{21}H_{18}O_{12}$
41	41.40	Kaempferol-3-O-glucoside*	sk	447.0972	447(30), 285(100)	447	162	285	447.1012	4.0	$C_{21}H_{20}O_{11}$
42	41.56	trans-piceid*	sk	389.1246	227 (100), 185 (17), 143 (6)	389	162	227	389.1251	0.5	$C_{20}H_{22}O_8$
43	42.40	trans-resveratrol*	sk	227.0750	185 (13), 143 (100)	227			227.0786	3.6	$C_{14}H_{12}O_3$

* Comparison with standard. sk, skin; pu, pulp; se, seeds; PIS, product ion scan, NL, neutral loss; PrI, precursor ion scan; [M-H]⁻ mass found.
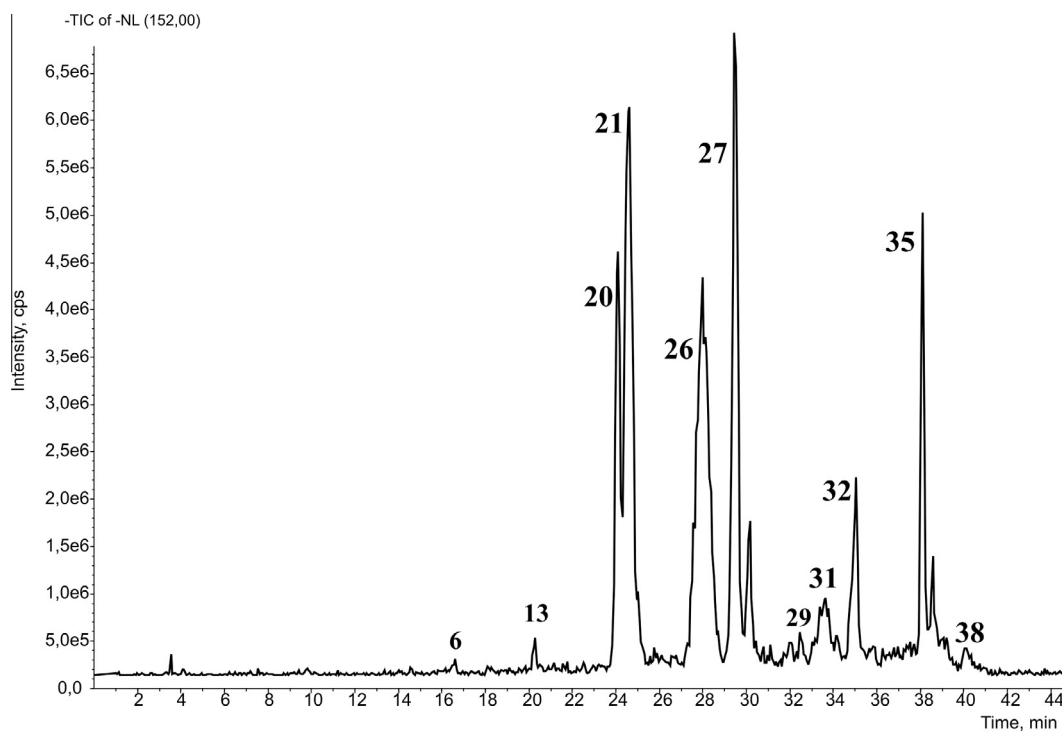[a] Obtained as theoretical mass – experimental mass.

Fig. 2. TIC of seed extract in neutral loss scan mode of 152 u. Peak identification: 6, procyanidin trimer C1; 13, (epi)catechin-hexose; 19, 20, 26 and 27, procyanidin dimers; 29, 31 and 32, (epi)catechin-(epi)catechingallate; 35, (−)-epicatechin-3-O-gallate; 38, dimer digallate.
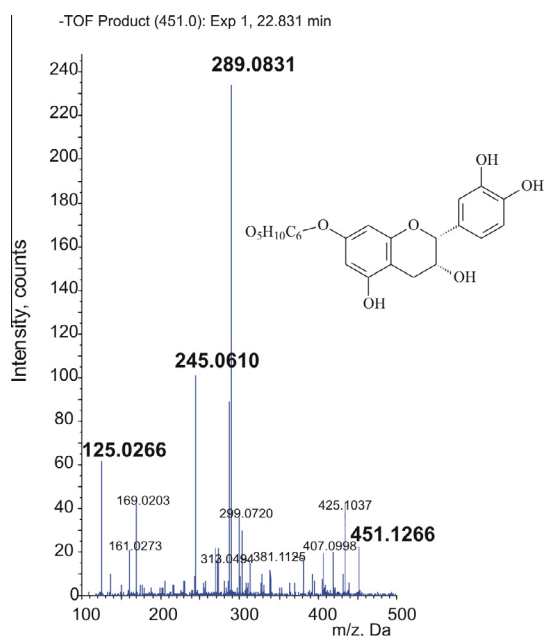

Fig. 3. The MS/MS product ion scan of m/z 451 (epi)catechin-hexose.

3.1.4.1. Flavanol hexosides. In Albariño seed, two flavan-3-ol hexoside isomers (peaks 13 and 16) at m/z 451 were tentatively identified as (+)-catechin or (−)-epicatechin-hexose. In PIS mode these flavanol hexosides showed a peak at m/z 289 due to the hexose moiety and the fragment at m/z 245 was attributed to (+)-catechin or (−)-epicatechin fragmentation (Fig. 2); the accurate mass measurements presented the same error for both compounds (2.1 mDa). The NL of 162 u and the PrI at m/z 289, in LC-ESI-QqQ-MS, confirmed the tentative identification of flavanol hexosides.

To the best of our knowledge, this is the first time that flavanol monomer hexosides have been detected in grapes, although they have been reported in other plants. Two research groups previously described the presence of flavanol hexose in barley (Wolfgang & Rudolf, 2002) and lentils (Dueñas, Sun, Hernández, Estrella, & Spranger, 2003).

Catechins are associated with health benefits, but they are unstable during storage, processing and during gut transit (Zho et al., 2002). However, recent evidence suggests that catechin-glucoside is more stable (between pH 4 and 8) than (+)-catechin. Raab et al., (2010) have shown that (+)-catechin-3'-O-β-D-glucopyranoside presents the greatest stability.

3.1.4.2. Flavan-3-ol galloylated derivatives. An ester derivative identified as (−)-epigallocatechin was found only in the skin. In fact the skin profile gave a peak at m/z 305, which represented a flavanolic unit; its confirmation was possible by comparing the chromatographic information with an authentic standard. As far as we know, flavan-3-ol galloylated derivatives have not been previously described in Albariño grape skin. Several monomeric and oligomeric flavanols linked to gallic acid were detected in the seed extract. Peak 9 showed an ion at m/z 457 which in PIS mode generated a preponderant fragment at m/z 305, probably produced by the loss of a galloyl group to epigallocatechin or gallocatechin. It was not possible to confirm the molecular structure of epigallocatechin-gallate or gallocatechin-gallate due to the lack of reference compounds. Three isomers of (epi)catechin-(epi)catechin-gallate, commonly known as dimer gallate (m/z 729), were also tentatively identified in seeds with mass errors below 1.5 mDa. The PIS of m/z 729 generated an ion at m/z 577 corresponding to the loss of gallic acid, while the more intense fragment at m/z 289 was due to the loss of (+)-catechin-gallate or a (−)-epicatechin-gallate unit. For the dimer gallates, the PrI by LC-ESI-QqQ-MS at m/z 289 showed peaks at m/z 729, thus providing further useful information for checking characteristic phenolic compounds of grape seed.

Another peak present only in the seed fraction at m/z 441 was identified as (−)-epicatechin-3-O-gallate as its retention time and mass spectra matched the standard. Moreover, the PIS showed two fragment ions resulting from the cleavage of the ester bond at m/z 289 for deprotonated (−)-epicatechin and at m/z 169 for a deprotonated gallic acid moiety. A prodelphynidin compound was also found in both seed and skin. LC-ESI-QqToF-MS analysis revealed the existence of a deprotonated molecule at m/z 593 and the PIS showed fragments at m/z 423, 305 and 289, which confirmed the presence of (epi)gallocatechin-(epi)catechin. In addition, another ion was detected in the seed extract at m/z 881, which was tentatively identified as (epi)catechin-(epi)gallocatechin-(epi)catechin or (epi)gallocatechin-(epi)catechin-(epi)catechin due to another (epi)catechin linked to the molecular structure. The PIS experiment suggests that the more abundant fragment was at m/z 729, which corresponds to the gallate unit moiety (Lazarus, Adamson, Hammerstone, & Schmitz, 1999). As depicted in Fig. 3, the total ion current of NL at 152 u could also be used for a fast screening of flavan-3-ol galloylated derivatives.

As described by Flamini (2003), dimer gallates were first identified in Niagara grapes. Other authors have confirmed that the grape seed phenolic profile is characterised by the presence of flavanol derivatives esterified with gallic acid and their occurrence can be considered typical of grape seeds (Santos-Buelga, Francis-Aricha, & Escribano-Bailón, 2005; Rodríguez Montealegre et al., 2006).

3.1.5. Flavonols

In the skin fraction three flavonol-O-hexosides (peaks 36, 37 and 41), two -O-glucuronides (peaks 34 and 40), one -O-rutinoside (peak 33) and one -O-pentoside (39) were plausibly identified (Fig. 1b and c). In this work, we found only -O-glycoside derivatives arising from cleavage of the glycosidic bond and loss of the sugar moieties (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2010). LC-ESI-QqToF-MS analysis of the skin phenolic extract revealed mass signals at m/z 609, 477 and 463, corresponding to deprotonated quercetin-3-O-rutinoside (also called rutin), quercetin-3-O-glucuronide and quercetin-3-O-glucoside (quercitrin), for peaks 33, 34 and 36, respectively. The deprotonated molecule at m/z 609 in PIS mode showed an intense fragment at m/z 301 due to the rutinoside moiety, and a fragment at m/z 151 typical for quercetin. Similar MS^2 experiments have been done for quercetin-3-O-glucuronide and quercetin-3-O-glucoside, showing the same fragment at m/z 301 for the glucuronide and glucoside moieties. The presence of the three quercetin derivatives was also checked by PrI at m/z 301 and by matching the retention time and mass spectra with data of available standard compounds. The presence of another quercetin derivative was detected by LC-ESI-QqToF-MS which showed a deprotonated molecular [M-H]$^-$ at m/z 433 and the PIS showed a fragment at m/z 301. In this case, NL and PrI experiments did not yield any information due to the low intensity of the peak. This compound was tentatively assigned to quercetin-3-O-pentose and, as far as we know, this is the first time that this phenolic compound has been reported in Albariño grape skin. The chromatogram of the skin extract in QqToF-MS also showed a deprotonated molecule at m/z 449 with an error of 2.0 mDa. The PIS of m/z 449 gave a product ion at m/z 303, which suggested the probable presence of diihydroquercetin-3-O-rhamnoside, as described by Masa and Vilanova (2008) for Albariño skin. Analysis of the skin phenolic profile also revealed the presence of two ions at m/z 461 and 447 corresponding to kaempferol-3-O-glucuronide and kaempferol-3-O-glucoside, respectively. The PIS in LC-ESI-QqToF-MS bore out these results, in both cases showing a preponderant fragment at m/z 285. Additionally, as described above, NL and PrI experiments characterised kaempferol-3-O-glucuronide and kaempferol-3-O-

glucoside, which were corroborated by comparison with the standard compounds (Table 1).

Flavonoid-C-glycosides, which show a different fragmentation pattern from -O-glycosides (Sanchez-Rabaneda et al., 2003; Han et al., 2008), were not detected in the Albariño grape.

3.1.6. Stilbenes

Stilbenes were eluted in the final part of the Albariño skin chromatogram. Peaks 42 and 43 showed deprotonated molecules at m/z 389 and 227 [M-H]$^-$, and were identified as *trans*-piceid and *trans*-resveratrol, respectively. Both stilbenes were corroborated by comparison with the reference compounds. The presence of resveratrol and its glucoside in red as well as white grapes has been ascribed to ultraviolet irradiation or stress, especially plant interaction with pathogens (Romero-Pérez, Lamuela-Raventós, Andreés-Lacueva, & de la Torre-Boronat, 2001).

Furthermore, as reported by Lamuela-Raventos, Romero-Pérez, Waterhouse and de la Torre-Boronat, (1995), *trans* isomers are transformed to the *cis* forms when grapes are exposed to UV radiation. Probably due to the extraction procedure, which was performed in a dark room with a red safety light, we did not detect cis isomer forms in Albariño skin. Stilbenes have been extensively studied as critical contributors to the health benefits of grapes and wine (Lamuela-Raventós & Waterhouse, 1999).

3.2. A nitrogen compound with phenolic structure: ʟ-tryptophan

A nitrogen compound identified as ʟ-tryptophan (peak 11) was found in all the anatomical parts of Albariño grapes. In LC-ESI-QqToF-MS, this peak showed an ion at m/z 203 and the PIS revealed two ions at m/z 116 and 142. The compound identity was confirmed by comparing its mass spectra with those of an authentic standard. ʟ-tryptophan can be present in white must and is ascribed to the metabolic pathway of 2-aminoacetophenone, a causal agent of an 'untypical ageing off-flavour' in wine. In another study, Mattivi, Vrhovšek, and Versini, (1999) found levels of ʟ-tryptophan in Chardonnay musts and wines ranging between 62 and 417 μg L^{-1}. (Mattivi et al., 1999).

3.3. Quantification of phenolic compounds found in skin, pulp and seed

The most abundant class of phenols found in Albariño grape berries were the monomeric and oligomeric form of flavan-3-ols, which were present in hypodermal layers of skin and in the soft parenchyma of the seed. The total content of flavanols was 611 mg $* 100$ g^{-1} of fresh matter (Table 2), while the compounds at the highest concentration were (+)-catechin and (−)-epicatechin.

Albariño grape skin exhibited a predominance of flavanols and flavonols but a considerable amount of hydroxycinnamates was also found. The major hydroxycinnamic acid present was *cis*-coutaric acid (see Table 2), followed by caftaric isomers. Flavonols were always found in glycoside form, principally as 3-glucosides; small amounts of rutinoside and glucuronide flavonols were also detected. The content of flavonols ranged between 0.39 and 12.4 mg $* 100$ g^{-1} of fresh matter for quercetin-3-O-rutinoside and quercetin-3-O-glucoside, respectively. As reported by Downey, Harvey, and Robinson (2004), the flavonol content cannot be considered as characteristic of a grape *cultivar* because the flavonol concentration is strongly affected by the degree of illumination of the grape cluster (Downey et al., 2004).

The phenolic content in the pulp was very low. Hydroxycinnamic acids were the most representative compounds, with a total content of about 1.63 mg $* 100$ g^{-1} of fresh matter. Small amounts of catechin, epicatechin and procyanidin B3 were also found.

Table 2
Content of phenolic compounds in the different anatomical parts of Albariño grape (mg * 100 g^{-1} of fresh matter). Mean values (±standard errors); nd, not detected.

Compounds	Skin	Pulp	Seed
Gallic acid	1.19	nd	1.92
Gallic acid hexose I	nd	nd	0.79
Gallic acid dihexose	1.13	nd	1.25
Protocatechuic acid-O-hexoside	nd	nd	0.45
Gallic acid hexose II	nd	nd	1.36
Protocatechuic acid-O-hexoside	nd	nd	1.54
Hydroxybenzoic acids	2.32 ± 0.1	nd	7.31 ± 0.3
cis-caftaric acid	2.53	0.11	nd
trans-caftaric acid	4.04	0.37	nd
cis-coutaric acid	6.23	nd	nd
trans-coutaric acid	0.27	nd	nd
Coumaric acid-O-hexose	2.27	1.03	nd
Fertaric acid	1.68	0.12	nd
p-coumaric acid	1.96	nd	nd
Hydroxycinnamic acids	18.98 ± 0.8	1.63 ± 0.1	nd
(epi)gallocatechin-(epi)catechin	nd	nd	3.58
Procyanidin trimer C1	nd	nd	12.65
(epi)gallocatechin-3-gallate	nd	nd	1.54
Epigallocatechin	2.09	nd	nd
(epi)catechin-3-hexose	nd	nd	1.49
(epi)catechin-3-hexose	nd	nd	3.52
Procyanidin B3	nd	0.57	44.65
Procyanidin B1	nd	nd	3.09
(+)-catechin	11.45	0.55	106.5
Procyanidin trimer I	nd	nd	31.43
Procyanidin trimer II	nd	nd	18.54
Procyanidin B4	8.04	nd	58.39
Procyanidin B2	8.65	nd	64.53
(−)-epicatechin	2.67	0.23	77.51
(epi)catechin-(epi)catechingallate I	nd	nd	26.76
Procyanidin trimer III	nd	nd	13.54
(epi)catechin-(epi)catechingallate II	nd	nd	23.73
(epi)catechin-(epi)catechingallate III	nd	nd	21.43
(−)-epicatechin-3-O-gallate	nd	nd	76.54
Dimer digallate	nd	nd	21.43
Flavanols	32.9 ± 2.7	1.35 ± 0.1	610.8 ± 35.8
Quercetin-3-O-rutinoside	0.42	nd	nd
Quercetin-3-O-glucuronide	0.98	nd	nd
Quercetin-3-O-glucoside	12.43	nd	nd
dihydroquercetin-3-O-rhamnoside	5.65	nd	nd
Quercetin-3-O-pentoside	0.23	nd	nd
Kaempferol-3-O-glucuronide	3.21	nd	nd
Kaempferol-3-O-glucoside	8.43	nd	nd
flavonols	31.45 ± 1.6	nd	nd
trans-piceid	6.93	nd	nd
trans-resveratrol	1.43	nd	nd
Stilbenes	8.36 ± 0.4	nd	nd
Total	94.21 ± 5.1	2.98 ± 0.2	618.1 ± 36.1

As described by other authors, the concentration of phenolics and their profile in grapes, depends on the grapevine variety as well as intrinsic factors such as genetics and extrinsic aspects linked to viticulture and the environment. The degree of ripeness and berry size are also influential (Rodríguez Montealegre et al., 2006). In accordance with previous papers, the results presented in this study demonstrate that grape berries generally present a very high polyphenolic content, which contributes to their value as an agricultural crop (Rodríguez Montealegre et al., 2006; Dietmar et al., 2004).

4. Conclusions

Using a combination of spectrometric techniques we were able to identify up to 43 compounds, two of which, (+)-catechin or (−)-epicatechin hexosides, as far as we know, have never been reported before in the grape berry. The QqToF-MS was very useful for its combination of high sensitivity, high resolution and high mass accuracy, also allowing the characterisation of deprotonated molecules from PIS experiments in HRMS mode. Good fits were ob-

tained for all investigated ions, with errors ranging from 0.2 to 4.5 mDa. The QqQ system was effective for obtaining information about the phenolic composition of grapes through NL and PrI experiments that allowed a first screening of families of compounds. In particular, an NL of 152 u was found to be helpful for a rapid screening of procyanidin dimers and trimers and gallate flavanols.

A wide range of phenolic compounds was found diversely distributed in every part of the Albariño grape berries but mainly in the skin and seed. Thus, this investigation resulted in an exhaustive characterisation of the phenolic profile of the different anatomical parts of the Albariño grape, and provides useful information for selecting suitable by-products for the extraction of potential health-promoting compounds.

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