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Phenolic profile of Sercial and Tinta Negra *Vitis vinifera* L. grape skins by HPLC–DAD–ESI-MS^n
Novel phenolic compounds in *Vitis vinifera* L. grape

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ABSTRACT

This study represents the first phytochemical research of phenolic components of Sercial and Tinta Negra *Vitis vinifera* L. The phenolic profiles of Sercial and Tinta Negra *V. vinifera* L. grape skins (white and red varieties, respectively) were established using high performance liquid chromatography–diode array detection–electrospray ionisation tandem mass spectrometry (HPLC–DAD–ESI-MS^n), at different ripening stages (*véraison* and maturity). A total of 40 phenolic compounds were identified, which included 3 hydroxybenzoic acids, 8 hydroxycinnamic acids, 4 flavanols, 5 flavanones, 8 flavonols, 4 stilbenes, and 8 anthocyanins. For the white variety, in both ripening stages, hydroxycinnamic acids and flavonols were the main phenolic classes, representing about 80% of the phenolic composition. For red variety, at *véraison*, hydroxycinnamic acids and flavonols were also the predominant classes (71%), but at maturity, anthocyanins represented 84% of the phenolic composition. As far as we know, 10 compounds were reported for the first time in *V. vinifera* L. grapes, namely protocatechuic acid-glucoside, *p*-hydroxybenzoyl glucoside, caftaric acid vanilloyl pentoside, *p*-coumaric acid-erythroside, naringenin hexose derivate, eriodictyol-glucoside, taxifolin-pentoside, quercetin-glucuronide-glucoside, malylated kaempferol-glucoside, and resveratrol dimer. These novel *V. vinifera* L. grape components were identified based on their MS^n fragmentation profile. This data represents valuable information that may be useful to oenological management and to valorise these varieties as sources of bioactive compounds.

1. Introduction

Grapes from *Vitis vinifera* L. belong to the world's largest fruit crops, and are consumed by population and applied, mainly, on wine production (Ross, Hoye, & Fernandez-Plotka, 2011). The emphasis placed by European Commission on enhancing the nutrient content of food crops confirms the importance of phenolic compounds in terms of health benefits to the international community (Beer, Joubert, Gelderblom, & Manley, 2002). Moreover, grape phenolic compounds play an important role in wine organoleptic characteristics (e.g., colour, astringency, bitterness, and interaction with proteins during wine oxidation, among others) (Kelebek, Canbas, Jourdes, & Teissedre, 2010), and their regular consumption on a diet has been associated with beneficial effects for human health (La Torre, Saitta, Vilasi, Pellicanò, & Dugo, 2006). Reduction of the incidence of cardiovascular diseases, inhibition of the oxidation of human low-density lipoproteins, and possible anti-carcinogenic and anti-ageing effects, due to their antioxidant and anti-inflammatory properties, among others, have been reported (Flamini, 2003; Xia, Deng, Guo, & Li, 2010).

Phenolic compounds are secondary plant metabolites that could be classified in flavonoid and non-flavonoid compounds, based on their carbon skeleton. Flavonoids are phenolic compounds with diphenylpropane (C6–C3–C6) skeletons. According to the modifications on the central C-ring, they can be divided into different structural classes including flavonols (e.g., quercetin, kaempferol), flavones (e.g., apigenin, luteolin), flavan-3-ols (e.g., (+)-catechin, (−)-epicatechin), flavanones (e.g., taxifolin and naringenin), and anthocyanidins (e.g., cyanidin and malvidin). Non-flavonoids comprise C6–C1 phenolic acids (e.g., gallic and protocatechuic acids), C6–C3 hydroxycinnamic acids (e.g., *p*-coumaric and caffeic acids) and their conjugated derivatives, and polyphenolic C6–C2–C6 stilbenes (e.g., *trans*-resveratrol and *trans*-piceid) (Flamini, 2003; Xia et al., 2010).

The grape phenolic profile, which comprises a detailed qualitative and quantitative data, is influenced by the grape variety, plant

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yield, leaf area/berry ratio, and by other factors that modulate the berry development, such as soil, geographic origin, and climatic conditions (Fanzone, Zamora, Jofré, Assof, & Pen~a-Neira, 2011). The grape phenolic profile changes during ripening, however, distinct evolution patterns, depending on grape variety and phenolic class were observed (Mazza, Fukumoto, Delaquis, Girard, & Ewert, 1999; Pérez-Magariño & González-San José, 2005). Moreover, the knowledge of grape phenolics profile during ripening offers a mean of evaluating the period of time when the maximum potential of phenolic compounds is exhibited. The impact of phenolic components in wine organoleptic properties and their potential human health benefits explain the growing interest on the study of phenolic compounds of several food related matrices.

The phenolic composition and properties of grapes and wines has been extensively studied by spectrophotometric methods and by high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) or diode array (DAD) detectors (Alonso Borbalán, Zorro, Guillén, & García Barroso, 2003; Bravo, Silva, Coelho, Boas, & Bronze, 2006; Fanzone et al., 2011; Jin, He, Bi, Cui, & Duan, 2009; Jordão, Ricardo-da-Silva, & Laureano, 2001; Kammerer, Claus, Carle, & Schieber, 2004; La Torre et al., 2006; Mateus, Proença, Ribeiro, Machado, & Freitas, 2011; Mazza et al., 1999; Obreque-Slier et al., 2010; Pérez-Magariño & González-San José, 2005; Silva, Pereira, Wouter, Giró, & Câmara, 2011; Sun, Liang, Bin, Li, & Duan, 2007; Sun, Ribes, Leandro, Belchior, & Spranger, 2006). These previous studies demonstrated that grapes represent a potential source of phenolic compounds, such as anthocyanins, hydroxycinnamic acids, flavanols, and flavonol glycosides (Alonso Borbalán et al., 2003; Fanzone et al., 2011; Jin et al., 2009; Kammerer et al., 2004; Mateus et al., 2011; Obreque-Slier et al., 2010), which are the most important phenolic classes due to their biological activities (Xia et al., 2010).

The aim of this research is to establish the phenolic profile of a Sercial (white) and Tinta Negra (red) *V. vinifera* L. varieties, at two different ripening stages (*véraison* and maturity), in order to gain information that may be useful to oenological management and to search potential bioactive compounds. This study was focused only on the skins as phenolic compounds were reported to be mainly on this tissue (Alonso Borbalán et al., 2003; Hollecker et al., 2009; La Torre et al., 2006; Xia et al., 2010). Firstly, the total phenolic content of each variety was determined at *véraison* and maturity using the Folin–Ciocalteu method. Then, the phenolic profile of each variety was established by HPLC–DAD–ESI-MS^n.

2. Materials and methods

2.1. Chemicals

The Folin–Ciocalteu reagent (FR, 2 N), gallic acid monohydrate (98%, purity), and glacial acetic acid (99%) were purchased from Fluka (Buchs, Switzerland). Methanol (99%), dichloromethane (99%), and DL-tartaric acid (99%) were purchased from Sigma–Aldrich (Madrid, Spain). Anhydrous sodium carbonate (99.8%), sodium hydroxide (NaOH, 98%) and ethanol (99.5%) were supplied from Panreac (Barcelona, Spain). Several phenolic standards were used for identification and quantification purposes, namely ferulic acid (98%), cinnamic acid (99%), (−)-epicatechin (⩾95%), and rutin (95%) supplied from Fluka (Buchs, Switzerland), protocatechuic acid (98%), kaempferol (⩾ 97%), and *trans*-resveratrol (99%) supplied by Sigma–Aldrich (St. Louis, MO, USA), and quercetin from Riedel-de-Haën (98%, Seelze, Germany). Malvidin-3-glucoside (95%, isolated from grapes) was used as standard. HPLC grade acetonitrile (CH_3CN, 99%) were obtained from LabScan (Dublin, Ireland), whereas formic acid (HCOOH, ⩾98%) from Fischer Scientific (Loughborough, UK). Solvents were filtered using a Solvent Filtration Apparatus 58061 from Supelco (Bellefonte, PA, USA). The ultra-pure water was obtained from Milli-Q ultrapure water system (Millipore, Bedford, USA).

2.2. Grape samples

Two Portuguese grape varieties (*V. vinifera* L.), namely Sercial (white grape) and Tinta Negra (red grape) were considered, as Tinta Negra is the main variety cultivated in the Madeira Island (around 90%), and Sercial is a noble variety. Both varieties are used to produce the world-famous Madeira wine. Tinta Negra grapes are also consumed by the population of Madeira Island. As far as we know, no information was available about phenolic profile of these *V. vinifera* L. grapes.

Healthy state Sercial and Tinta Negra *V. vinifera* L. grapes were harvested at different ripening stages in 2008, from two experimental vineyards, property of Regional Secretary of Agriculture. Sercial was harvested in Estreito da Calheta (Quinta das Vinhas vineyard, 32°44′0.09″N, 17°11′14.80″W) vineyard; Tinta Negra was harvested in Estreito de Câmara de Lobos (Vila Afonso vineyard, 32°39′50.59″N, 16°58′48.28″W), both located in the south of Madeira Island (Portugal). To evaluate the phenolic profile of these two varieties, a sampling strategy was designed that includes five sampling moments for Sercial, from August 2 (*véraison* – day 0) to September 20 (post-maturity – day 49), and four sampling moments for Tinta Negra, from July 19 (*véraison* – day 0) to August 30 (post-maturity – day 42) (Table 1). For each sampling moment and variety, *ca.* 1000 g of grape berries were picked randomly throughout the vineyard, taking into account the number of berries per bunch, and the balance between shadow and sun exposure. This strategy, following a z shaped pattern to avoid edge and centre effects, contributed to the understanding of the intrinsic and natural variability of the fruit and allowed to validate the data obtained. *Véraison* (day 0) was defined according to blossom, bloom, berry texture and change in berry skin colour, which indicates the beginning of ripening, whereas maturity was established based on maximum sugar content and minimum titratable acidity. For Sercial and Tinta Negra grapes, the maturity data was established at day 42 and 35 after *véraison*, respectively (Table 1). Samples were transported immediately under refrigeration (*ca.* 2–5 °C) to the laboratory and were stored at −20 °C until analysis. Each sample of grapes (*ca.* 1000 g) harvested for each variety, at each sampling moment was separated into two sub-samples to be used in the following sample preparation step (Section 2.3).

2.3. Sample preparation

2.3.1. To determine sugar content and titratable acidity

For each sampling moment, 200 g of grape berries (sub-sample from the *ca.* 1000 g) were defrosted at 4 °C overnight and then crushed in a *turbo blender* (Moulinex – LM600E, Ecully, France) during 2 min. The juice suspension was centrifuged at 5000 rpm (Sigma 4K10 Braun, Melsungen, Germany) for 15 min, at room temperature to obtain a clarified juice. The juice was then filtered through 0.45 μm pore size membrane filters and stored at −20 °C until analysis.

2.3.2. To determine the total phenolic content and establish the phenolic profile by HPLC–DAD–ESI-MS^n

The grapes of each variety stage were manually peeled, and the skins were separated from the pulp. In the current research, the extraction of grape phenolics from lyophilized skins were performed using a fast protocol, with some modifications (Santos, Pinto, Silvestre, & Neto, 2010). Briefly, the grapes of each variety and maturity stage were manually pealed, and the skins were separated from the pulp. About 50 g of grape skins (sub-sample from

Table 1
Sugar content, titratable acidity, and total phenolic content (TPC) during ripening of Sercial and Tinta Negra *Vitis vinifera* L. grapes.

Days after *véraison*	Samples	Sugar content (g/l)[a]	Titratable acidity (g/l)[a]	TPC/dried skin (mg/kg)[a]
Sercial				
0 (*Véraison*)	August 2	90.1	24.2	7770
14	August 16	123.9	18.8	–
35	September 6	138.2	10.9	–
42 (Maturity)	September 13	161.5	8.3	3568
49	September 20	152.0	8.0	
Tinta Negra				
0 (*Véraison*)	July 19	143.2	14.5	3147
14	August 2	170.4	13.2	–
35 (Maturity)	August 23	206.8	8.6	13,812
42	August 30	185.1	8.4	–

[a] R.S.D% values were lower than 5% for all assays.

ca. 1000 g) was freeze-dried using VirTis benchTop K (SP Industries, NY, USA), and 2.5 g of dried skins was submitted to a soxhlet extraction with dichloromethane during 6 h to remove the lipophilic fraction. The dried skins lipophilic free fraction were then submitted to extraction with 100 ml of ethanol or methanol (49.5% aqueous solutions (v/v) with 1% acetic acid, pH 2), in order to evaluate their extraction efficiency. The best extraction solvent mixture was chosen based on the extraction yield (g/kg of dried grape skin) and total phenolic content (TPC) determined as described below. The extraction yield obtained by ethanol:H_2O:acetic acid (33.3 g/kg dried grape skin) was slightly higher than that obtained with methanol:H_2O:acetic acid (26.4 g/kg dried grape skin), whereas TPC was similar for both extracts, 3068 and 3288 mg/kg, respectively. Based on these results, ethanol:H_2O:acetic acid was selected for further assays, as in terms of toxicity, a mixture with ethanol is better from user healthy perspective. Furthermore, this data is in agreement with other grape phenolic extractions (Jensen, Demiray, Egebo, & Meyer, 2008; Montealegre, Peces, Vozmediano, Gascuena, & Romero, 2006), as no remarkable differences were observed between aqueous ethanol and methanol mixture, and the solvent acidification also improved the extraction efficiency.

After this previous assay, the lipophilic free dried skins were extracted using ethanol:H_2O:acetic acid, during 24 h, under constant stirring (400 rpm), at room temperature (25 °C) to ensure the extensive extraction and avoid phenolic compounds degradation. The suspension was filtered, the organic solvent removed by low-pressure evaporation and the aqueous solution was freeze-dried (phenolic residue).

2.4. Methods

2.4.1. Chemical analysis of grapes

Sugar content and titratable acidity were determined during ripening of *V. vinifera* L. grapes using European Union recommended methodologies for wine sectors (EU Council Regulation 2676/90/EEC of September 17th, 1990). Sugar content was determined based on the corresponding °Brix, which was measured using an Atago RX-1000 (Tokyo, Japan) digital refractometer. Titration of total acidity was conducted with NaOH (0.1 N) until pH 8.1, and the results were expressed as g tartaric acid/L (TA, predominant organic acid in grapes). All analyses were performed in triplicate.

2.4.2. Total phenolic content by Folin–Ciocalteu method

Total phenolic content (TPC) was determined spectrophotometrically using Folin–Ciocalteu method, with some modifications (Pérez-Magariño & González-San José, 2006). Briefly, the phenolic residues obtained from Sercial and Tinta Negra skins. Briefly, accurately weighed aliquots of phenolic extracts dissolved in 1 ml of water (corresponding to concentration ranges between 75 to 139 mg of residue/ml) were mixed with 3 ml of Folin–Ciocalteu reagent (1:10 v/v, in Milli-Q water), and then shook for 10–15 s. After 3 min, 2.4 ml of saturated sodium carbonate (7.5% w/v) aqueous solution was added. The reaction mixture was kept in dark for 30 min, and its absorbance measured at 765 nm against water in a UV–vis spectrophotometer (MutiSpec-1501, Shimadzu, Japan). A calibration curve was plotted with gallic acid standard solutions (GA, 10–200 mg/l, $A_{765} = 0.0044\text{GAE}\ (\text{mg/L}) + 0.044$; $r^2 = 0.995$). The analyses were performed in triplicate, and the results expressed as mg of gallic acid equivalent (GAE)/kg of dried skin.

2.4.3. Phenolic profile by HPLC–DAD–ESI-MS^n

The quantitative analysis of the phenolic compounds was carried out on a HPLC system of Dionex ultimate 3000 series (Sunnyvale, CA) instrument equipped with binary pump, diode array detector (DAD), autosampler and column compartment. The equipment was equipped with an Atlantis dC_{18} column (250 mm × 4.6 mm i.d. × 5 μm) supplied from Waters (Milford, Ma, USA) at controlled temperature (25 °C). The elution was performed using mobile phase A (10% CH_3CN and 0.1% HCOOH in aqueous solution), and mobile phase B (0.1% HCOOH in CH_3CN). The flow rate was 0.4 ml/min, and the detection range from 210 to 520 nm. The gradient program was used as follows: 0 min 100% A; 3 min 100% A; 10 min 90% A; 30 min 80% A; 35 min 75% A; 50 min 50% A; 60 min 100% A. The phenolic residue of each variety/stage was dissolved in the initial HPLC mobile phase A and the solutions were filtered through 0.45 μm micropore membranes prior to injection into HPLC system (injection volume 10 μl). The detection by DAD was conducted by scanning between 210 to 520 nm, with a resolution of 1.2 nm, and the semiquantification was conducted at 280 nm for the lower molecular weight phenolic compounds, and at 320, 360, and 520 nm for stilbenes, flavonols, and anthocyanins, respectively. As not all the phenolic compounds of grapes are commercially available, and following a frequently adopted approach (Fanzone et al., 2011; Jin et al., 2009; Kammerer et al., 2004), nine standards, representative of the chemical classes under study were selected (Fig. 1). The selected chemical standards were used to perform calibration curves (Table 2), and the results for each target phenolic compound were expressed in equivalents of the standard used. For each of the nine standards, an ethanolic stock solution was prepared (500 μg/ml). All solutions were stored at −20 °C. Working solutions were prepared by diluting adequate amounts of each stock solution in the mobile phase A. Seven different levels, covering the concentration range expected for each phenolic compound (Alonso Borbalán et al., 2003; Fanzone et al., 2011; Hollecker et al., 2009; Obreque-Slier et al., 2010) were prepared (Table 2). All analyses were performed in triplicate.

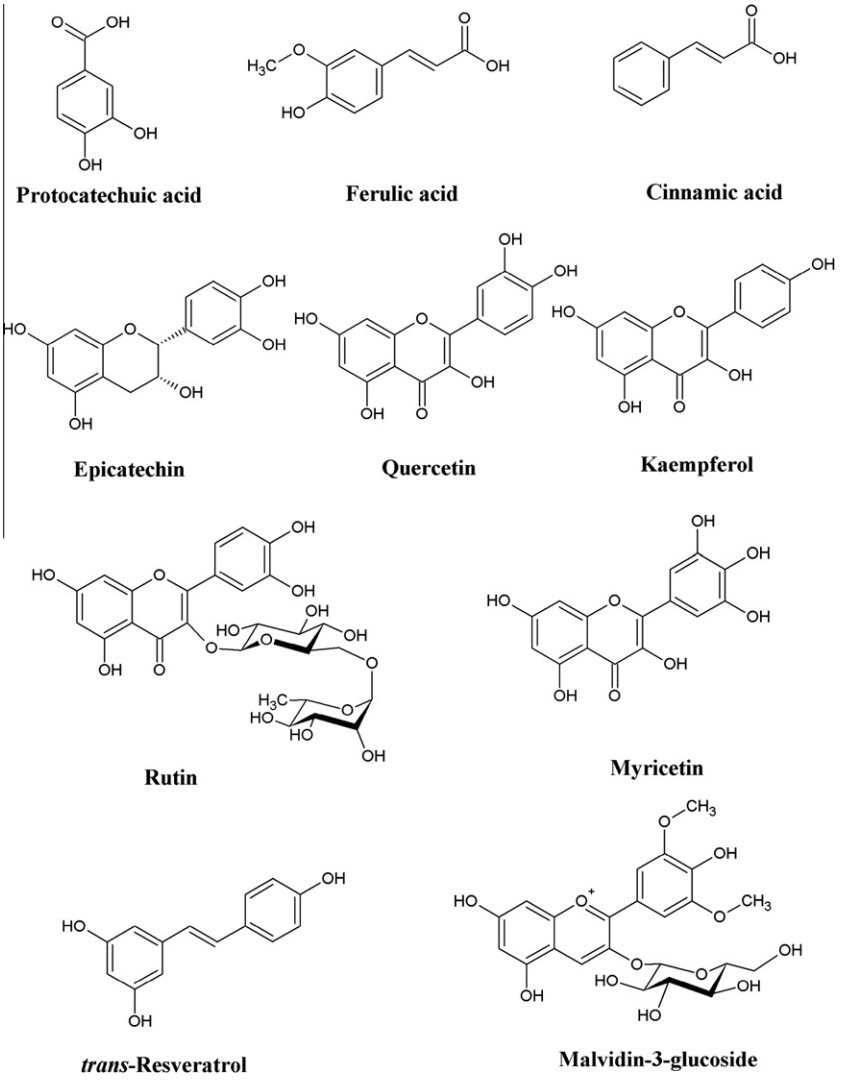

Fig. 1. Structures of the phenolic compounds used as standards for qualitative and quantitative analysis.

Table 2
Calibration data used for the HPLC–DAD semiquantification of phenolic compounds.

Compound name	RT (min)	λ (nm)	Phenolic class	Concentration range (μg/ml)	Calibration curve	r^2	LOD (μg/ml)	LOQ (μg/ml)
Protocatechuic acid	13.50	280	Hydroxybenzoic acid	0.74–49.60	$y = 0.37x - 0.18$	0.999	0.17	0.58
(−)-Epicatechin	19.89	280	Flavan-3-ol	0.50–252.00	$y = 0.14x + 0.40$	0.998	0.15	0.51
Malvidin-3-glucoside	21.98	520	Anthocyanin	0.20–342.86	$y = 0.31x - 0.99$	0.999	0.03	0.12
Rutin	25.45	360	Flavonol	1.70–212.00	$y = 0.30x - 0.25$	0.997	0.14	0.48
Ferulic acid	28.70	320	Hydroxycinnamic acid	0.83–82.80	$y = 1.06x + 0.59$	0.999	0.24	0.80
trans-Resveratrol	39.41	320	Stilbenes	0.14–312.40	$y = 1.62x - 1.08$	0.999	0.03	0.09
Quercetin	44.27	360	Flavonol	1.03–206.00	$y = 0.83x - 1.71$	0.995	0.05	0.17
Cinnamic acid	46.52	280	Cinnamic acid	0.56–42.00	$y = 1.38x - 0.17$	0.999	0.03	0.10
Kaempferol	48.79	360	Flavonol	0.20–60.00	$y = 0.69x + 0.78$	0.996	0.06	0.20

λ – detection wavelength (nm), RT – retention time, r^2 – correlation coefficients, LOD – limit of detection, LOQ – limit of quantification.

For identification purposes, mass spectrometry analysis was performed using a Bruker Esquire model 6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionisation (ESI) source. Data acquisition and processing were performed using Esquire control software. The mass spectrometer was operated in the negative mode, and the mass range from 50 to 1000 *m*/*z*, under the following conditions: nebulizer gas pressure of 50 psi, drying gas flow of 10 ml/min, desolvation temperature of 350 °C, cone voltage between 30 and 50 V, collision energy set between 10 and 45 V, and the capillary voltage ranged from 2.6 to 2.9 kV.

3. Results and discussions

3.1. Phenolic content of Sercial and Tinta Negra V. vinifera L. grapes

Table 1 shows the TPC at *véraison* and maturity stages of Sercial and Tinta Negra *V. vinifera* L. grapes. For Sercial, TPC values ranged

from 3568 to 7770 mg/kg, and for Tinta Negra from 3147 to 13812 mg/kg. The TPC values decreased from *véraison* (day 0) to maturity (day 42), for Sercial, whereas for Tinta Negra an increase, from *véraison* (day 0) to maturity (day 35), was observed. In general, it is known that the TPC increases throughout grape ripening (Pérez-Magariño & González-San José, 2006). Although, in some cases, this tendency was not reported (Alonso Borbalán et al., 2003), as observed for Sercial. According to a previous study (Alonso Borbalán et al., 2003), at the start of maturation, the grapes are smaller, the ratio of skins and seeds to the pulp is higher, with the phenolic compounds being therefore more concentrated. In fact, at *véraison*, Sercial grapes are smaller than Tinta Negra, but during ripening, their size increase is higher than that observed for Tinta Negra. The TPC values of Sercial, at *véraison*, were twofold higher than those found for Tinta Negra, whereas, at maturity, the TPC values of Tinta Negra were fourfold higher than those of Sercial. TPC is a screening approach to estimate the total content of phenolics, however, as it does not give any detailed information about phenolic fraction, therefore, the phenolic profile was established by HPLC–DAD–ESI-MS^n.

3.2. Phenolic identification by HPLC–DAD–ESI-MS^n

3.2.1. General

To establish the phenolic profiles two different ripening stages were selected: *véraison*, not a harvesting stage, but according to previous studies may present high level of phenolics (Alonso Borbalán et al., 2003; Obreque-Slier et al., 2010), and maturity, a harvesting stage for oenological practice. Fig. 2 shows the HPLC–DAD chromatogram of phenolic compounds at different ripening stages (*véraison* and maturity) recorded at signal of 360 nm. The peak assignment of phenolic compounds extracted from *V. vinifera* L. grape skins was carried out by comparison of their retention time and MS^n fragmentation profiles with standards, analyzed under the same experimental conditions, and/or with published data. As observed in Table 3, 40 phenolic compounds were tentatively identified, that include 3 hydroxybenzoic acids, 8 hydroxycinnamic acids, 4 flavanols, 5 flavanones, 8 flavonols, 4 stilbenes, and 8 anthocyanins.

3.2.2. Hydroxybenzoic acids

Compounds **1**, **3** and **5** with $[M-H]^-$ ions at *m*/*z* 191, 315 and 299, respectively, were identified as quinic acid, protocatechuic acid-glucoside and *p*-hydroxybenzoyl glucoside, respectively, comparing their MS^2 profiles with previously publish data (Aaby, Ekeberg, & Skrede, 2007; Fang, Yu, & Prior, 2002; Santos et al., 2010). Compounds **3** and **5** showed a common MS^2 fragmentation pattern $[M-H-glucose]^-$, which yield product ions at *m*/*z* 153 and 137, explained by the elimination of a glucose unit (162 amu).

3.2.3. Hydroxycinnamic acids

The identification of caffeoylshikimic acid (compound **2**, $[M-H]^-$ at *m*/*z* 335), caftaric acid (compound **6**, $[M-H]^-$ at *m*/*z* 311), *cis*-coutaric acid (compound **11**, $[M-H]^-$ at *m*/*z* 295), and *trans*-coutaric acid (compound **12**, $[M-H]^-$ at *m*/*z* 295) has been carried out by comparing the obtained MS^2 profiles with publish data (Alonso Borbalán et al., 2003; Bravo, Goya, & Lecumberri, 2007). The identification of *m*-coumaric acid (compound **31**, $[M-H]^-$ at *m*/*z* 163) and cinnamic acid (compound **40**, $[M-H]^-$ at *m*/*z* 147) was confirmed by their $[M-H]^-$ ion and retention time with the corresponding standards analyzed in the same HPLC conditions.

Compounds **7** and **14** were tentatively identified as a caftaric acid vanilloyl pentoside, and *p*-coumaric acid erythroside, respectively, based on their MS^n data as described below. The proposed fragmentation pathways for compounds **7** and **14** are illustrated in Fig. 3. Compound **7** showed a $[M-H]^-$ ion at *m*/*z* 577, and when fragmented led a MS^2 product ion at *m*/*z* 443, which corresponds to a loss of $C_8H_6O_2$ moiety (134 amu), corresponding to vanillin. Further, fragmentation of product ion (*m*/*z* 443) produced a MS^3

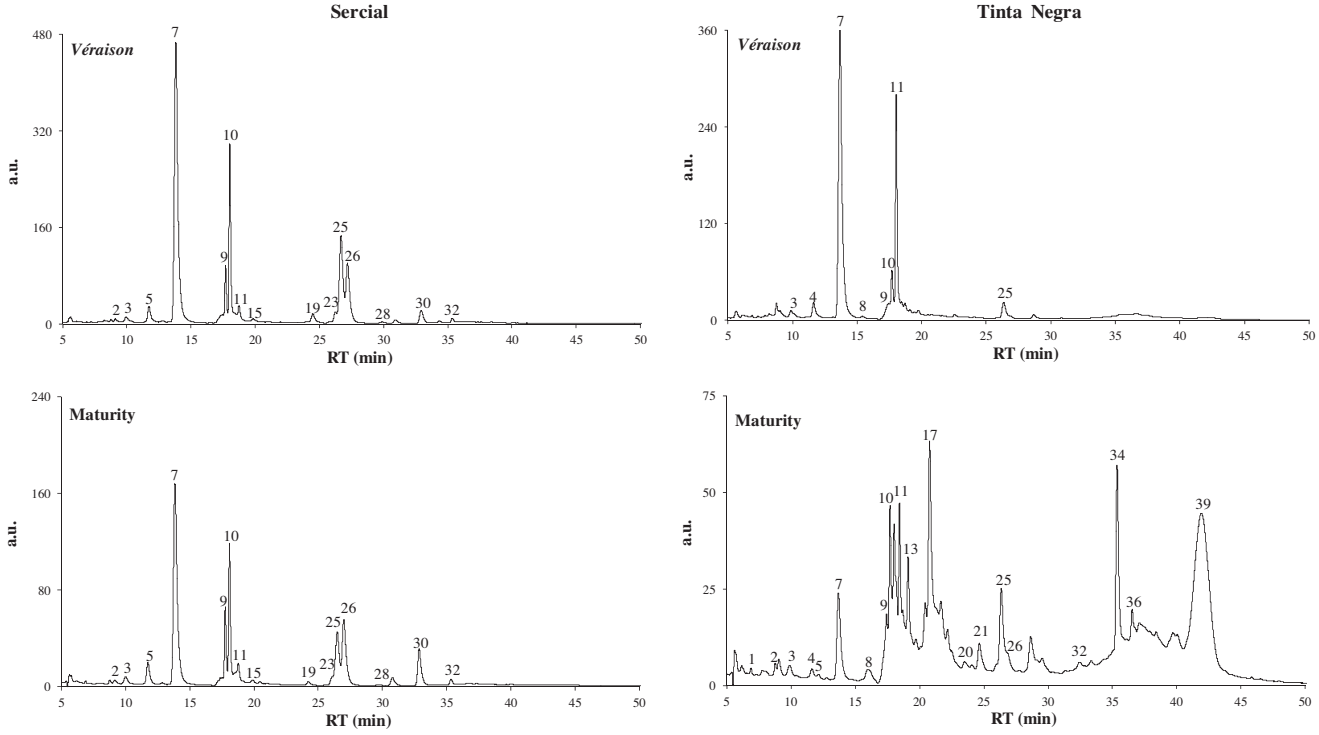

Fig. 2. HPLC–DAD chromatograms in Sercial and Tinta Negra grape skins at different ripening stages (*véraison* and maturity) recorded at signal of 360 nm. Peak numbers are reported in Table 3. a.u. – arbitrary unit.

Table 3
Characterisation and semiquantification of phenolic compounds of Sercial and Tinta Negra *Vitis vinifera* L. grape skins at different ripening stages (*véraison* and maturity) using HPLC–DAD–ESI-MS^n.

t_R (min)	Compound No.	Compound name	[M−H] (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 ions (*m*/*z*)	Identification	Phenolic content (mg/kg dried skin)			
							Sercial		Tinta Negra	
							Véraison	Maturity	*Véraison*	Maturity
		Hydroxybenzoic acids								
6.93	1	Quinic acid[a]	191	173, 127, **111**[A], 85		Santos et al. (2010)[B]	25.5 (2)	8.1 (8)	<LOD	10.5 (5)
10.14	3	Protocatechuic acid-glucoside[a,C]	315	**153**, 109		Fang, Yu, and Prior (2002)	264.9 (6)	202.4 (7)	27.6 (13)	51.3 (13)
11.40	5	*p*-Hydroxybenzoyl glucoside[a,C]	299	239, 179, **137**		Aaby et al. (2007)	283.9 (10)	159.3 (19)	<LOD	28.6 (15)
		Sub-total (mg/kg dried skin)					574.3 (4)	369.8 (9)	27.6 (13)	90.4 (7)
		Hydroxycinnamic acids								
7.80	2	Caffeoylshikimic acid[a]	335	**179**, 161, 135		Bravo, Goya, and Lecumberri (2007)	14.9 (14)	8.8 (6)	<LOD	33.1 (17)
12.74	6	Caftaric acid[b]	311	**179**, 135		Alonso Borbalán et al. (2003)	8.0 (15)	<LOQ	<LOD	<LOQ
13.68	7	Caftaric acid vanilloyl pentoside[b,C]	577	**443**	**311**, 179	MS	1839.8 (4)	526.4 (5)	700.0 (5)	65.9 (18)
18.42	11	*cis*-Coutaric acid[b]	295	**163**		Alonso Borbalán et al. (2003)	573.3 (4)	172.1 (8)	227.9 (6)	92.6 (18)
18.61	12	*trans*-Coutaric acid[b]	295	**163**		Alonso Borbalán et al. (2003)	122.1 (12)	79.5 (22)	<LOQ	<LOQ
19.70	14	*p*-Coumaric acid-erythroside[a,C]	265	205, **163**	119	MS	49.1 (14)	23.2 (7)	27.7 (1)	23.9 (6)
32.88	31	*m*-Coumaric acid[b]	163	**119**		CO	<LOQ	<LOQ	<LOD	<LOD
46.52	40	Cinnamic acid	147	-		CO	<LOD	<LOD	<LOD	6.2 (3)
		Sub-total (mg/kg dried skin)					2607.2 (4)	810.0 (7)	955.6 (5)	221.7 (15)
		Flavanols								
11.11	4	Epigallocatechin[c]	305	221, 219, **179**		Sun et al. (2007)	<LOQ	<LOQ	1.5 (18)	<LOQ
17.62	9	Proanthocyanidin dimer[c]	577	**425**, 407, 289		Sun et al. (2007)	227.7 (16)	63.8 (16)	86.5 (2)	115.2 (8)
18.02	10	Catechin[c]	289	**245**, 205, 179		Sun et al. (2007)	48.9 (10)	28.7 (19)	<LOD	<LOD
20.16	15	Epicatechin	289	**245**, 205, 179		Sun et al. (2007), CO	<LOQ	<LOQ	<LOD	37.6 (17)
		Sub-total (mg/kg dried skin)					276.6 (13)	92.5 (18)	88.0 (9)	152.8 (14)
		Flavanones								
17.12	8	Taxifolin[d]	303	177, 151, **125**		Sun et al. (2007)	<LOD	<LOD	35.9 (2)	37.1 (4)
21.46	16	Naringenin hexose derivate[d,C]	597	**435**	**271**, 177, 151, 119	MS	34.1 (1)	<LOD	<LOD	<LOD
22.73	19	Eriodictyol-glucoside[d,C]	449	**287**	**151**, 135	Hvattum (2002)	34.7 (3)	26.3 (1)	<LOD	<LOD
23.50	20	Taxifolin-pentoside[d,C]	435	**303**, 285, 151		Hvattum (2002)	<LOD	<LOD	25.6 (15)	45.5 (3)
35.86	35	Naringenin[d]	271	177, **151**, 119		Sun et al. (2007)	31.5 (2)	<LOD	26.8 (1)	32.1 (1)
		Sub-total (mg/kg dried grape)					100.3 (2)	26.3 (1)	88.3 (12)	114.7 (3)
		Flavonols								
22.36	18	Quercetin-glucuronide-glucoside[d,C]	639	**477**, 301	179, 151	Mullen, Edwards, and Crozier (2006)	34.7 (5)	<LOD	<LOD	<LOD
25.45	23	Rutin	609	**301**, 271, 255, 179		Hollecker et al. (2009), CO	416.6 (7)	61.6 (11)	280.9 (10)	428.0 (12)
26.63	25	Quercetin-glucuronide[d]	477	**301**	179, 151	Hollecker et al. (2009)	1140.9 (4)	280.2 (9)	15.3 (1)	24.7 (1)
27.23	26	Quercetin-glucoside[d]	463	**301**	179, 151	Alonso Borbalán et al. (2003)	871.3 (3)	370.4 (9)	56.2 (6)	245.5 (5)
29.93	28	Kaempferol-rutinoside[e]	593	**285**	257	Hollecker et al. (2009)	22.8 (11)	23.6 (17)	<LOD	<LOD
32.44	30	Quercetin-pentoside[d]	433	**301**	179, 151	Lopes-Lutz, Dettmann, Nimalaratne, and Schieber (2010)	151.9 (3)	138.7 (8)	<LOD	27.4 (1)
33.55	32	Malylated kaempferol-glucoside[e,C]	563	**447**	**285**, 257	MS	10.5 (15)	<LOQ	<LOQ	<LOQ
34.49	33	Kaempferol-glucoside[e]	447	**285**	257	Hollecker et al. (2009)	30.4 (13)	3.8 (7)	<LOD	<LOD
		Sub-total (mg/kg dried skin)					2679.1 (6)	878.3 (8)	352.4 (8)	725.6 (8)

(*continued on next page*)

Table 3 (*continued*)

t_R (min)	Compound No.	Compound name	[M−H] (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 ions (*m*/*z*)	Identification	Phenolic content (mg/kg dried skin)			
							Sercial		Tinta Negra	
							Véraison	Maturity	*Véraison*	Maturity
		Stilbenes								
24.07	21	Resveratrol dimer like restrysol[f,C]	471	377, 349, **255**		Jean-Denis et al. (2006)	<LOD	<LOD	8.5 (3)	14.3 (12)
26.03	24	*trans*-Piceid[f]	389	**227**		Hollecker et al. (2009)	16.2 (13)	7.3 (1)	<LOD	<LOD
39.41	37	*trans*-Resveratrol	227	**185**		Hollecker et al. (2009), CO	11.6 (3)	8.1 (3)	5.1 (1)	9.9 (1)
41.60	38	*cis*-Resveratrol[f]	227	**185**		Hollecker et al. (2009)	<LOD	7.4 (1)	5.2 (1)	8.0 (2)
		Sub-total (mg/kg dried skin)					27.8 (8)	22.8 (2)	18.8 (1)	32.2 (6)
		Anthocyanins								
19.28	13	Malvidin-glucoside (hydrated form)[g]	509	347, **329**		Mazerolles et al. (2010)	<LOD	<LOD	54.3 (1)	603.7 (3)
21.98	17	Malvidin-3-glucoside	491	**329**		Mazerolles et al. (2010), CO	<LOD	<LOD	136.9 (2)	3143.9 (4)
24.84	22	Delphinidin-coumarylglucoside (hydrated form)[g]	627	319, **301**		MS	<LOD	<LOD	33.3 (3)	153.7 (17)
29.32	27	Petunidin acetylglucoside[g]	519	**315**		MS	<LOD	<LOD	27.2 (2)	89.4 (13)
31.36	29	Delphinidin acetylglucoside[g]	505	**301**		MS	<LOD	<LOD	<LOD	42.2 (1)
35.30	34	Malvidin acetylglucoside[g]	533	**329**		Mazerolles et al. (2010)	<LOD	<LOD	<LOD	583.9 (16)
37.41	36	Malvidin coumarylglucoside[g]	637	**329**		MS	<LOD	<LOD	<LOD	310.1 (1)
42.07	39	Malvidin coumarylglucoside (hydrate form)[g]	655	347, **329**		MS	<LOD	<LOD	64.6 (1)	2087.9 (9)
		Sub-total (mg/kg dried skin)					<LOD	<LOD	316.3 (3)	7014.8 (16)
		Total (mg/kg dried skin)					6265.3 (4)	2199.7 (6)	1847.0 (5)	8352.2 (3)

<LOD – not detected.
<LOQ – not quantified.
CO – identified by co-injection and ESI fragmentation of standard.
MS – identified based on MS^n fragmentation pattern experimentally achieved.
[A] Ions in boldface indicate the more abundant *m*/*z* ion.
[B] Identified by comparing the ESI fragmentation with the published data.
[C] Compounds identified for the first time in *Vitis vinifera* L. grapes.
[a] Expressed in equivalents of protocatechuic acid.
[b] Expressed in equivalents of ferulic acid.
[c] Expressed in equivalents of epicatechin.
[d] Expressed in equivalents of quercetin.
[e] Expressed in equivalents of kaempferol.
[f] Expressed in equivalents of *trans*-resveratrol.
[g] Expressed in equivalents of malvidin-3-glucoside.

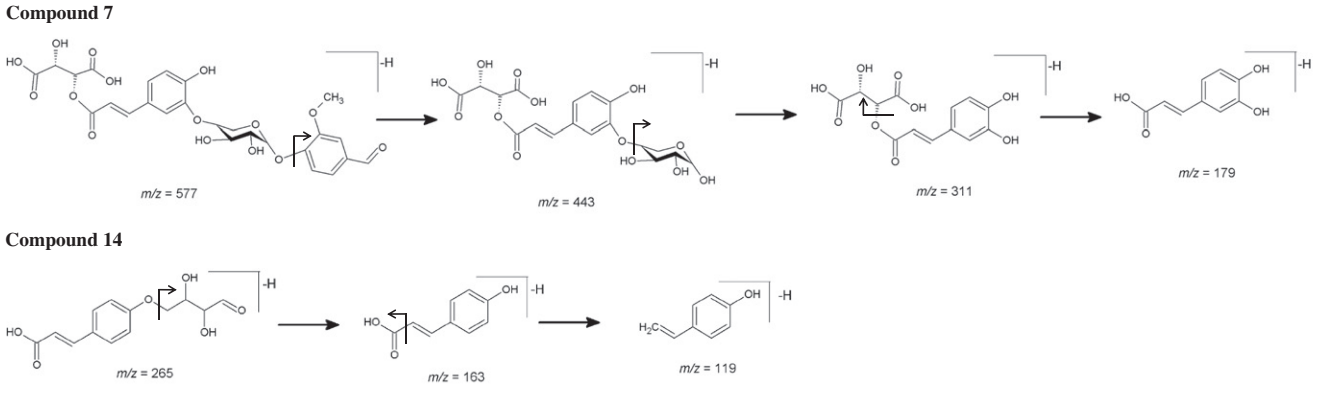

Fig. 3. Proposed fragmentation pathways for the caftaric acid vanilloyl pentoside (compound **7**) and *p*-coumaric acid-erythroside (compound **14**).

ion at *m*/*z* 311 and 179, which as reported above are common to caftaric acid (Alonso Borbalán et al., 2003). Compound **14** tentatively identified as *p*-coumaric acid-erythroside, showed a $[M-H]^-$ at *m*/*z* 265, and upon fragmentation lead to a MS^2 product ion at *m*/*z* 163, corresponding to a loss of *m*/*z* 102, which can be tentatively attributed to erythrose. Further, fragmentation of *m*/*z* 163

produced a MS^3 product ion at *m/z* 119, allowing the assignment of *m/z* 163 fragment to *p*-coumaric acid.

3.2.4. Flavanols

Based on MS^2 fragmentation pattern obtained and on published data, (Sun et al., 2007) four flavanols were identified: epigallocatechin (compound **4**, $[M-H]^-$ at *m/z* 305), a proanthocyanidin dimer (compound **9**, $[M-H]^-$ at *m/z* 577), catechin (compound **10**, $[M-H]^-$ at *m/z* 289), and epicatechin (compound **15**, $[M-H]^-$ at *m/z* 289). Epigallocatechin (compound **4**) $[M-H]^-$ fragmentation led to ions at *m/z* 221, 219 and 179, due to the cleavage of the A-ring of flavan-3-ol and heterocyclic ring fission, respectively. Compound **9** with a $[M-H]^-$ ion at *m/z* 577 has been previously identified as a proanthocyanidin dimer (Aaby et al., 2007), and exhibited MS^2 ions at *m/z* 425, 407, and 289. This pattern was recognized as a proanthocyanidin dimer of (epi)catechin–(epi)catechin type (Kajdžanoska, Gjamovski, & Stefova, 2010). The identification of catechin (compound **10**) and epicatechin (compound **15**) was confirmed by MS^2 product ions and retention times of corresponding standards.

3.2.5. Flavanones

Taxifolin (compound **8**, $[M-H]^-$ at *m/z* 303), eriodictyol-glucoside (compound **19**, $[M-H]^-$ at *m/z* 449), taxifolin-pentoside (compound **20**, $[M-H]^-$ at *m/z* 435), and naringenin (compound **35**, $[M-H]^-$ at *m/z* 271) were identified by comparing the obtained MS^2 fragmentation patterns with publish data (Hvattum, 2002; Sun et al., 2007). Compound **16** was tentatively identified as a naringenin hexose derivate, based on its MS^2 fragmentation pattern. This phenolic compound showed a $[M-H]^-$ at *m/z* 597, and its MS^2 fragmentation yielded the product ion at *m/z* 435, resulting from the loss of a glucose moiety (162 amu). Furthermore, fragmentation of *m/z* 435 produced a MS^3 ion at *m/z* 271, characteristic of naringenin, resulting from the loss of 164 amu ($C_9H_8O_3$). It was not possible to unambiguously identify the structure of this last moiety. However, the mass is compatible with aromatic structures for example of syringaldehyde type.

3.2.6. Flavonols

The flavonol fractions of Sercial and Tinta Negra grape skins are mainly composed by quercetin and kaempferol derivates. Five glycosides of quercetin, namely quercetin-glucuronide-glucoside (compound **18**, $[M-H]^-$ at *m/z* 639), quercetin-rutinoside (compound **23**, $[M-H]^-$ at *m/z* 609), quercetin-glucuronide (compound **25**, $[M-H]^-$ at *m/z* 477), quercetin-glucoside (compound **26**, $[M-H]^-$ at *m/z* 463), and quercetin-pentoside (compound **30**, $[M-H]^-$ at *m/z* 433) were identified based on MS^2 and MS^3 fragmentation patterns, by comparison with published data (Alonso Borbalán et al., 2003; Hollecker et al., 2009; Lopes-Lutz, Dettmann, Nimalaratne, & Schieber, 2010; Mullen, Edwards, & Crozier, 2006). All compounds showed a MS^2 fragment ion at *m/z* 301, which corresponds to the cleavage of a glycosidic linkage with concomitant H rearrangement. Compound **23** was identified as quercetin-rutinoside (rutin) after comparing their $[M-H]^-$ ion, MS^2 product ions, and retention time with the corresponding standard.

In addition, three kaempferol glycosides were also detected, namely kaempferol-rutinoside (compound **28**, $[M-H]^-$ at *m/z* 593), malylated kaempferol-glucoside (compound **32**, $[M-H]^-$ at *m/z* 563), and kaempferol-glucoside (compound **33**, $[M-H]^-$ at *m/z* 447). Compounds **28** and **33** were identified by comparing the obtained MS^2 profiles with publish data (Hollecker et al., 2009), whereas malylated kaempferol-glucoside (compound **32**) was identified based on the MS^n fragmentation pattern, as illustrated in Fig. 4. This compound showed a $[M-H]^-$ at *m/z* 563, and MS^2 led a product ion at *m/z* 447, which corresponded to the loss of malic acid ($C_4H_4O_4$, 116 amu). Furthermore, fragmentation of *m/z* 447 produced a MS^3 ion at *m/z* 285, which could be originated by the loss of glucose unit (162 amu). Finally, the ion at *m/z* 285 corresponds to kaempferol aglycone in the negative ionisation mode.

3.2.7. Stilbenes

Compounds **21**, **24**, **37** and **38** were identified as a resveratrol dimer ($[M-H]^-$ ion at *m/z* 471), a resveratrol glycoside (*trans*-piceid, $[M-H]^-$ ion at *m/z* 389), *trans*-resveratrol ($[M-H]^-$ ion at *m/z* 227), and *cis*-resveratrol ($M-H]^-$ ion at *m/z* 227), based on their characteristic $[M-H]^-$ ions and MS^2 fragmentation patterns (Hollecker et al., 2009; Jean-Denis, Pezet, & Tabacchi, 2006). According to the MS fragmentation pattern of the resveratrol dimer detected, it is proposed that this dimer could be a resveratrol dimer like restrytsol, which was previously reported in grapevine leaves (Jean-Denis et al., 2006). *trans*-Resveratrol identification was also confirmed by comparison with a chemical standard. As reported above for other glycoside derivatives, *trans*-piceid, a resveratrol glycoside (compound **24**) was also identified by its $[M-H]^-$ ion and product ion at *m/z* 227, due to the loss of glucose moiety (Hollecker et al., 2009).

3.2.8. Anthocyanins

This phenolic class is preferentially detected using positive mode, which are found as favylium cations under acidic conditions, whereas the negative mode is usually preferred for carboxylic acids and uncharged flavonoids, which are detected as the deprotonated $[M-H]^-$ species (Kammerer et al., 2004; Mazerolles et al., 2010). Nevertheless, the negative mode has been successfully applied to identify this phenolic class in red wines (Mazerolles et al., 2010). In the present study, using negative mode, eight anthocyanins (compounds **13**, **17**, **22**, **27**, **29**, **34**, **36**, and **39**) were identified, and a similar fragmentation pattern was observed, which comprises the corresponding ($[M-H]^-$ ions, and MS^2 fragments with characteristic *m/z* values of each aglycone (*m/z* 329, 315, and 301 for malvidin, petunidin and delphinidin respectively), indicating the loss of the sugar moiety. Compounds **13** ($[M-H]^-$ ion at *m/z* 509) and **17** ($[M-H]^-$ ion at *m/z* 491) were identified as malvidin-glucoside in hydrated form and malvidin-3-glucoside, respectively, by comparing the obtained MS^2 profiles with publish data (Mazerolles et al., 2010). Furthermore, malvidin-3-glucoside identification was also confirmed by comparison with the retention

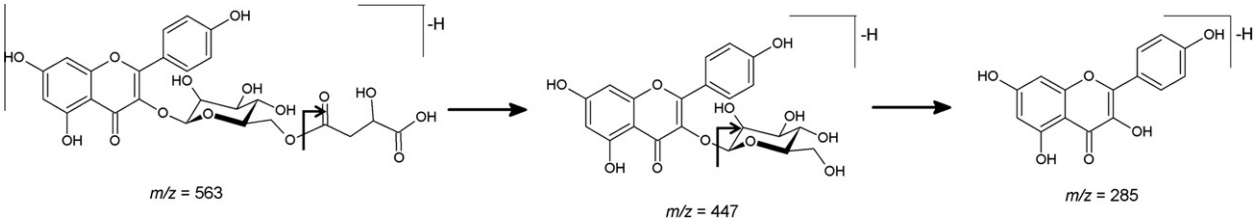

Fig. 4. Proposed fragmentation pathway for the malylated kaempferol-glucoside (compound **32**).

time and fragmentation profile of a chemical standard. Compounds **34** ([M−H]$^-$ at *m*/*z* 533), **36** ([M−H]$^-$ at *m*/*z* 637), and **39** ([M−H]$^-$ at *m*/*z* 655) were tentatively identified as malvidin acetylglucoside, malvidin coumarylglucoside, and malvidin coumarylglucoside in hydrated form, respectively; in all cases MS^2 led to the detection of a product ion at *m*/*z* at 329, corresponding to malvidin aglycone. The MS^2 product fragments corresponding to the loss of an acetylglucose unit (204 amu), and coumarylglucoside (308 amu) moiety were observed for compounds **34** and **36** respectively. The petunidin acetylglucoside (compound **27**) was tentatively identified based on its [M−H]$^-$ ion at *m*/*z* 519, and MS^2 exhibited a product ion at *m*/*z* 315, indicating the loss of acetylglucoside moiety (204 amu). Delphinidin-coumarylglucoside in hydrated form (compound **22**, [M−H]$^-$ at *m*/*z* 627) and delphinidin acetylglucoside (compound **29**, [M−H]$^-$ at *m*/*z* 505) were also detected, and in both cases MS^2 studies showed a product ion at *m*/*z* 301, corresponding to delphinidin aglycone.

3.3. Phenolic profile of Sercial and Tinta Negra V. vinifera L. grapes

The quantification of phenolic compounds was performed by external calibration curves, using a set of nine reference compounds selected based on the principle of structure related target analyte/standard (chemical structure or functional group). The relevant data concerning the calibration plots is shown in Table 2, and a good linearity was obtained with a regression coefficient (r^2) higher than 0.995. The limits of detection (LOD) and quantification (LOQ) were calculated on the basis of the standard deviation of the replicate analyses concerning the phenolic standards with lowest concentration level, where LOD and LOQ are three and ten times of standard deviation, respectively. Therefore, the LOD values range from 0.03 to 0.24 μg/ml, whereas the LOQ values range from 0.09 to 0.80 μg/ml. The qualitative and quantitative data about the identified phenolic compounds are reported in Table 3, and it was conducted at 280 nm for the phenolic acids, and at 320, 360, and 520 nm for stilbenes, flavonols, and anthocyanins, respectively.

Sercial and Tinta Negra grape skins exhibited different phenolic profiles, concerning the type of compounds identified and their corresponding content. At *véraison*, a total of 27 and 22 phenolic compounds were detected in Sercial and Tinta Negra grape skins, respectively, whereas at maturity a total of 25 and 32 phenolic compounds was identified. Moreover, for Sercial, at *véraison* and maturity the quantified phenolic compounds accounted for 6265 and 2200 mg/kg, respectively. For Tinta Negra, the quantified phenolic compounds accounted for 1847 and 8352 mg/kg, at *véraison* and maturity, respectively.

Caftaric acid vanilloyl pentoside, quercetin-glucuronide, quercetin-glucoside, *cis*-coutaric acid, protocatechuic acid-glucoside, and *p*-hydroxybenzoyl glucoside were the predominant phenolic compounds identified in Sercial, which represent about 78% of phenolic composition, in both ripening stages. For Tinta Negra, at *véraison*, the predominant phenolic compounds detected were caftaric acid vanilloyl pentoside, *cis*-coutaric acid, procyanidin dimer, rutin, and malvidin-3-glucoside (73% of the phenolic composition), whereas at maturity malvidin-3-glucoside, malvidin-3-glucoside (hydrated form), malvidin acetylglucoside, malvidin coumarylglucoside (hydrated form), and rutin represent 82% of the phenolic composition. Other phenolic compounds were only detected in Sercial grape skins, namely *m*-coumaric acid, catechin, quercetin-glucuronide-glucoside, kaempferol-rutinoside, kaempferol-glucoside, and *trans*-piceid, whereas epicatechin, resveratrol dimer like restrysol, and anthocyanins were only detected in Tinta Negra. As expected, no anthocyanins were detected in Sercial white variety, as the methodology applied in this research was not the most suitable to determine traces of anthocyanin, characteristic of white varieties. From the total of 40 phenolic compounds detected, and as far we know, 10 are detected for the first time in *V. vinifera* L. grapes (see Table 3). These phenolic compounds represent about 40% of the phenolic profile for Sercial grape skins, at both ripening stages, and for Tinta Negra at *véraison*. Moreover, for Tinta Negra at maturity, these phenolic compounds represent only 0.8% of the phenolic composition.

The phenolic compounds contents of Sercial and Tinta Negra varieties reported here were compared with German (Carbenet Mitos, Lemberger, Spätburgunder, Schwazriesling, Trollinger, Weisser Riesling) (Kammerer et al., 2004), Italian (Chardonnay and Sauvignon) (Hollecker et al., 2009), and Portuguese varieties (Alfrocheiro, Jaen, Tinta Roriz, Touriga Nacional, and Touriga Francesa) (Mateus et al., 2011; Novak, Janeiro, Seruga, & Oliveira-Brett, 2008). This comparison showed that flavanols concentration in Sercial and Tinta Negra grape skins was lower than Weisser Riesling (Kammerer et al., 2004), whereas the flavonols concentration is quite similar. Moreover, flavanols and flavonols content in Sercial and Tinta Negra were quite similar to those reported for Italian (Hollecker et al., 2009), and higher than for other Portuguese varieties (Novak et al., 2008). The anthocyanins concentration in Tinta Negra was quite similar to those observed for German (Kammerer et al., 2004), and higher than for Portuguese varieties (Mateus et al., 2011; Novak et al., 2008). Despite the fact that this study was carried out only over one harvesting period, the results reported above are noteworthy, as previous studies demonstrated that phenolic profile of grapes do not change significantly through harvests (Fanzone et al., 2011; Kammerer et al., 2004; Mazza et al., 1999). Thus, the data obtained about phenolic composition of Sercial and Tinta Negra *V. vinifera* L. grape skins indicated that these varieties are well-situated, based on phenolic compounds contents, when compared to world-famous varieties.

4. Conclusions

This study represents the first phytochemical research of phenolic components of Sercial and Tinta Negra *V. vinifera* L. varieties used to produce Madeira wine. A total of 40 phenolic compounds were identified in Sercial and Tinta Negra grape skins using HPLC–DAD–ESI-MS^n. From these, as far as we know, 10 are reported for the first time in *V. vinifera* L. grapes, namely protocatechuic acid-glucoside, *p*-hydroxybenzoyl glucoside, caftaric acid vanilloyl pentoside *p*-coumaric acid-erythroside, naringenin hexose derivate, eriodictyol-glucoside, taxifolin-pentoside, quercetin-glucuronide-glucoside, malylated kaempferol-glucoside, and resveratrol dimer like restrysol. It is important to point out that, at maturity, this set of ten compounds represent 40% of the phenolic composition of Sercial, whereas for Tinta Negra only 0.8%. At maturity, hydroxycinnamic acids and flavonols were the predominant classes for Sercial (about 80%), whereas Tinta Negra was mainly composed by anthocyanins (84%).

Sercial grape skins, in both ripening stages, and Tinta Negra, at *véraison*, may be considered as a potential source of hydroxycinnamic acids, as well as of flavonols, which are known to be responsible for the bitter and astringent properties of wine. Tinta Negra grape skins, at maturity, may be considered as an anthocyanins source, which are responsible for the bluish-red and purple colour of grapes and wines (Tian et al., 2009; Vidal et al., 2004). The final contribution of these components for the wine organoleptic properties is ruled by the winemaking process. For example, for Tinta Negra grapes, the maceration time could be optimized in order to increase the anthocyanins concentration in wine.

From human health benefits point of view, some considerations can be taken into account. Considering the antioxidant activity order previously reported (procyanidin dimer > flavanol > flavonol > hydroxycinnamic acids > simple phenolic acids) (Soobrattee,

Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005), and the Sercial and Tinta Negra phenolic profiles, these grape varieties may be considered a potential sources of natural antioxidants.

In vivo study using different cancer cell lines reported that quercetin-3-glucoside, one of the major phenolic compounds of Sercial skins, presented notable growth-inhibitory effects in colon, breast, hepatocellular, and lung cancer cells. Rutin, one of the major Tinta Negra phenolic compounds, showed antiproliferative effect on five epithelial cancer cells (You, Ahn, & Ji, 2010). Furthermore, several studies have demonstrated a wide range of anthocyanins biological activities (Xia et al., 2010). Malvidin-3-glucoside, the major compound in Tinta Negra, at maturity, was reported as reducer of oxidative stress, and showed also beneficial effects on cardiovascular diseases, and chronic inflammation associated with nitric oxide (Wang & Mazza, 2002). In addition, some studies reported the physiological and biochemical outcomes after supplementation with grape extracts, a potential source of phenolic compounds (Falchi et al., 2006; Frederiksen et al., 2007).

Finally, the data obtained on the present study represents valuable information specially helping the winemaker support decision, as some empirical knowledge may be now sustained by objective data (i.e. high astringency of Sercial variety). This type of information is fundamental to evaluate the winemaking adequacy for each variety, allowing the improvement of the wine quality and respecting the specificities of Madeira wine. This evaluation should be included in the innovation strategy, as an essential factor in the sustainable oenology of each Appellation. Finally, the valorisation of these varieties may also be explored regarding the presence of potential bioactive compounds. Future *in vivo* assays, especially using models closed to human, must be performed using phenolic fractions of Sercial and Tinta Negra grape skins to understand their potential human health benefits.

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