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Identification of *Vitis vinifera* L. grape berry skin color mutants and polyphenolic profile

Vanessa Ferreira [a,b], Fátima Fernandes [c], Olinda Pinto-Carnide [d], Patrícia Valentão [c], Virgílio Falco [e], Juan Pedro Martín [f], Jesús María Ortiz [f], Rosa Arroyo-García [b], Paula B. Andrade [c], Isaura Castro [a,*]

[a] Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal
[b] Centro de Biotecnología y Genómica de Plantas (CBGP), Campus de Montegancedo, Autovía M40, km38, 28223 Pozuelo de Alarcón, Madrid, Spain
[c] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, R. Jorge Viterbo Ferreira, n° 228, 4050-313 Porto, Portugal
[d] Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal
[e] Centro de Química de Vila Real (CQ-VR), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal
[f] Dpto. de Biotecnología-Biología Vegetal, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitária s/n, 28040 Madrid, Spain

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ABSTRACT

A germplasm set of twenty-five grapevine accessions, forming eleven groups of possible berry skin color mutants, were genotyped with twelve microsatellite loci, being eleven of them identified as true color mutants. The polyphenolic profiling of the confirmed mutant cultivars revealed a total of twenty-four polyphenols, comprising non-colored compounds (phenolic acids, flavan-3-ols, flavonols and a stilbene) and anthocyanins.

Results showed differences in the contribution of malvidin-3-*O*-glucoside to the characteristic Pinot Noir anthocyanins profile. Regarding the two Pique-Poul colored variants, the lighter variant was richer than the darker one in all classes of compounds, excepting anthocyanins. In Moscatel Galego Roxo the F3′H pathway seems to be more active than F3′5′H, resulting in higher amounts of cyanidin, precursor of the cyanidin derivatives.

As far as we are aware, this is the first time that a relationship between the content of polyphenolic compounds is established in groups of grape berry skin color mutant cultivars.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most cultivated fruit plants and an economically important crop worldwide. Grapes, consumed either as fresh fruit or as products derived from them (wine, juice and others), are a rich source of polyphenolic compounds (Fraige, Pereira-Filho, & Carrilho, 2014). These compounds are one of the main quality factors of grapes and wine, due to their contribution to wine color, oxidation reactions, interactions with proteins, aging behavior of wines and sensorial characteristics, such as bitterness and astringency (Figueiredo-González,

* Corresponding author.
E-mail address: icastro@utad.pt (I. Castro).

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Martínez-Carballo, et al., 2012). In red wines, the perception of astringency has been mainly attributed to proanthocyanidins, also known as condensed tannins, which are involved in copigmentation processes with the anthocyanins and the formation of new pigments, which contribute to the stability and definition of red wine color (Quijada-Morín et al., 2012).

In addition, polyphenols are confirmed to have a variety of effects on human health, such as anti-inflammatory, antimicrobial and anti-aging, and also to play a preventing role against cardiovascular diseases (Ivanova et al., 2011). They are considered to be the key compounds responsible for the antioxidant potential of grapes and wine (Burns et al., 2000).

Polyphenolic compounds are mainly present in skin and seeds of grape berries and can be classified in two groups, flavonoids and non-flavonoids, based on the primary chemical structures of hydroxybenzenes (Gómez Gallego, Gómez García-Carpintero, Sánchez-Palomo, Hermosín-Gutiérrez, & González Viñas, 2011; Liang, Owens, Zhong, & Cheng, 2011). The majority of the flavonoids found in grapes include flavan-3-ols, flavonols and anthocyanins, three structural classes divided according to the oxidation degree of their heterocyclic ring. Non-flavonoids are mainly hydroxycinnamic and hydroxybenzoic acids, stilbenes and volatile phenols (Figueiredo-González, Martínez-Carballo, et al., 2012; Liang et al., 2011; Teixeira, Eiras-Dias, Castellarin, & Gerós, 2013).

Among these classes of polyphenolic compounds, hydroxycinnamic and hydroxybenzoic acids play critical roles in developing the bitterness and astringency properties of wine. The synthesis of hydroxycinnamic acids occurs mainly before *véraison* and they are commonly accumulated in berry skin and flesh of white and red varieties. Their concentration decreases with the increase of fruit size and dilution solutes during ripening. Particularly in white wines, hydroxycinnamic acids contribute to color browning under oxidation with non-phenolic molecules. Compared to the amount of hydroxycinnamic acids, hydroxybenzoic derivatives levels are commonly low in wine (Teixeira, Eiras-Dias, Castellarin, & Gerós, 2013).

Anthocyanins are the most abundant polyphenolic compounds in colored grapes, being responsible for red, purple and blue pigmentation of the grape berries and, consequently, of the red wine. The second most abundant class of flavonoids is flavan-3-ols. In grape, flavan-3-ols exist as monomers or linked forming condensed tannins. Flavan-3-ols have a direct impact on the complexity of wine taste and mouthfeel, being responsible for bitterness of wine and also associated with astringency (Liang et al., 2011; Teixeira et al., 2013). Flavonols are found in grapes and wine as glycosides. Flavonols are yellow pigments that directly contribute to the color of white wines, being masked by anthocyanins in red wines. However, flavonols are also important cofactors for color enhancement, affecting red wine color by means of copigmentation (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2010; Liang et al., 2011).

Because of the role of polyphenols in the overall quality and, therefore, the market value of grapes and grape products, there has been considerable interest and research in determining the composition and contents of polyphenolic compounds in grape cultivars (Liang et al., 2011) and wines, particularly by relating the polyphenolic content and profile with specific features, such as color, astringency and sweetness (Figueiredo-González, Cancho-Grande, & Simal-Gándara, 2013; Figueiredo-González, Cancho-Grande, et al., 2014; Figueiredo-González, Regueiro, Cancho-Grande, & Simal-Gándara, 2014; Quijada-Morín et al., 2012). Although polyphenolic compounds biosynthesis in *V. vinifera* L. grapes is under genetic control and can be affected by several factors, as grape variety, ripening stage, climate, soil, light, place of growing and vine cultivation, the differences among grape cultivars are sometimes enough to make possible to use grape polyphenolic composition as a tool for cultivar authenticity and differentiation (Castillo-Muñoz et al., 2010).

Grape variety identification can be achieved by accurate genetic methods (Polymerase Chain Reaction – PCR), usually molecular markers. However, such methods are not currently available for grape berry color mutant cultivars discrimination. As so, the aim of this study was to provide data about the characteristic profiles of polyphenolic compounds of ripe grapes, by determining the compounds on a group of color and non-color related berried grapevine cultivars, derived from single varieties identified and selected by Simple Sequence Repeat (SSR) molecular markers.

2. Materials and methods

2.1. Standards and reagents

Acetic acid, acetonitrile, methanol (MeOH), ethyl acetate, formic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and hydrochloric acid from Pronalab (Lisboa, Portugal). The polyphenolic compounds used as reference were purchased from Sigma–Aldrich (Steinheim, Germany) (caftaric acid, gallic acid, *p*-coumaric acid, quercetin-3-*O*-glucoside, epigallocatechin gallate, resveratrol-3-*O*-glucoside and syringic acid) and Extrasynthése (Genay, France) (catechin, epigallocatechin, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, (−)-epicatechin, epicatechin gallate, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside and syringetin-3-*O*-glucoside).

The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Solid-phase extraction (SPE) was performed with Chromabond C18 non-endcapped (NEC) columns (50 μm particle size, 60°A porosity; 10 g sorbent mass/70 mL reservoir volume) from Macherey–Nagel (Düren, Germany).

2.2. Grape samples

Twenty-five grapevine accessions of different varieties were harvested in 2011 in the same plot, in the experimental vineyard of the University of Trás-os-Montes and Alto Douro, Vila Real (41°19′N, 7°44′W, 500 m above mean sea level), Baixo Corgo sub-region of the Demarcated Douro Region, northern Portugal. The cultivation practices followed (spraying of crop protectants, weed control, shoot guiding) were the same for all vines. The mean annual temperature in the region was 13.3 °C; and total annual rainfall was 721 mm (IPMA, 2015).

Sampling was performed by picking young leaves and optimum ripeness berries randomly distributed throughout each plant. Samples represent a putative berry color mutant pool, which includes samples with similar designation, differing only in relation to the name of its skin berry color.

After harvest, the entire grapes were stored at −20 °C and freeze-dried in a Labconco Freezone 4.5 apparatus (Kansas City, MO, US). The lyophilized samples were then powdered in an appliance mill (model A327R1, Moulinex, Spain). The powdered material was kept in a desiccator, in the dark, until analysis.

Voucher specimens were deposited at Laboratório de Farmacognosia, Faculdade de Farmácia, Universidade do Porto, and at Laboratório de Marcadores Moleculares, University of Trás-os-Montes and Alto Douro under the following designations: AL092011_B (Alvarelhão), ALB092011_W (Alvarelhão Branco), B092011_B (Bastardo), BR092011_R (Bastardo Roxo), BB092011_W (Bastardo Branco), CB092011_W (Carrega Branco), CT092011_B (Carrega Tinto), MF092011_W (Malvasia Fina),

MFR092011_R (Malvasia Fina Roxo), MR092011_W (Malvasia Rei), MRT092011_B (Malvasia Rei Tinto), MGB092011_W (Moscatel Galego Branco), MGR092011_R (Moscatel Galego Roxo), MGT092011_B (Moscatel Galego Tinto), M092011_B (Mourisco), MB092011_W (Mourisco Branco), PB092011_W (Pinot Blanc), PG092011_R (Pinot Gris), PN092011_B (Pinot Noir), PPG092011_R (Pique-Poul Gris), PPN092011_B (Pique-Poul Noir), TB092011_W (Touriga Branco), TT092011_B (Touriga Tinto), G092011_W (Gouveio), GR092011_R (Gouveio Roxo).

2.3. DNA extraction and nuclear microsatellite amplification

Accessions were genotyped by amplifying a set of twelve microsatellite markers, including the *Organisation Internationale de la Vigne et du Vin* (OIV) core set: VVS2, VVMD5, VVMD7, VVMD27, ssrVrZAG62, ssrVrZAG79, that correspond to OIV801 to OIV806 descriptors (OIV., 2009), established by the European Project GENRES#81 for the identification of grapevine cultivars and part of the OIV 'Descriptor List for Grapevine Varieties and *Vitis* Species' along with VVMD28, VVMD32 (Bowers, Dangl, & Meredith, 1999), VVIv37, VVIv67, VVIp31 (Merdinoglu et al., 2005) and VMC4f3 (Di Gaspero, Peterlunger, Testolin, Edwards, & Cipriani, 2000).

Young leaves genomic DNA was extracted using DNeasy® Plant Mini Kit (QIAGEN, Düren, Germany) purification kit, according to the manufacturer's instructions. Extracted genomic DNA was quantified using a UV spectrometer (Nanodrop® ND-1000, Fisher Scientific, Wilmington, Delaware, USA), followed by quality check in a 1.0% agarose gel electrophoresis. Necessary dilutions were done (approximately 10 ng/μL) and kept at 4 °C for further utilization.

Each 20 μL PCR mixture contained 0.2 mM of deoxynucleotide triphosphate (dNTP), 2 mM of $MgCl_2$, 10 ng of template DNA, various concentrations of primer and 1 U of *Taq* DNA polymerase in the manufacturer's buffer. One primer of each pair was fluorescently labeled with 6-fluorescein amidite (FAM) (blue), tetrachlorofluorescein succinimidyl ester (TET) (green) or hexachlorofluorescein succinimidyl ester (HEX) (yellow). PCR amplifications were performed in a T-100™ Thermal Cycler (BIORAD). The program comprised an initial denaturation step (95 °C/5 min), followed by 40 cycles of 94 °C/45 s, 50 °C/60 s and 72 °C/90 s.

Two multiplex PCRs were carried out with the OIV simple Sequence Repeats (SSR) core set, the first one involving VVS2, VVMD5 and VVMD7 (set A), and the second VVMD27, ssrVrZAG62 and ssrVrZAG79 (set B).

Set A multiplex reactions contained 0.2 μM of VVS2, 0.5 μM of VVMD5 and 0.25 μM of the VVMD7 primer pairs. Set B reactions included 0.5 μM of VVMD27 and of ssrVrZAG79, and 0.1 μM of ssrVrZAG62 primer pairs. Individual reactions were performed with the remaining six primer pairs (VVMD28, VVMD32, VVIv37, VVIv67, VVIp31 and VMC4f3), with a primer concentration of 0.5 μM.

The amplicons were separated in 3% (w/v) agarose gel electrophoresis in Tris–borate–EDTA (TBE) buffer, for 2 h at a constant voltage of 120 V, followed by ethidium bromide staining to verify the existence of amplicons, and then by capillary electrophoresis (ABI PRISM model 310, PE Applied Biosystems, CA, USA). GENESCAN-350 TAMRA (PE Applied Biosystems, CA, USA) was included as internal sizing standard and labeled products were analyzed and sized using Peak Scanner V1.0 software (PE Applied Biosystems, CA, USA).

2.4. Extraction of polyphenolic compounds

Healthy berries from each accession (ca. 5 g) were extracted with 100 mL of 80% MeOH for 2 h, under stirring (300 rpm) after flushing with nitrogen in order to prevent oxidations during extraction. The extract was centrifuged (10 min, 4000 rpm) and the material was re-extracted with 100 mL of 80% MeOH (15 min). The combined supernatants were evaporated to dryness under reduced pressure, at 30 °C. The residue was dissolved in 50 mL of deionized water and applied on the SPE cartridge, preconditioned with 20 mL of ethyl acetate, 20 mL of methanol and 20 mL of 0.01 N HCl. Non-colored phenolics (fraction I) and anthocyanins (fraction II) were eluted with 20 mL of ethyl acetate and 40 mL of methanol containing 0.1% HCl, respectively. The eluates were concentrated under reduced pressure and the residues obtained were redissolved in appropriate volume of methanol (fraction I) and acidified water (pH 3.0) (fraction II), membrane-filtered (0.45 μm) and an aliquot of 20 μL was injected into an HPLC-DAD system.

2.5. HPLC-DAD analysis of polyphenolic compounds

Non-colored phenolic compounds and anthocyanins were analyzed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column (25.0 cm × 0.46 cm, 5 μm particle size; Waters, Milford, MA, USA).

2.5.1. Non-colored compounds

The mobile phase solvents consisted of 2% (v/v) acetic acid in water (eluent A) and 0.5% (v/v) acetic acid in water and acetonitrile (50:50, v/v, eluent B) using a gradient program as follows: from 10% to 24% B (20 min), from 24% to 30% B (20 min), from 30% to 55% B (20 min), from 55% to 70% B (5 min), from 70% to 80% B (5 min), from 80% to 100% (5 min), 100% B isocratic (5 min). Flow rate was 1.0 mL/min. Chromatograms were registered at 280, 320 and 350 nm. Compounds were identified by comparing their retention times and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008). Quantification was performed by external standard method. Flavan-3-ols, syringic and gallic acids were determined at 280 nm, hydroxycinnamic derivatives and resveratrol-3-*O*-glucoside were quantified at 320 nm and flavonols at 350 nm. Coutaric acid was determined as *p*-coumaric acid and the other compounds as themselves. Standards and samples were analyzed in triplicate.

2.5.2. Anthocyanins

The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v, eluent A; 40:10:50, v/v/v; eluent B) using a gradient program as follows: from 10% to 25% B (10 min), from 25% to 31% B (5 min), from 31% to 40% (5 min), from 40% to 50% B (10 min), from 50% to 100% B (10 min). Flow rate was 0.8 mL/min. Detection was performed at 500 nm. Compounds were identified by comparing their chromatographic behavior and UV spectra with those of authentic standards and with literature data (Dopico-García et al., 2008). Quantification was performed by external standard method. Petunidin-3-*O*-*p*-coumaroylglucoside and petunidin-3-*O*-glucoside were quantified as petunidin; peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside were determined as peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside, respectively. The other compounds were determined as themselves. Standards and samples were analyzed in triplicate.

2.6. Statistical analysis

Principal component analysis (PCA) was carried out using SPSS® 21.0 software (IBM, NY, USA). PCA was applied for reducing the number of variables (24 variables corresponding to each identified phenolic compound: gallic acid, caftaric acid, coutaric acid, catechin, syringic acid, epicatechin, epigallocatechin gallate, epicatechin gallate, resveratrol-3-*O*-glucoside, myricetin-3-*O*-glucoside,

quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, syringetin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, petunidin-3-*O*-*p*-coumaroylglucoside, peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside) to a smaller number of the new derived variables (principal components, PCs) that adequately summarize the original information, i.e., the phenolic composition of the grape berry skin color mutant cultivars. PCA method shows similarities between samples projected on a plan and makes it possible to identify which variables determine these similarities and in what way.

3. Results and discussion

3.1. Microsatellite analysis for grape berry color mutants identification

In the last years, developments in DNA analysis for varieties discrimination through microsatellite fingerprinting in viticulture has become the technique of choice for grape varietal identification and distinction (Bowers, Dangl, Vignani, & Meredith, 1996; Sefc, Regner, Turetschek, Glossl, & Steinkellner, 1999). Several grapevine varieties develop color mutants, originating new cultivars, in which phenotypic identification can be difficult before the fruit setting. However, these cultivars have the same profile than the original variety if they are analyzed by using microsatellite markers, thus facilitating the identification of the true grape berry color mutants. The European project GENRES#081 established a set of six microsatellite markers, which was included in the 'Descriptor List for Grapevine Varieties and *Vitis* Species' and used in this study (OIV, 2009). Although these six microsatellite loci are considered as the minimal standard marker set for grapevine cultivar identification, one other group of six microsatellite loci were amplified in order to proceed to a more accurate grape cultivar authentication (Table 1).

In this study, eleven groups of two or three accessions, with names suggesting the existence of berry skin color mutation were characterized with microsatellites. By comparison with published profiles, identification of most of the varieties was confirmed, and six new genotypes were detected (Table 1).

As a consequence of the microsatellite analysis, eleven of the twenty-five accessions were selected and identified as true berry skin color mutants, belonging to five distinct varieties: Malvasia Fina, Gouveio, Moscatel Galego, Pinot, and Pique-Poul (shaded in gray in Table 1), which were used for polyphenols composition analysis. The remaining cases, Alvarelhão, Carrega, Mourisco, and Touriga, the supposed color mutation were discarded.

Bastardo Branco, Moscatel Galego Tinto, the two Malvasia Fina and Gouveio, and the three Pinot profiles were confirmed by comparison with previously published results (Pinto-Carnide et al., 2003; Veloso et al., 2010) (Table 1). Carrega Tinto and Touriga Tinto were identified as Tinta Grossa (Veloso et al., 2010) and Touriga Franca (Martín et al., 2006), respectively (Table 1). Furthermore, Mourisco was identified as Marufo (Castro, Martín, Ortiz, & Pinto-Carnide, 2011).

Moreover, synonymies with Spanish cultivars were also confirmed, namely Alvarelhão with Brancellao; Alvarelhão Branco with Prieto Picudo Blanco I; Gouveio and Gouveio Roxo with Godello; Moscatel Galego Branco and Moscatel Galego Roxo with Moscatel de Grano Menudo (Martín, Borrego, Cabello, & Ortiz, 2003), Malvasia Rei with Palomino Fino (EU-VITIS, 2015) and Pique-Poul Gris and Pique-Poul Noir with Picapoll Negro (Cabello et al., 2012) (Table 1). As it can be observed, microsatellites profiles were the same for color mutants (Table 1).

The three Bastardo accessions showed different profiles although Bastardo and Bastardo Branco had always at least one allele of each loci in common, indicating a possible parentage relationship among them. The same occurred with Malvasia Rei and Malvasia Rei Tinto, and also with Moscatel Galego Tinto and Moscatel Galego Branco.

3.2. Polyphenolic compounds

Twenty-four polyphenolic compounds, distributed by colored and non-colored compounds, were identified (Figs. 1 and 2) and quantified (Table 2) in all berry skin color mutants selected by SSRs.

3.2.1. Non-colored compounds

Among non-colored polyphenolic compounds, four phenolic acids, eleven flavonoids and one stilbene were identified (Table 2).

3.2.1.1. Phenolic acids. Gallic (**1**), syringic (**5**), caftaric (**2**) and coutaric (**3**) acids were the four phenolic acids identified in all studied mutants (Fig. 1, Table 2). Phenolic acids constituted one of the less represented group of non-colored compounds, both in colored and non-colored variants (Table 2), as also previously observed by Liang et al. (2011).

Among hydroxybenzoic acids, with exception of Pinot Gris, the content of gallic acid (**1**) was higher than that of syringic acid (**5**). Caftaric acid (**2**) was the main hydroxycinnamic acid.

Pinot Noir revealed the highest amount of both hydroxybenzoic (ca. 71 mg/kg dry berry) and hydroxycinnamic acids (ca. 58 mg/kg dry berry) (Table 2).

3.2.1.2. Flavan-3-ols. Four flavan-3-ols were identified: catechin (**4**), epicatechin (**6**), epigallocatechin gallate (**7**) and epicatechin gallate (**8**) (Fig. 1, Table 2). Epigallocatechin gallate (**7**), which is negatively correlated with astringency (Quijada-Morín et al., 2012), was not detected in five of the berry skin color mutants (Table 2), namely in the black mutant variants, Pinot Noir and Pique-Poul Noir and also in Pinot Gris, Gouveio and Gouveio Roxo (Table 2). Despite, this compound was detected in the remaining mutants, in general, its representativeness was reduced when compared with the other three flavan-3-ols. Catechin (**4**) gave the highest contribution for the amount of this class of flavonoids, representing more than 62% of total flavan-3-ols in all berry skin color mutants (Table 2). The highest concentrations of flavan-3-ols are generally found in green grapes and decrease during ripening (Mulinacci et al., 2008). Although these compounds are located in both grapes seed and skin, concentrations are much lower in the last. In addition, their composition is also different, skin containing both flavan-3-ols and their galloylated forms, whereas seed presents mainly the first (González-Manzano, Rivas-Gonzalo, & Santos-Buelga, 2004). Catechin usually is the main flavanol in both skin and seed, although epicatechin is also well represented; however, in some grape cultivars, these monomers are found at similar levels or the amount of epicatechin is higher (Dopico-García et al., 2008; Escribano-Bailón, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1995; Santos-Buelga, Francia-Aricha, & Escribano-Bailón, 1995).

Pinot Gris was the richest mutant in flavan-3-ols (ca. 2127 mg/kg dry berry), showing even higher quantities than its black variant, Pinot Noir (1415 mg/kg dry berry) (Table 2). Among white mutants, the one corresponding to Pinot variety (Pinot Blanc) also revealed high content of flavan-3-ols (ca. 871 mg/kg dry berry). In opposition, Malvasia Fina was the poorest in this kind of compounds, considering both its white and red variants (ca. 121 mg/kg of dry berry and ca. 132 mg/kg of dry berry, respectively) (Table 2).

Table 1
Results of the analysis with twelve SSR loci in eleven groups of *V. vinifera* L. accessions, each group including two or three accessions that presumably could be skin color mutants.

Cultivar name	Berry color	SSR loci																								Identified SSR profile
		VVS2		VVMD5		VVMD7		VVMD27		VrZAG62		VrZAG79		VMC4f3		VVMD28		VVMD32		VVIp31		VVIv37		VVIv67		
Alvarelhão	N	130	150	218	222	237	237	181	185	187	193	249	257	171	177	231	255	237	253	186	190	163	173	355	360	Brancellao (a)
Alvarelhão Branco	B	140	156	232	236	237	237	175	181	185	185	249	249	181	185	233	255	249	259	174	188	155	155	353	360	Prieto Picudo Blanco I (a)
Bastardo	N	140	148	222	234	255	255	171	185	187	187	243	245	165	177	231	245	237	253	178	188	159	167	368	371	New genotype
Bastardo Roxo	R	130	140	224	232	245	251	175	181	187	193	243	249	171	187	233	255	253	259	180	194	159	173	NA	NA	New genotype
Bastardo Branco	B	140	148	222	234	255	255	171	185	187	187	243	249	177	177	231	231	237	253	188	190	159	161	371	371	Bastardo (b)
Carrega Branco	B	132	142	224	232	245	245	182	181	191	193	237	249	163	171	231	243	249	259	192	194	167	173	360	368	New genotype
Carrega Tinto	N	140	148	230	234	237	251	175	191	187	199	249	255	165	204	245	255	249	269	174	178	157	159	361	368	Tinta Grossa (b)
Gouveio	B	150	156	222	234	237	241	181	185	185	187	249	249	177	185	231	255	249	269	178	188	159	167	363	368	Gouveio, Godello (a, b)
Gouveio Roxo	R	150	156	222	234	237	241	181	185	185	187	249	249	177	185	231	255	249	269	178	188	159	167	363	368	Gouveio, Godello (a, b)
Malvasia Fina	B	140	142	222	236	237	255	175	191	187	187	245	249	165	204	231	233	249	253	188	188	157	159	368	371	Malvasia Fina (b, c)
Malvasia Fina Roxo	R	140	142	222	236	237	255	175	191	187	187	245	249	165	204	231	233	249	253	188	188	157	159	368	371	Malvasia Fina (b, c)
Malvasia Rei	B	140	142	224	236	237	247	181	191	187	193	249	255	173	204	233	245	253	255	186	188	159	163	360	361	Malvasia Rei, Palomino Fino (d)
Malvasia Rei Tinto	N	130	142	224	236	237	247	181	185	187	193	241	249	171	173	231	245	253	269	188	190	159	163	353	361	New genotype
Moscatel Galego Branco	B	130	130	224	232	231	247	175	191	185	195	249	253	165	204	243	265	261	269	182	186	159	161	360	371	Moscatel de Grano menudo (a)
Moscatel Galego Roxo	R	130	130	224	232	231	247	175	191	185	195	249	253	165	204	243	265	261	269	182	186	159	161	360	371	Moscatel de Grano menudo (a)
Moscatel Galego Tinto	N	130	148	222	224	237	247	175	185	185	187	249	253	171	204	243	255	237	261	182	186	159	173	360	371	Moscatel Galego Tinto (b)
Mourisco	N	140	142	224	228	237	241	179	191	187	191	245	255	173	181	241	251	237	249	174	190	155	159	353	361	Marufo (e)
Mourisco Branco	B	130	140	222	222	NA	NA	179	185	187	103	245	249	177	181	225	245	237	249	178	186	159	163	360	371	New genotype
Pinot Blanc	B	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pinot Gris	R	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pinot Noir	N	134	148	224	234	237	241	181	185	187	193	237	243	171	177	215	233	237	269	178	182	149	159	360	368	Pinot (b)
Pique-Poul Gris	R	130	130	222	228	237	241	175	185	187	187	249	249	171	204	231	233	237	259	178	182	159	161	358	360	Picapoll Negro (f)
Pique-Poul Noir	N	130	130	222	228	237	241	175	185	187	187	249	249	171	204	231	233	237	259	178	182	159	161	358	360	Picapoll Negro (f)
Touriga Branco	B	132	142	224	232	245	245	181	181	191	193	237	249	163	171	231	243	249	253	192	194	167	173	360	368	New genotype
Touriga Tinto	N	140	150	222	224	237	241	177	179	191	193	243	245	173	204	231	251	237	269	174	182	155	159	353	363	Touriga Franca (g)

Berry color: N = black; B = white; R = red. Gray-shaded groups are considered as true skin color mutants, based on the coincidence of microsatellite profiles. Right hand column identify the cultivars based on the references: (a) Martín et al. (2003); (b) Veloso et al. (2010); (c) Pinto-Carnide et al. (2003); (d) http://www.eu-vitis.de; (e) Castro et al. (2011); (f) Cabello et al. (2012); (g) Martín et al. (2006). NA = not amplified.

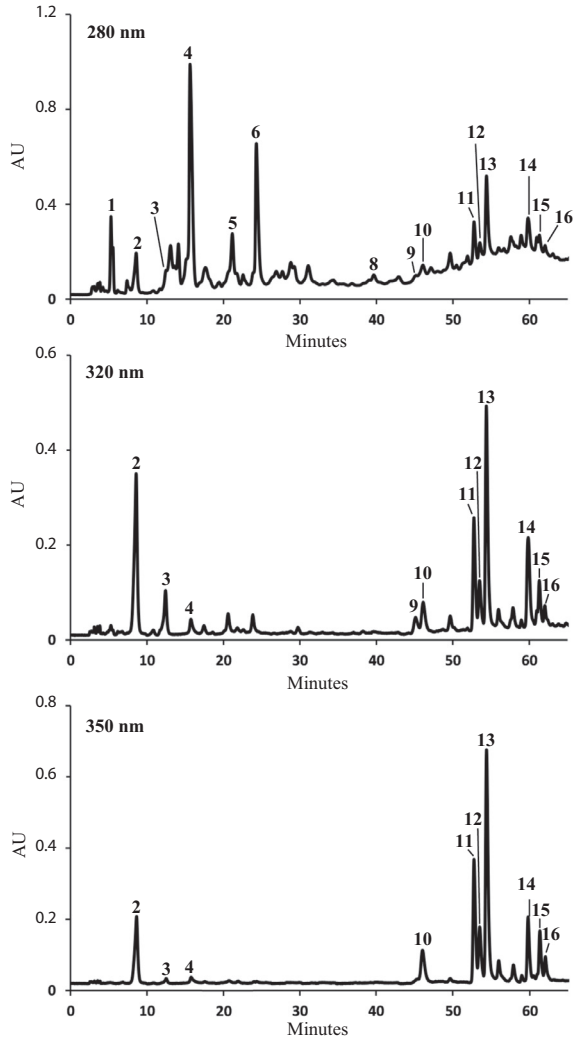

Fig. 1. HPLC-DAD chromatogram of non-colored phenolics of *V. vinifera* cv. Pinot Noir grapes hydromethanolic extract. (**1**) Gallic acid; (**2**) Caftaric acid; (**3**) Coutaric acid; (**4**) Catechin; (**5**) Syringic acid; (**6**) Epicatechin; (**7**) Epigallocatechin gallate; (**8**) Epicatechin gallate; (**9**) Resveratrol-3-*O*-glucoside; (**10**) Myricetin-3-*O*-glucoside; (**11**) Quercetin-3-*O*-galactoside; (**12**) Quercetin-3-*O*-rutinoside; (**13**) Quercetin-3-*O*-glucoside; (**14**) Kaempferol-3-*O*-glucoside; (**15**) Isorhamnetin-3-*O*-glucoside; (**16**) Syringetin-3-*O*-glucoside.

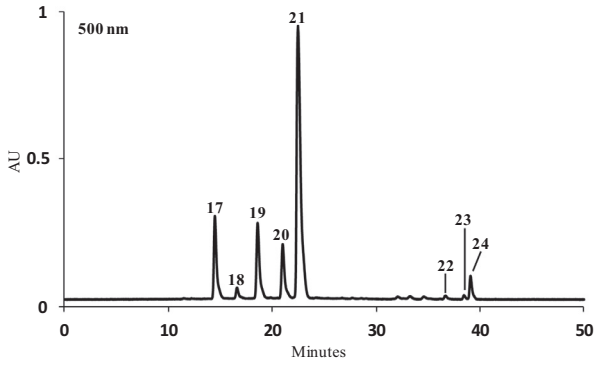

Fig. 2. HPLC-DAD chromatogram of anthocyanins from *V. vinifera* cv. Pinot Noir grapes hydromethanolic extract. (**17**) Delphinidin-3-*O*-glucoside; (**18**) Cyanidin-3-*O*-glucoside; (**19**) Petunidin-3-*O*-glucoside; (**20**) Peonidin-3-*O*-glucoside; (**21**) Malvidin-3-*O*-glucoside; (**22**) Petunidin-3-*O*-*p*-coumaroylglucoside; (**23**) Peonidin-3-*O*-*p*-coumaroylglucoside; (**24**) Malvidin-3-*O*-*p*-coumaroylglucoside.

3.2.1.3. Flavonols. Several flavonols, including 3-*O*-glycosides of myricetin (**10**), quercetin (**11**–**13**), kaempferol (**14**), isorhamnetin (**15**) and syringetin (**16**), were found (Table 2). Myricetin-3-*O*-glucoside (**10**) was only detected in Pinot Noir (Fig. 1, Table 2). Moreover, Moscatel Galego Roxo, Pinot Gris and Pinot Noir were the only mutants that presented syringetin-3-*O*-glucoside (**16**).

Pique-Poul Gris revealed the highest flavonols content (ca. 258 mg/kg dry berry), quercetin-3-*O*-galactoside (**11**) accounting for 39% of the flavonols determined in this skin color mutant variant (Table 2). In fact, quercetin derivatives were the most represented flavonols in all mutant variants, ranging between 66% and 98% of the compounds in Gouveio and Pique-Poul Noir, the poorest and richest mutant variants, respectively.

3.2.1.4. Stilbenes. Only one stilbene, resveratrol-3-*O*-glucoside (**9**), was found, being present in all berry skin color mutants (Fig. 1, Table 2). With the exception of Pinot Noir, that was the richest mutant in this stilbene (Table 2), red berry-grapes showed higher resveratrol-3-*O*-glucoside levels than their white and black berry-grapes variants. However, resveratrol-3-*O*-glucoside (**9**) did not represent more than 4% of total non-colored compounds content in all berry skin color mutants.

3.2.2. Anthocyanins

In this study, eight anthocyanins were identified (Fig. 2, Table 2). The detected compounds were monoglucoside derivatives of five anthocyanidins, (delphinidin (**17**), cyanidin (**18**), petunidin (**19** and **22**), peonidin (**20** and **23**) and malvidin (**21** and **24**)), some of them being also acyl derivatives (compounds **22**, **23** and **24**).

Anthocyanins are synthesized in grape cells at the cytosolic surface of the endoplasmic reticulum, by a multienzyme complex *via* the flavonoid pathway (Boss, Davies, & Robinson, 1996). The genes encoding the early steps enzymes of the polyphenolic biosynthetic pathway, namely chalcone synthase, chalcone isomerase and flavanone-3-hydroxylase, belong to multicopy families and the different number gene copies may have temporal and spatial partitioned expression profiles that sometimes coincide with the biosynthesis of a particular flavonoid (Kuhn et al., 2014).

As expected, these colored polyphenols were only found in black and red mutant variants. However, two exceptions were observed, namely in Malvasia Fina Roxo and Gouveio Roxo. Despite Malvasia Fina Roxo revealed some red pigmentation in its skin berry, it did not presented detectable amounts of anthocyanins. The anthocyanins composition is affected by the expression of flavonoid 3′5′-hydroxylase (F3′5′H) and flavonoid 3′-hydroxylase (F3′H) genes. F3′5′H genes are present in highly redundant copy numbers and only two copies of F3′H genes are found in the grape genome, being one copy expressed and the other transcriptionally silent (Kuhn et al., 2014). The prevalence of F3′5′H over F3′H in black cultivars would lead to the presence of more delphinidin, the precursor of blue/purple petunidin and malvidin derivatives, and, in contrast, it would yield less cyanidin, the precursor of the red peonidin derivatives (Kuhn et al., 2014). On this way, the lack of detectable amounts of anthocyanins in Malvasia Fina Roxo suggests a lack of activity of both F3′5′H and F3′H genes. Another possibility that can influence and be behind the color observed in Malvasia Fina Roxo is the synthesis of other compounds, such as carotenoids, which are lipid soluble pigments found in many vegetable crops (Jackson, 2008) usually masked by the presence of anthocyanins that donates the dominant color. Moreover, in the field no detectable skin color development was observed for Gouveio Roxo during ripening, which suggests that these compounds were not being synthesized. The variation of anthocyanin composition and concentration in grapes is a consequence of many factors, such as cultivar, climate (like sunlight exposure, UV irradiation, temperature), canopy management,

Table 2
Quantification of polyphenolic compounds in hydromethanolic extracts of 11 skin color mutant cultivars (mg/kg berry, dry basis)[a]. Within parenthesis standard deviations of three determinations. MF – Malvasia Fina; G – Gouveio; MGB – Moscatel Galego Branco; PB – Pinot Blanc; MFR – Malvasia Fina Roxo; GR – Gouveio Roxo; MGR – Moscatel Galego Roxo; PPG – Pique-Poul Gris; PG – Pinot Gris; PPN –Pique-Poul Noir; PN – Pinot Noir.

	White cultivars				Red cultivars					Black cultivars	
	MF	G	MGB	PB	MFR	GR	MGR	PPG	PG	PPN	PN
Phenolic acids											
Hydroxybenzoic acids											
(**1**) Gallic acid	5.7 (0.1)	8.1 (0.1)	19.2 (0.5)	18.3 (0.0)	11.6 (0.6)	9.8 (0.2)	19.6 (0.1)	24.4 (0.3)	16.1 (0.3)	15.1 (0.3)	38.4 (0.0)
(**5**) Syringic acid	5.5 (0.0)	7.0 (0.1)	11.4 (0.3)	17.2 (0.1)	9.7 (0.8)	7.0 (0.0)	5.7 (0.0)	10.8 (0.9)	20.2 (1.1)	6.8 (0.1)	32.8 (2.0)
Hydroxycinnamic acids											
(**2**) Caftaric acid	22.8 (0.2)	10.5 (0.2)	24.0 (0.3)	28.1 (0.2)	15.8 (0.1)	14.9 (0.1)	45.4 (0.5)	44.2 (0.3)	12.1 (0.4)	10.1 (0.1)	53.9 (0.2)
(**3**) Coutaric acid	2.1 (0.1)	0.7 (0.1)	4.2 (0.0)	2.7 (0.1)	2.2 (0.0)	3.0 (0.0)	5.7 (0.0)	4.7 (0.0)	3.3 (0.0)	1.1 (0.0)	4.6 (0.1)
∑	36.1 (0.4)	26.3 (0.5)	58.8 (1.1)	66.3 (0.4)	39.3 (1.5)	34.7 (0.3)	76.4 (0.6)	84.1 (1.5)	51.7 (1.8)	33.1 (0.5)	129.7 (2.3)
Flavonoids											
Flavan-3-ols											
(**4**) Catechin	88.5 (1.1)	391.1 (19.7)	408.1 (7.2)	589.8 (2.5)	83.8 (5.5)	341.7 (3.1)	379.8 (10.2)	261.6 (1.7)	1615.5 (34.2)	212.5 (1.0)	977.0 (4.5)
(**6**) Epicatechin	24.0 (0.0)	133.6 (2.1)	206.0 (3.1)	254.2 (4.6)	36.2 (0.7)	118.9 (0.2)	130.0 (3.2)	104.0 (0.7)	474.8 (9.5)	119.2 (6.7)	423.6 (2.9)
(**7**) Epigallocatechin gallate	5.0 (0.1)	–	9.0 (0.6)	12.7 (2.1)	7.2 (0.5)	–	5.3 (0.0)	6.9 (0.1)	–	–	–
(**8**) Epicatechin gallate	3.3 (0.0)	10.3 (0.1)	19.9 (0.2)	14.4 (0.2)	4.4 (0.7)	13.9 (0.0)	11.5 (0.1)	23.4 (1.7)	36.4 (5.0)	11.7 (0.0)	14.4 (0.1)
∑	120.8 (1.2)	535.0 (21.9)	643.0 (11.1)	871.1 (9.4)	131.6 (7.4)	474.5 (3.3)	526.6 (13.5)	395.9 (4.2)	2126.7 (48.7)	343.4 (7.7)	1415.0 (7.5)
Flavonols											
(**10**) Myricetin-3-*O*-gluc	–	–	–	–	–	–	–	–	–	–	20.3 (0.8)
(**11**) Quercetin-3-*O*-galactoside	44.6 (0.3)	52.4 (0.4)	68.2 (1.7)	19.7 (0.6)	59.7 (0.7)	45.9 (1.2)	82.2 (0.7)	100.6 (1.5)	86.8 (1.2)	41.9 (0.1)	33.8 (0.4)
(**12**) Quercetin-3-*O*-rutinoside	21.6 (0.4)	30.6 (0.1)	23.5 (0.5)	3.1 (0.0)	28.0 (0.7)	20.1 (0.1)	24.8 (0.2)	27.6 (0.3)	25.8 (0.1)	16.0 (0.2)	30.5 (0.0)
(**13**) Quercetin-3-*O*-glucoside	48.4 (0.3)	81.3 (0.5)	51.6 (1.1)	5.9 (0.0)	70.7 (1.2)	54.4 (0.7)	52.7 (0.6)	82.3 (1.7)	63.8 (0.7)	39.3 (0.2)	67.2 (1.0)
(**14**) Kaempferol-3-*O*-glucoside	34.9 (0.6)	78.6 (0.2)	66.8 (1.4)	4.5 (0.2)	37.0 (1.1)	46.5 (0.2)	60.3 (0.9)	47.4 (1.0)	32.6 (0.7)	19.4 (0.2)	25.8 (0.3)
(**15**) Isorhamnetin-3-*O*-glucoside	1.4 (0.1)	4.4 (0.2)	4.2 (0.1)	1.5 (0.1)	1.6 (0.1)	5.4 (0.1)	2.9 (0.0)*	–	9.2 (0.1)	1.8 (0.1)	13.0 (0.0)
(**16**) Syringetin-3-*O*-glucoside	–	–	–	–	–	–		–	3.2 (0.3)	–	8.6 (0.2)
∑	150.9 (1.7)	247.3 (1.4)	214.3 (4.8)	34.7 (0.9)	197.0 (3.8)	172.3 (2.3)	222.9 (2.4)	257.9 (4.5)	221.4 (3.1)	118.4 (0.8)	199.2 (2.7)
Anthocyanins											
(**17**) Delphinidin-3-*O*-glucoside	–	–	–	–	–	–	1.6 (0.1)	–	0.6 (0.0)	1.5 (0.1)	81.9 (0.6)
(**18**) Cyanidin-3-*O*-glucoside	–	–	–	–	–	–	5.1 (0.1)	0.7 (0.1)	0.1 (0.0)	4.3 (0.3)	9.9 (0.4)
(**19**) Petunidin-3-*O*-glucoside	–	–	–	–	–	–	0.4 (0.0)	–	2.8 (0.4)	2.7 (0.0)	94.8 (0.5)
(**20**) Peonidin-3-*O*-glucoside	–	–	–	–	–	–	1.2 (0.2)	0.6 (0.0)	7.8 (0.2)	13.4 (0.1)	52.2 (0.5)
(**21**) Malvidin-3-*O*-glucoside	–	–	–	–	–	–	0.9 (0.1)	0.7 (0.2)	79.5 (0.1)	25.7 (0.8)	448.7 (1.5)
(**22**) Petunidin-3-*O*-*p*-coumaroylglucoside	–	–	–	–	–	–	–	–	–	1.4 (0.4)	4.3 (0.1)
(**23**) Peonidin-3-*O*-*p*-coumaroylglucoside	–	–	–	–	–	–	–	0.3 (0.0)	–	2.9 (0.1)	3.6 (0.0)
(**24**) Malvidin-3-*O*-*p*-coumaroylglucoside	–	–	–	–	–	–	–	0.3 (0.0)	–	3.7 (0.1)	27.4 (0.1)
∑	–	–	–	–	–	–	9.2 (0.5)	2.6 (0.3)	90.8 (0.7)	55.6 (1.9)	722.8 (3.2)
Stilbenes											
(**9**) Resveratrol-3-*O*-glucoside	1.4 (0.0)	1.1 (0.0)	0.9 (0.0)	1.7 (0.0)	1.9 (0.0)	2.0 (0.1)	1.2 (0.0)	3.7 (0.0)	2.1 (0.1)	0.7 (0.0)	5.5 (0.1)
Total phenols	309.2 (3.3)	809.7 (23.8)	917.0 (17.0)	973.8 (10.7)	369.8 (12.7)	683.5 (6.0)	836.3 (17.0)	744.2 (10.5)	2492.7 (54.4)	551.2 (10.9)	2472.2 (15.8)

"–": not detected.
[a] ∑: sum of the identified polyphenolic compounds.
* Isorhmnetin-3-*O*-glucoside and syringetin-3-*O*-glucoside were quantified together.

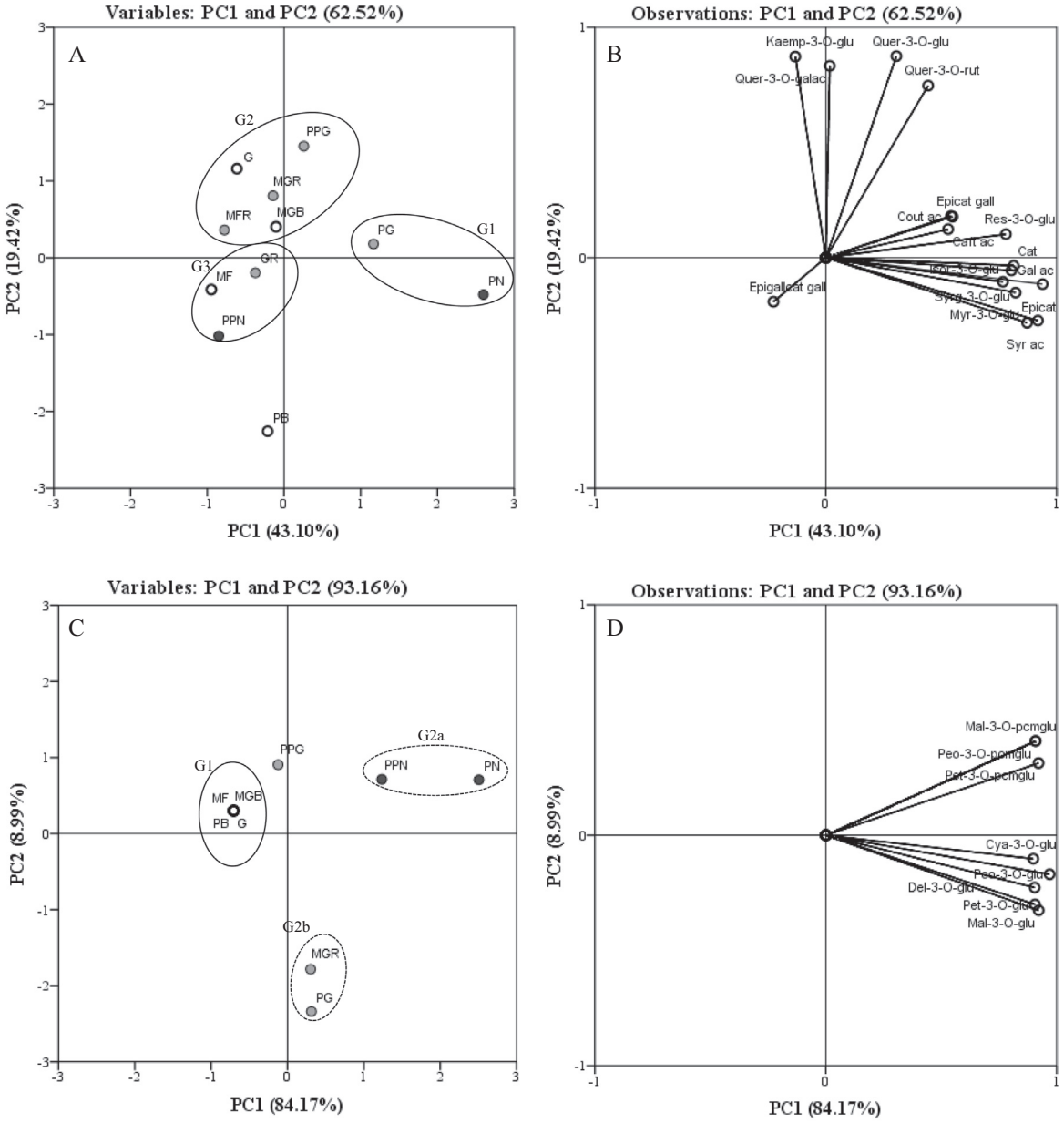

Fig. 3. Projection of grape berry skin color mutants (A and C) (variables: Malvasia Fina (MF); Gouveio (G); Moscatel Galego Branco (MGB); Pinot Blanc (PB); Malvasia Fina Roxo (MFR); Gouveio Roxo (GR); Moscatel Galego Roxo (MGR); Pique-Poul Gris (PPG); Pinot Gris (PG); Pique-Poul Noir (PPN); Pinot Noir (PN)) and loadings by (B) non-colored polyphenols and (D) anthocyanins composition (variables: gallic acid (Gal ac); caftaric acid (Caft ac); coutaric acid (Cout ac); catechin (Cat); syringic acid (Syr ac); epicatechin (Epicat); epigallocatechin gallate (Epigallcat gall); epicatechin gallate (Epicat gall); resveratrol-3-*O*-glucoside (Res-3-*O*-glu); myricetin-3-*O*-glucoside (Myr-3-*O*-glu); quercetin-3-*O*-galactoside (Quer-3-*O*-galac); quercetin-3-*O*-rutinoside (Quer-3-*O*-rut); quercetin-3-*O*-glucoside (Quer-3-*O*-glu); kaempferol-3-*O*-glucoside (Kaemp-3-*O*-glu); isorhamnetin-3-*O*-glucoside (Isor-3-*O*-glu); syringetin-3-*O*-glucoside (Syr-3-*O*-glu); delphinidin-3-*O*-glucoside (Del-3-*O*-glu); cyanidin-3-*O*-glucoside (Cya-3-*O*-glu); petunidin-3-*O*-glucoside (Pet-3-*O*-glu); peonidin-3-*O*-glucoside (Peo-3-*O*-glu); malvidin-3-*O*-glucoside (Mal-3-*O*-glu); petunidin-3-*O*-*p*-coumaroylglucoside (pet-3-*O*-pcmglu), peonidin-3-*O*-*p*-coumaroylglucoside (peon-3-*O*-pcmglu), malvidin-3-*O*-*p*-coumaroylglucoside (pet-3-*O*-pcmglu)) into the plane composed by the principal components PC1 and PC2 containing 62.52% and 93.16% of the total variance for non-colored polyphenols and anthocyanins composition, respectively.

fertilizers and water regimes, affecting both the expression of the structural and regulatory genes (Downey, Dokoozlian, & Krstic, 2006; He et al., 2010). Recently, some studies addressed the effects of anti-fungal treatments on the color and phenolic profile of red wines, concluding that, in general, the anti-fungal substances had different effects depending on the cultivar and on the phenolic compound analyzed, usually resulting in less colorful wines (Briz-Cid, Figueiredo-González, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2014, 2015). Therefore, the accession analyzed and designated as Gouveio Roxo, despite presenting the profile of Gouveio variety, as confirmed by the molecular analysis (Table 1), it does not correspond to a red variant of Gouveio evidenced by the lack of anthocyanins.

Qualitative differences among berry skin color mutants of the same variety were also seen (Table 2). Pinot Gris did not show the three acylated anthocyanins (compounds **22**, **23** and **24**) present in Pinot Noir (Table 2).

Delphinidin-3-*O*-glucoside (**17**), petunidin-3-*O*-glucoside (**19**) and petunidin-3-*O*-*p*-coumaroylglucoside (**22**) were not detected in Pique-Poul Gris, but were found in the other red mutant variants, as well as in its black variant Pique-Poul Noir. However, Pique-Poul Gris presented peonidin-3-*O*-*p*-coumaroylglucoside

(**23**) and malvidin-3-*O*-*p*-coumaroylglucoside (**24**) that were not detected in the remaining red-colored mutant variants (Table 2). As so, the presence or absence of these compounds could be a specific feature of Pique-Poul Gris, allowing to easily distinguishing this red color variant from the other red variants studied.

Quantitatively, Pinot Noir was clearly the mutant with the highest amount of anthocyanins (ca. 723 mg/kg dry berry), about eight times more than its red variant, Pinot Gris (ca. 91 mg/kg dry berry) (Table 2). Liang et al. (2011) described that during the whole course of maturation, the skin cells of colored grapes accumulate anthocyanins and, consequently, the color of the berry is progressively darkened.

Malvidin-3-*O*-*p*-glucoside (**21**) was the major compound in the black mutant variants and in Pinot Gris, although at notably higher concentration in Pinot Noir (ca. 449 mg/kg dry berry) (Table 2). Pinot Noir is an international cultivar, being much studied concerning to its anthocyanin composition. Dimitrovska, Bocevska, Dimitrovski, and Murkovic (2011) and Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006 reported the anthocyanin profile of Pinot Noir, both showing malvidin-3-*O*-*p*-glucoside as the main anthocyanin. However, our results also showed the presence of three acylated derivatives (Fig. 2, Table 2), petunidin-3-*O*-*p*-coumaroylglucoside, peonidin-3-*O*-*p*-coumaroylglucoside and malvidin-3-*O*-*p*-coumaroylglucoside (compounds **22**, **23** and **24**), which, as far as we know, are reported for the first time in this cultivar. This observation provides evidence about the influence of the environmental factors on the anthocyanin pattern.

Our results suggest that different proportions of individual anthocyanins, in addition to their total amount, can affect the color types of grape berry skin color mutants. Considering Pinot Noir, besides malvidin-3-*O*-glucoside (**21**), other anthocyanins, namely delphinidin-3-*O*-glucoside (**17**), petunidin-3-*O*-glucoside (**19**) and peonidin-3-*O*-glucoside (**20**), should be highlighted because of their contribution to the total anthocyanins content of this grape (Table 2).

Several authors reported cyanidin derivatives as the minor group of anthocyanins in colored cultivars (Figueiredo-González, Simal-Gándara et al., 2012; Núñez, Monagas, Gomez-Cordovés, & Bartolomé, 2004). Furthermore, taking into account that cyanidin is the precursor of others anthocyanins, it is usual to find low concentrations of its derivatives in red colored grapes (Núñez et al., 2004). Nevertheless, this was not observed on Moscatel Galego Roxo, suggesting that the F3′H pathway is more active than F3′5′H, resulting in higher amounts of cyanidin-3-*O*-glucoside.

Previous studies described the UDPglucose:flavonoid-3-*O*-glucosyltransferase (UFGT) activity as critical for anthocyanins biosynthesis (Boss et al., 1996; Zheng et al., 2013). The control of the biosynthetic step mediated by UFGT in the anthocyanin pathway is mainly affected by *Myb* genes family. The presence of *Gret1* retrotransposon, in the promotor region of MYBA1, is associated with white-fruited cultivars when present in a homozygous state (Kobayashi, Goto-Yamamoto, & Hirochika, 2004). Additional polymorphisms in this gene are also strongly associated with a red or pink-fruited phenotype (This, Lacombe, Cadle-Davidson, & Owens, 2007), which can possibly explain some of the color differences among the different sets of berry color mutants studied.

3.3. Principal components analysis (PCA)

To study the relationship between colored and non-colored related berried cultivars and their polyphenolic composition, PCA was applied to the content (mg/kg dry grape) of non-colored compounds (Fig. 3A and B) and anthocyanins (Fig. 3C and D).

PCA of normalized non-colored dataset explained 62.52% of total variations, PC1 accounting for 43.10% of the variance and PC2 for 19.42% (Fig. 3A and B). As shown in Fig. 3A, three groups could be clearly distinguished (Fig. 3A and B). One group (G1) includes the color-related berried Pinot Gris and Pinot Noir. These mutants appeared in the positive plan of PC1, due to the absence of epigallocatechin gallate, but highest content in flavano-3-ols (ca. 2127 and 1415 mg/kg dry grape, respectively), namely in catechin, as well as the high content of phenolic acids, namely syringic acid. Despite being included in the same group, Pinot Gris appeared in the positive plan of the PC2 and Pinot Noir in the negative one due to the presence of myricetin-3-*O*-glucoside in the last one. Group G2 included the white variants Gouveio and Moscatel Galego Branco and the red variants Malvasia Fina Roxo, Moscatel Galego Roxo and Pique-Poul Gris due to their high content in glucoside derivatives of kaempferol and quercetin, with particular relevance for Pique-Poul Gris, the richest one. The low amounts of flavonols and phenolic acids in Malvasia Fina, Gouveio Roxo and Pique-Poul Noir led to the inclusion of them in another group (G3) in the negative parts of PC1 and PC2. Pinot Blanc was clearly separated from the other accessions due to its low levels in flavonols (Fig. 3A and B).

PCA of anthocyanins explained 93.16% of total variation, where PC1 accounts for 84.17% of the variance and PC2 for 8.99% (Fig. 3C and D). Due to the absence of anthocyanins in their composition, white variants, such as Malvasia Fina, Gouveio, Moscatel Galego Branco and Pinot Blanc, were grouped together (G1) in the negative part of PC1 (Fig. 3C and D).

PCA confirmed that the anthocyanin profile was related to the grape skin color. Among color-berried mutants, two subgroups were established (Fig. 3C and D). One group (G2a) that appears in the positive plan of PC1 and PC2 includes the black mutants Pique Poul Noir and Pinot Noir for their content in *p*-coumaroyl derivatives of petunidin, peonidin and malvidin. Another group (G2b) included Moscatel Galego Roxo and Pinot Gris, in which no *p*-coumaroyl derivatives were identified. The red variant Pique-Poul Gris, in which the content of non-acylated anthocyanins was higher than that of acylated ones, was the poorest mutant regarding anthocyanins, appearing in the positive part of PC2, close to the group of white mutant variants (G1).

4. Conclusions

The variation of polyphenolic compounds in groups of grape berry skin color mutants, including related black, red and white-berried mutant variants derived from a single variety identified by nuclear microsatellite analysis, was investigated, for the first time. The grape berry skin color mutants were distinguished according to their phenolic acids, flavan-3-ols, flavonols and anthocyanins composition. Molecular and chemical approaches complemented each other in the correct identification of the grape berry skin color mutants.

As expected, anthocyanins were the main group of compounds that allowed a clear division among color and non-colored related mutant variants. The results also revealed differences in the contribution of different anthocyanins to distinguish berry skin color mutants.

The observed chemical richness and differences among related mutant cultivars derived from a single variety encourage the use of berry skin color somatic variants, not only for the development of new cultivars with interesting characteristics, namely concerning the color feature, but also to improve knowledge on colored and non-colored cultivars and understanding the evolutionary events behind their origin.

The study of genes involved in the polyphenolic biosynthesis may help elucidating the genetics behind grape berry skin color and understand how this kind of compounds affected the skin pigmentation of the studied grape berry skin color mutants.

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