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Bioactive compounds of red grapes from Dão region (Portugal): Evaluation of phenolic and organic profile

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ABSTRACT

Objective: To improve the knowledge on the metabolite profile of five red grapes from Dão region (Portugal), concerning to the phenolic characteristics (coloured and non-coloured phenolics) and organic acid composition.

Methods: Five red grapes collected from Dão region were studied. The profiles of phenolic compounds and organic acids were estimated by high-performance liquid chromatography with diode-array detection and high-performance liquid chromatography with UV detector, respectively.

Results: Totally 24 phenolic compounds were identified, and distributed by several classes: 8 anthocyanins, 1 hydroxybenzoic acid, 4 hydroxycinnamic acids, 1 stilbene, 4 flavan-3-ols, 6 flavonols. Additionally, 10 organic acids were detected in all samples. Total contents of each phenolic class and organic acids amounts varied significantly among the different grape cultivars investigated. The principal components analysis differentiates the Touriga Nacional from the other varieties due to their high contents in anthocyanins, non-coloured phenolics and organic acids. Touriga Nacional is an important red grape cultivar, highly esteemed in Dão region for its ability to produce high quality wines.

Conclusions: The results suggest that the red grapes from Dão region present a good composition in bioactive compounds, being important for the production of wines with superior quality.

1. Introduction

The tradition of wine production in Portugal dates back to centuries of history, with enviable potential for producing high quality wines. This country is responsible for producing of the best wines in the world, being the first to have a demarcated region (region of Douro, where it is produced the Port wine), which ensures the production of genuine wines originated in a particular region. Dão is a unique region with important viticulture traditions, located in north central Portugal, from which the excellent edapho-climatic conditions are turned to advantage for vineyard culture, which corresponds to 20 000 ha. The Dão region presents a temperate climate, although cold and rainy in winter and frequently is very hot in summer. Dão wines with Denomination d'Origine Contrôlée arise from vineyards established in granite land, between 400 and 500 m altitude.

Grapes from *Vitis vinifera* L. belong to the world's largest fruit crops, being a variety known for the best quality wines [1]. Grapes and products derived from them constitute an important

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factor worldwide. Viticulture is one of the activities with the highest impact in the Portuguese economy, representing about 15% of all agricultural production, and placing Portugal among the largest producers of wine worldwide.

Grape ripening is a physiological period that influences the composition of the grapes and determines varietal characteristics, which have influence on the future of wine quality. Grapes undergo many changes during the ripening process which involves a number of physical and biochemical modifications, like weight, volume, rigidity, sugar, acidity, colour and aroma [2]. The optimum level of harvesting can be determined by the level of soluble solids, berry weight, titratable acidity, as well as full flavour characteristics [2]. To harvest the grapes at ideal maturity, it is necessary to investigate their profile and composition in phenolics and organic acids in the field throughout maturation.

Chemical composition is one of the most quality criteria for fruit products. The grapes content in phenolic compounds and organic acids is of great importance for the organoleptic characteristics of grapes and wines, being related with the degree of grape ripening [2,3].

Grapes are a rich source of phenolic compounds (mainly in skin and seed), which play an important role in oenology due to their influence on some important sensory properties of grapes and wines, such as colour, stability, bitterness and astringency [1,4]. Due to their antioxidant and anti-inflammatory properties, phenolic compounds are associated with several beneficial physiological effects that are derived from moderate wine consumption [5]. The study of phenolic composition of grapes may allow the establishment of one or more biomarkers specific for a particularly type of grapes, allowing to assess their chemical evolution during growth and maturation [1,5–7].

The most abundant non-coloured phenolics in skin are flavonols, while flavan-3-ols monomer such as (+)-catechin and (−)-epicatechin, as well as dimers, trimmers and polymeric forms, also called procyanidins (2–10 units), are present mainly in grape seeds. These compounds may contain subunits of gallic acid, epigallocatechin of epicatechin gallate linked by an interflavan bond [4,5,8–10].

Anthocyanins are the main pigments of red grapes located in skins and appeared manly during the ripening, which are mainly responsible for the colour of red wine [1,10]. The major anthocyanins found in grapes are derived from cyanidin, peonidin, delphinidin, petunidin and malvidin, and they generally occur as glycosides and acylglycosides; malvidin-3-*O*-glucoside is the most abundant [5,6,10,11].

The organic acids composition of grapes and wines is important, because they have influence on the organoleptic properties (flavor, colour and aroma) and on the stability and microbiological control of the products. Tartaric and malic acids are the predominant organic acids in grapes juices, on the other hand, succinic and citric acids are present in minor proportion [6,12].

Therefore, in this work we aimed to contribute to the knowledge of the metabolite profile of the main red grapes from Dão region (Portugal), and determine simultaneously their non-coloured and coloured phenolics and organic acids contents. The phenolic compounds were determined by high-performance liquid chromatography with diode-array detection (HPLC/DAD) and organic acids by high-performance liquid chromatography with ultraviolet detection (HPLC/UV). Then, the principal components analysis (PCA) was used to analyze the results previously obtained.

2. Materials and methods

2.1. Standards and reagents

All chemicals used were of analytical grade. The standard compounds were purchased from various suppliers: oxalic, aconitic, citric, ketoglutaric, tartaric, malic, quinic, succinic, shikimic, fumaric, cafeic, *p*-coumaric and ferulic acids were from Sigma–Aldrich (St. Louis, MO, USA). Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside were from Extrasynthèse (Genay, France). *trans*-Caffeoyltartaric acid (*t*-CAFTA) and *trans*-*p*-coumaroyltartaric acid (*t*-COUTA) were kindly supplied by Dr. C. Garcia-Viguera (CEBAS-CSIC, Murcia, Spain). Epigallocatechin, catechin, epicatechin, epigallocatechin gallate, epicatechin gallate, resveratrol-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, laricitrin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside and syringetin-3-*O*-glucoside were from Extrasynthèse (Genay, France); gallic acid was from Fluka (Buchs, Switzerland). Water was deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Grape samples

Five samples of five red grapes varieties from Dão region (Portugal) were harvested during September of 2012, in Quinta das Camélias, located in Sabugosa: Tondela (Portugal). The varieties under study were: “Jaen”, “Touriga Nacional”, “Alfrocheiro”, “Tinta Roriz” and “Syrah”. After harvested, the grapes were preserved at −20 °C and dried in a lyophilizer apparatus (Labconco 4.5, Kansas City, MO, USA).

2.3. Phenolic compounds

2.3.1. Extraction

The non-coloured and coloured phenolic compounds were extracted according to the procedure described by Oszmianski and Lee [13]. Aliquots of 5 g of powder sample were weighed and extracted with 100 mL of MeOH (80%) along 2 h under stirring after flushing with nitrogen to avoid oxidations. Then, the extract was centrifuged for 10 min at 4000 r/min. Continuing the material was again extracted during 15 min with 100 mL of MeOH (80%). The both supernatants were evaporated to dryness under reduced pressure at 30 °C. The resultant extract was dissolved with 50 mL of deionised water and placed into the column. The solid-phase extraction cartridge was preconditioned with 20 mL of ethyl acetate, 20 mL of methanol and 20 mL of 0.01 mol/L HCl. After passage of the sample, the column was washed with 3 mL of 0.01 mol/L HCl. Then, the fraction I, designed by non-coloured phenolics was eluted with 20 mL of ethyl acetate. The fraction II, designed by anthocyanins was eluted with 40 mL of methanol containing 0.1% HCl. The fractions I and II were evaporated under reduced pressure, and the dried extracts obtained were re-dissolved with 1 mL of methanol (non-coloured phenolics) and in 20 mL of acidified water, pH 3.0 (anthocyanins), using a membrane-filtered (0.45 μm).

2.3.2. HPLC-DAD analysis

The extracts 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).

2.3.2.1. Anthocyanins

The conditions described by Kammerer *et al.* were employed [14]. The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and water/formic acid/acetonitrile (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), from 100% to 10% B (5 min). Total run time was 50 min. Flow rate was 0.8 mL/min. The injection volume was 20 μL. Detection was achieved with a Gilson diode array (DAD) detector. The compounds in each sample were identified by comparing their retention times and UV–Vis spectra in the 200–600 nm range with the library of spectra previously compiled by the authors. Peak purity was checked by means of the Gilson 160 SpectraViewer Software Contrast Facilities. Anthocyanin quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Anthocyanins quantification was achieved using a calibration plot of external standard at 500 nm. Malvidin-3-*O*-*p*-coumaroylglucoside and petunidin-3-*O*-*p*-coumaroylglucoside were quantified as malvidin-3-*O*-glucoside. Additionally, the peonidin-*O*-*p*-coumaroylglucoside was quantified as peonidin-3-*O*-glucoside.

2.3.2.2. Non-coloured phenolics

The method for quantification of the non-coloured phenolic was previously described by Dopico-García *et al.* [6]. The mobile phase used is composed by 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). The solvent system starting with 10% of B, and installing a gradient to obtain (24% B at 20 min, 30% B at 40 min, 55% B at 60 min, 70% B 65 min, 80% B at 70 min), 100% B at 75 min, and maintain 100% B isocratic during 5 min (80 min). A solvent flow rate was 1.0 mL/min. The injection volume was 20 μL. Detection was achieved with a Gilson diode array detector (DAD). Spectral data from all peaks were accumulated in the range of 200–400 nm and chromatograms were recorded at 280, 320 and 350 nm. The data were processed on Unipoint System Software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. The quantification of phenolic compounds was archived by the absorbance recorded in the chromatograms relative to external standard at 350 nm for quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, myricetin-3-*O*-rhamnoside and isorhamnetin-3-*O*-glucoside, at 320 nm for *p*-coumaric acid, caffeic acid, ferulic acid and resveratrol-3-*O*-glucoside. 280 nm was used for gallic acid, epigallocatechin, catechin, epicatechin, epigallocatechin gallate and epicatechin gallate. As the available amounts of *t*-CAFTA and *t*-COUTA standards were not enough, these compounds were quantified as caffeic and *p*-coumaric acids, respectively. Syringetin-3-*O*-glucoside and laricitrin-3-*O*-glucoside were quantified as myricetin-3-*O*-rhamnoside.

2.4. Organic acids

2.4.1. Extraction

Organic acids extraction was performed according to a described procedure [3]. A total of 0.25 g of dried grapes were mixed with 50 mL of H_2SO_4 0.005 mol/L under stirring (300 r/min) for 30 min. The extracts were filtered and then passed through a C18 solid-phase extraction column (70 mL/10000 mg; Macherey–Nagel), previously conditioned with 30 mL of methanol and 70 mL of acid water (pH 2 with HCl). The aqueous extract, containing the organic acids, was evaporated until dryness under reduced pressure (30 °C) and redissolved in 0.005 mol/L H_2SO_4 (1 mL) for HPLC/UV analysis (20 μL).

2.4.2. HPLC/UV analysis

The separation and quantification of organic acids were carried out according to a procedure described by Silva *et al.* [15] in analytical HPLC unit (Gilson), using an ion exclusion Nucleogel Ion 300 OA (300 mm × 7.7 mm) (Germany) column, in conjunction with a column heating device set at 30 °C. Detection was performed with a Gilson UV–vis detector at 214 nm. Organic acids quantification was achieved by measuring the absorbance recorded in the chromatograms relative to external standards. This procedure was performed in triplicate.

2.5. Statistical analysis

All data were recorded as mean ± SD of triplicate determinations. Mean values were compared using Two-way ANOVA and Bonferroni test, as *post-hoc* test, was used to determine differences with statistical significance. Differences were considered significant for $P < 0.05$. Statistical analysis was carried out using Graphpad Prism 5 Software (San Diego, CA, USA). PCA was carried out using XLSTAT 2013.5 software. The PCA method shows similarities between samples projected on a plane and makes it possible to identify which variables determine these similarities and in what way.

3. Results

3.1. Anthocyanins

The analysis of red grapes from Dão region (Portugal) by HPLC/DAD allowed the identification of eight anthocyanins: delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, petunidin-3-*O*-*p*-coumaroyl-glucoside, peonidin-3-*O*-*p*-coumaroyl-glucoside, malvidin-3-*O*-*p*-coumaroyl-glucoside (Table 1 and Figure 1). All compounds were previously described in the red grapes of Touriga Nacional and Syrah [10,11,16–18]. Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and malvidin-3-*O*-glucoside were previously described in Jaen and Alfrocheiro varieties from Dão region [18], being the other anthocyanins described herein for the first time in Jaen, Alfrocheiro and Tinta Roriz grape varieties.

Table 1
Anthocyanin contents of red "Dão" grape samples (mg/kg of lyophilized sample).

Anthocyanins		Grapes				
		Jaen	Touriga Nacional	Alfrocheiro	Tinta Roriz	Syrah
1	Delphinidin-3-*O*-glucoside	92.6 ± 3.5	609.1 ± 4.0[a]	111.5 ± 1.1[b]	425.7 ± 13.6[a,b,c]	160.0 ± 7.2[a,b,c,d]
2	Cyanidin-3-*O*-glucoside	16.5 ± 0.5	75.9 ± 0.1[a]	21.3 ± 0.1[b]	45.4 ± 1.7[a,b]	19.1 ± 1.4[b]
3	Petunidin-3-*O*-glucoside	112.2 ± 4.1	644.8 ± 2.5[a]	146.0 ± 0.9[b,b,a]	376.5 ± 13.7[a,b,c]	216.3 ± 9.5[a,b,c,d]
4	Peonidin-3-O–glucoside	129.6 ± 4.4	427.1 ± 7.9[a]	244.7 ± 2.1[a,b,a]	107.6 ± 6.3[c]	183.1 ± 7.1[a,b,c,d]
5	Malvidin-3-*O*-glucoside	842.6 ± 21.6	2800.2 ± 11.6[a]	1128.6 ± 5.1[a,b,a]	1260.4 ± 39.5[a,b,c]	1223.6 ± 50.6[a,b,c,d]
6	Petunidin-3-*O*-*p*-coumaroyl-glucoside	13.4 ± 0.2	91.1 ± 0.9[a]	12.3 ± 0.3[b]	77.3 ± 1.8[a,c]	64.7 ± 3.0[a,b,c]
7	Peonidin-3-*O*-*p*-coumaroyl-glucoside	20.2 ± 0.5	114.5 ± 0.3[a]	30.3 ± 0.2[b]	22.7 ± 0.9[b]	70.7 ± 2.7[a,b,c,d]
8	Malvidin-3-*O*-*p*-coumaroyl-glucoside	132.5 ± 3.5	573.6 ± 0.7[a]	132.9 ± 0.1[b]	287.4 ± 8.5[a,b,c]	360.1 ± 14.3[a,b,c,d]
Total		1359.6	5336.3	1827.6	2603.0	2297.6

Significant results ($P < 0.05$) are indicated as: [a] - *vs* Jaen; [b] - *vs* Touriga Nacional; [c] - *vs* Alfrocheiro; [d] - *vs* Tinta Roriz.

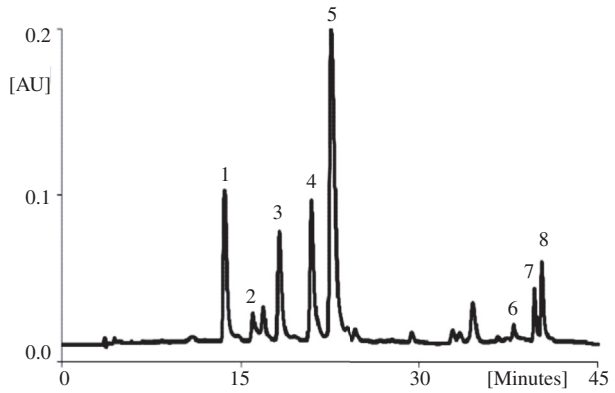

Figure 1. HPLC/DAD anthocyanins profile of Touriga Nacional "Dão" red grapes from Quinta das Camélias, harvested in 2012 (detected at 500 nm).

Despite the difference observed in the contents of each anthocyanin, all the analysed samples exhibited a similar profile, with amounts ranging between 1359.6 and 5336.3 mg/kg of lyophilized grapes (Table 1). Jaen was the poorest variety and Touriga Nacional showed remarkable higher amounts, which was at least more than twice higher than the other analised varieties (Table 1). This fact can be visualized by the colour of the skins and the must produced by maceration of these grapes.

3.2. Non-coloured phenolics

The analysis by HPLC/DAD allowed the identification and quantification of 16 non-coloured phenolics: one hydroxybenzoic acid (1), four hydroxycinnamic acids (2, 3, 6, 8), one stilbene (10), four flavan-3-ols (4, 5, 7, 9) and six flavonols (11–16) (Table 2 and Figure 2).

All of these compounds were previously reported in black grapes [6,9]. Nevertheless, the five grape varieties showed different qualitative and quantitative composition (Table 2). From the identified compounds, epigallocatechin gallate was not detected in Jaen; whereas epicatechin was not found in Touriga Nacional. The ferulic acid was absent in Tinta Roriz, Alfrocheiro and Syrah. Finally, isorhamnetin-3-*O*-glucoside was not found in Alfrocheiro and Syrah (Table 2).

The phenolic contents of the five analysed grape varieties ranged between 343.8 and 1328.3 mg/kg of lyophilized material, being Touriga Nacional the richest one. Jaen presented the lowest amounts in non-coloured phenolic compounds (Table 2).

Table 2
Non-coloured phenolic composition of red "Dão" grape samples (mg/kg of lyophilized sample).

Non-coloured phenolics		Grapes				
		Jaen	Touriga Nacional	Alfrocheiro	Tinta Roriz	Syrah
1	Gallic acid	1.5 ± 0.1	13.4 ± 0.3	3.9 ± 0.0	11.8 ± 0.1	6.4 ± 0.2
2	*t*-CAFTA	9.7 ± 0.2	23.5 ± 0.0[a]	82.9 ± 0.4[a,b]	21.0 ± 0.2[c]	4.5 ± 0.0[b,c,d]
3	*t*-COUTA	3.0 ± 0.0	107.6 ± 2.1[a]	8.6 ± 0.0[b]	9.5 ± 0.1[b]	19.2 ± 0.9[a,b]
4	Epigallocatechin	99.0 ± 4.2	128.7 ± 6.7[a]	84.9 ± 2.1[a,b]	61.1 ± 0.2[a,b,c]	159.6 ± 1.0[a,b,c,d]
5	Catechin	29.1 ± 1.9	267.9 ± 40.9[a]	117.6 ± 0.7[a,b]	96.6 ± 9.7[a,b,c]	117.2 ± 1.7[a,b,d]
6	Caffeic acid	4.2 ± 0.0	29.2 ± 0.9[a]	15.2 ± 0.1[b]	9.9 ± 0.2[b]	12.0 ± 0.2[b]
7	Epicatechin	21.1 ± 0.8	nd	18.6 ± 1.5[b]	14.5 ± 0.9[b]	15.2 ± 0.8[b]
8	Ferulic acid	5.3 ± 0.2	8.8 ± 0.1	nd	nd	nd
9	Epicatechin gallate	1.4 ± 0.0	16.1 ± 0.2[a]	6.9 ± 0.0	11.5 ± 1.3	24.0 ± 0.4[a,c]
10	Polydatin	43.3 ± 0.6	93.0 ± 9.6[a]	nd	31.4 ± 0.5[b,c]	58.8 ± 0.2[a,b,c,d]
11	Quercetin-3-*O*-galactoside	24.3 ± 0.3	97.6 ± 9.2[a]	32.1 ± 0.5[b]	29.8 ± 1.5[b]	84.2 ± 0.4[a,b,c,d]
12	Quercetin-3-*O*-rutinoside	7.3 ± 0.6	100.5 ± 4.2[a]	9.9 ± 0.6[b]	20.1 ± 1.0[a,b]	71.2 ± 0.4[a,b,c,d]
13	Quercetin-3-*O*-glucoside	56.4 ± 1.1	325.1 ± 5.8[a]	33.0 ± 0.8[a,b]	61.7 ± 3.5[b,c]	229.8 ± 1.4[a,b,c,d]
14	Laricitrin-3-*O*-glucoside	18.8 ± 0.4	59.8 ± 2.4[a]	7.0 ± 0.0[b]	11.5 ± 0.5[b]	29.6 ± 1.2[b,c,d]
15	Isorhamnetin-3-*O*-glucoside	12.2 ± 0.6	1.9 ± 0.5	nd	7.4 ± 0.3	nd
16	Syringetin-3-*O*-glucoside	7.2 ± 0.3	55.2 ± 1.5[a]	16.8 ± 0.7[b]	7.7 ± 0.4[b]	36.1 ± 2.4[a,b,c,d]
Total		343.8	1328.3	437.4	405.4	867.8

nd: not detected; Significant results ($P < 0.05$) are indicated as: [a] - *vs* Jaen; [b] - *vs* Touriga Nacional; [c] - *vs* Alfrocheiro; [d] - *vs* Tinta Roriz.

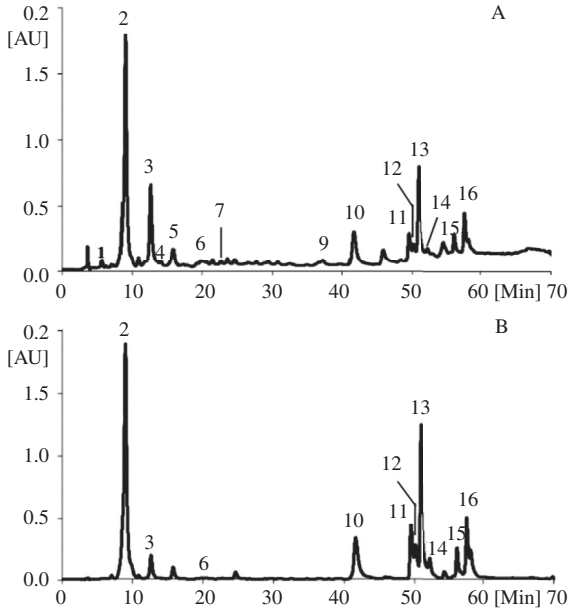

Figure 2. HPLC/DAD non-coloured phenolics profile of Touriga Nacional "Dão" red grapes from Quinta das Camélias, harvested in 2012.
A: Detected at 280 nm; B: Detected at 350 nm.

3.3. Organic acids

The HPLC/UV analysis of red grapes extracts revealed a profile composed by 10 identified organic acids: oxalic, aconitic, ketoglutaric, citric, tartaric, malic, quinic, succinic, shikimic and fumaric acids (Table 3). The total amounts of organic acids in the analysed samples ranged from ca. 20.5 to 69.0 g/kg of lyophilized grape samples, Touriga Nacional being the variety that showed high levels (Table 3).

3.4. PCA analysis

All data of anthocyanins, non-coloured phenolic compounds and organic acids were analysed by PCA, being expressed as mass by weight of lyophilized grapes. Figure 3 shows the projection of variables, grouped by chemical classes obtained for the five grape varieties in the plane composed by principal axes F1 and F2, containing 85.1% of the total variance (Figure 3).

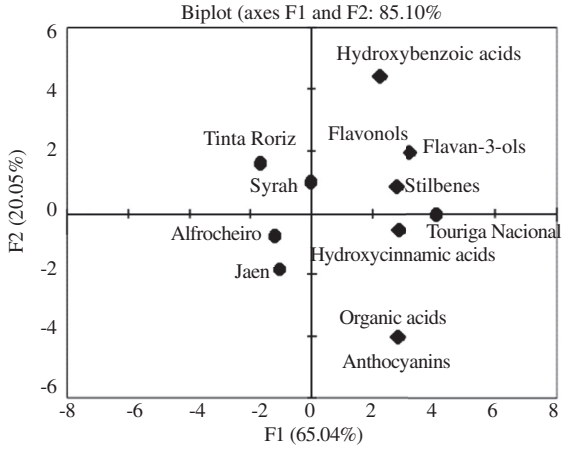

Figure 3. PCA of all phenolic compounds and organic acids in red "Dão" grapes.

4. Discussion

Grapes including a number of polyphenolic constituents have shown interesting biological properties, related to their antioxidant capacities [1,5,11,16]. Among them, anthocyanins, which are extracted from the skins of black grapes, are natural colorants which have aroused growing interest of researchers due their extensive range colour, innocuous and beneficial health.

The results obtained for anthocyanins revealed that the malvidin-3-*O*-glucoside was the major compound representing ca. 48%–62% of total compounds. These results are in accordance with previous works, that reported malvidin-3-*O*-glucoside as the main compound in black grapes [6,9,16,19] and in Dão red wines [10]. Anthocyanins are characterized by antioxidant potential, anti-inflammatory activity and their cellular signaling activity [5].

In respect to non-coloured phenolic, gallic acid was the only hydroxybenzoic acid found in all analysed grapes (Table 2). It is the only benzoic acid that has been formally identified native state in grapes, found in the solid parts of the berry, either in free form of flavanol ester (*i.e.* epicatechin-3-*O*-gallate) [5].

Regarding the hydroxycinnamic acids, they corresponded to ca. 4.1%–24.4% of the total of non-coloured phenolic compounds in the analysed grape varieties, *t*-CAFTA being the major one in Jaen, Alfrocheiro and Tinta Roriz. Additionally,

Table 3
Organic acids composition of red "Dão" grape samples (mg/kg of lyophilized sample).

Organic acids		Grapes				
		Jaen	Touriga Nacional	Alfrocheiro	Tinta Roriz	Syrah
1	Oxalic	18 868.7 ± 219.3	8 369.6 ± 572.1^{a}	11 482.7 ± 127.2^{a,b}	836.8 ± 26.5^{a,b,c}	3 073.2 ± 970.1^{a,b,c,d}
2	Aconitic	31.5 ± 1.0	11.9 ± 1.2^{a}	16.3 ± 0.4^{a,b}	13.4 ± 0.1^{a,b,c}	14.1 ± 0.0
3	Ketoglutaric	nq	155.5 ± 2.2^{a}	309.4 ± 4.3^{a,b}	82.6 ± 4.8^{a,b,c}	0.1 ± 0.0^{a,b,c,d}
4	Citric	nq	nq	nq	233.8 ± 11.9^{a,b,c}	251.5 ± 12.9^{a,b,c}
5	Tartaric	10 813.7± 383.8	38 711.6 ± 1201.4^{a}	3 892.6 ± 272.8^{a}	12 903.6 ± 181.9^{a,b,c}	21 656.3 ± 652.5^{a,b,c,d}
6	Malic	3 832.1 ± 361.7	14 869.8 ± 154.9^{a}	14 461.7 ± 435.3^{a}	3 130.8 ± 4.2^{a,b,c}	2 590.2 ± 12.4^{a,b,c,d}
7	Quinic	3 112.6 ± 152.4	1 478.0 ± 0.2^{a}	1 064.3 ± 53.9^{a,b}	3 024.3 ± 4.7^{a,b,c}	2 661.2 ± 45.2^{a,b,c,d}
8	Succinic	14 161.6 ± 397.2	3 869.7 ± 33.7^{a}	5 379.0 ± 563.0^{a,b}	nd	nd
9	Shikimic	110.3 ± 2.0	99.0 ± 0.9^{a}	140.7 ± 1.2^{a,b}	39.5 ± 0.9^{a,b,c}	205.7 ± 1.9^{a,b,c,d}
10	Fumaric	1 937.9 ± 31.5	1 430.2 ± 16.1^{a}	1 858.9 ± 25.8^{a,b}	190.5 ± 2.1^{a,b,c}	99.0 ± 0.1^{a,b,c,d}
Total		52 868.4	68 995.3	38 605.6	20 455.3	30 551.3

nd: Not detected; nq: Not quantified; Significant results ($P < 0.05$) are indicated as: ^{a} - *vs* Jaen; ^{b} - *vs* Touriga Nacional; ^{c} - *vs* Alfrocheiro; ^{d} - *vs* Tinta Roriz.

t-COUTA was the biggest one in Touriga Nacional and Syrah (Table 2). The hydroxycinnamic acids are located in the vacuoles of the skin and pulp cells, essentially in the form of tartaric esters [5].

One stilbene compound, polydatin (resveratrol-3-*O*-glucoside) was identified in all samples, except in Alfrocheiro (Table 2). The highest level in polydatin was found in Touriga Nacional (93.0 mg/kg of determined phenolics), followed by Syrah, Jaen and Tinta Roriz (58.8, 43.3 and 31.4 mg/kg of determined phenolics, respectively). Stilbenes are phytoalexins synthesized by plant, especially in skins, leaves and roots in response to fungal infections and UV light, being the grapes and their derived products considered as the most important dietary sources [5,20]. Resveratrol and their derivatives have been reported once that could prevent some kinds of cancer, cardioprotective and neuroprotective effects, protection against obesity, among others [20].

In respect to flavonoids (flavan-3-ols and flavonols), they showed percentages that ranged from 74.4% to 88.4%. Epigallocatechin was the most abundant in Jaen and Syrah (28.8% and 18.4% of determined phenolics, respectively). Quercetin-3-*O*-glucoside was the major one in Touriga Nacional (24.5% of determined phenolics), and catechin showed higher amounts in Alfrocheiro and Tinta Roriz (26.9% and 23.8% of the determined phenolics, respectively) (Table 2). Flavonols are secondary metabolites present in almost all higher plants. They are considered to act as UV- and photo-protectors because they absorb strongly at both UV-A and UV-B wavelengths. Also, flavonols playing an important role in wine copigmentation together with anthocyanins, are useful markers in grape taxonomy, and are considered bioactive grape/wine compounds of possible importance for human health and nutrition [5].

The nature and concentration of organic acids are important factors influencing the organoleptic characteristics of fruit and vegetables. Acids are known to have a lower susceptibility to change during processing and storage than other compounds, such as pigments and flavour components [21]. They are primary metabolites, which can be found in great amounts in all plant materials. As phenolics, this class of compounds may also have a protective role against various diseases due to their antioxidant properties [22].

Oxalic acid was the most abundant in Jaen (35.7%). Oxalic acid is an organic acid present in various organisms, has shown some antioxidant activities and thus could play an important role in systemic resistance, programmed cell death, redox homeostasis in plants, and an anti-senescence effect in harvested fruits [23].

Additionally, tartaric acid was the main compound in Touriga Nacional, Tinta Roriz and Syrah (56.1%, 63.1% and 70.9%, respectively) and malic acid was the main one in Alfrocheiro (37.5%) (Table 3). Tartaric and malic acids are the most abundant organic acids in grapes and wines. The levels in which these acids are present are related to the chemical and biological stability of wines. Their levels in grapes are the data frequently used to determine the harvesting date, particularly since each acid presents a different behaviour during grape ripening process. Malic acid shows a continuous decrease during ripening whereas tartaric acid remains almost unchanged. Therefore, different ratios can be obtained during ripening and the optimum harvest date can be established from their ratio [3,24].

The PCA analysis showed that the Touriga Nacional is clearly separated from the other varieties, being projected into the plane formed by F1 positive and F2 neutral axis due to their high concentration in phenolic compounds (anthocyanins and non-coloured) and organic acids.

Tinta Roriz and Syrah are presented into the planes formed by F1 negative and F2 positive axis. On the other hand, Alfrocheiro and Jaen were projected into the planes formed by F1 and F2 negative axis (Figure 3). This different position between the last both groups is due the high amounts in hydroxybenzoic acids, flavonols and anthocyanins in Tinta Roriz and Syrah (Tables 1 and 2). Otherwise, Alfrocheiro and Jaen are richer in organic acids (Table 3). These results agree with those observed in other studies, which indicate that it is possible to differentiate between grape varieties with PCA of their chemical constituents [6,25].

Despite the anthocyanin profile revealed to be more suited to characterize the red "Dão" grape varieties, our results revealed some relationships among the several varieties and their composition in anthocyanins and non-coloured phenolic compounds. This is very interesting that once the quality and typical characteristics of red wine are greatly dependent on the composition of the grapes in these bioactive compounds, they have influence on astringency, color, flavour, among others.

In summary, the non-coloured phenolics, anthocyanins and organic acids profiles were performed in five red varieties from "Dão" region (Portugal). Malvidin-3-*O*-glucoside was the most abundant anthocyanin. Concerning non-coloured phenolics, catechin and epigallocatechin were the main flavan-3-ols and quercetin-3-*O*-glucoside proved to be the flavonol found in higher amounts. In respect to organic acids, in general way, tartaric and malic acids were the main ones.

The PCA analysis revealed in more detail the relationship among the several red grapes and their chemical composition to perform the characterization of the evaluated varieties taking into account to their contents in hydroxybenzoic and hydroxycinnamic acids, stilbenes, flavan-3-ols, flavonols, anthocyanins and organic acids. Touriga Nacional proved to be distinctly different for the other varieties due their high contents in all chemical classes. These results justify the reason why this variety is the most important red grape cultivar in Dão region for its ability to produce high quality wines, being responsible for the prestige that Dão wines have acquired over the years.

Conflict of interest statement

We declare that we have no conflict of interest.

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