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Original Paper

Quantification of polyphenols with potential antioxidant properties in wines using reverse phase HPLC

A RP-HPLC method with photodiode array detection (DAD) was developed to separate, identify and quantify simultaneously the most representative phenolic compounds present in Madeira and Canary Islands wines. The optimized chromatographic method was carefully validated in terms of linearity, precision, accuracy and sensitivity. A high repeatability and a good stability of phenolics retention times (<3%) were obtained, as well as relative peak area. Also high recoveries were achieved, over 80.3%. Polyphenols calibration curves showed a good linearity ($r^2 > 0.994$) within test ranges. Detection limits ranged between 0.03 and 11.5 $\mu\text{g/mL}$ for the different polyphenols. A good repeatability was obtained, with intra-day variations less than 7.9%. The described method was successfully applied to quantify several polyphenols in 26 samples of different kinds of wine (red, rosé and white wines) from Madeira and Canary Islands. Gallic acid was by far the most predominant acid. It represents more than 65% of all phenolics, followed by *p*-coumaric and caffeic acids. The major flavonoid found in Madeira wines was *trans*-resveratrol. In some wines, (–)-epicatechin was also found in highest amount. Canary wines were shown to be rich in gallic, caffeic and *p*-coumaric acids and quercetin.

Keywords: Antioxidants / HPLC-photodiode array detection / Polyphenols / Wines

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1 Introduction

Phenolic compounds are secondary metabolites that are synthesized by plants [1] during normal development and in response to stress conditions. They are widely found in vegetables, fruits, seeds, juices, tea, coffee, chocolate, vinegar and wines [2, 3] and consequently are common components of the human diet [2]. Although structurally diverse, phenolics are classified into two groups: the flavonoids and the nonflavonoids. The flavonoid family includes the flavonols, like myricetin, quercetin and kaempferol, which exist both as aglycons and sugar conjugates; the flavan-3-ols, as (+)-catechin and (–)-epicatechin; and the anthocyanins such as malvidin-3-glucoside. The nonflavonoids encompass hydroxybenzoic acids

such as gallic acid; hydroxycinnamates, including *p*-coumaric, caffeic and cinnamic acids; and the stilbenes, like *trans*-resveratrol and *cis*-resveratrol [4].

Polyphenols are the principal compounds related to the wine consumption benefits due to antioxidant and free radical scavenging properties. These physiological effects are especially associated to flavonoids and stilbenes [5, 6], namely quercetin, (+)-catechin, gallic acid and *trans*-resveratrol [7]. Flavonoids have shown the inhibition of lower density lipoprotein (LDL) oxidation by macrophages and cupric ions [8], as increasing evidence shows that oxidized LDL and very LDL (VLDL) may be involved in the pathogenesis of atherosclerosis [9–11]. Phenolic acids and flavonoid-like compounds also acted as antioxidants in LDL oxidation by peroxyl radicals generated by an azo-initiator [12]. The stilbene *trans*-resveratrol has gained great attention and a number of scientific papers have appeared related to the moderate consumption of red wine, the ability to inhibit platelet aggregation and LDL oxidation and its beneficial effects in health. Soleas *et al.* [7] had related that *trans*-resveratrol may be the most effective anticancer polyphenol present in red wine. Flavanols, such as (–)-epicatechin have many beneficial health effects such as anti-tumorigenic, anti-

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Abbreviations: DAD, photodiode array detection; FC, Folin–Ciocalteu; GAEs, gallic acid equivalents; LDL, low density lipoprotein; PC, principal components

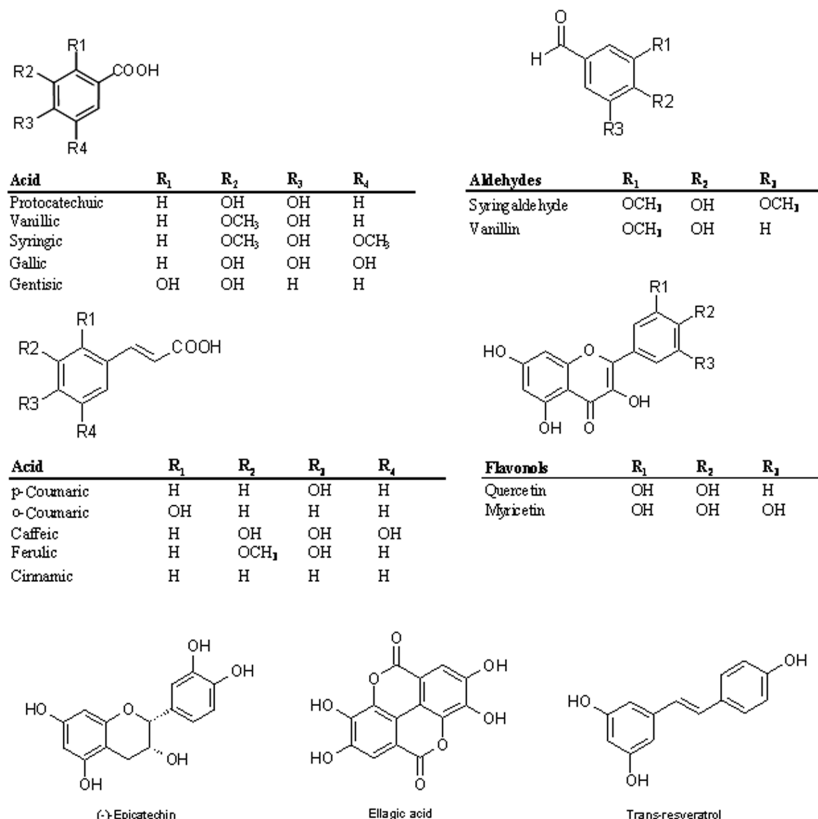


Figure 1. Chemical structures of main phenolic compounds found in studied wines.

mutagenic, anti-pathogenic and anti-oxidative properties and has attracted interest because their concentrations in red wine are higher than other flavonoids [13].

Wine is an excellent natural source of various polyphenol families [14]. They play a number of important roles in viticulture and oenology including UV protection, disease resistance, pollination, color, and defence against predation in plants [15], as well as haze formation, hue, and taste in wines [16]. In particular, tannins confer astringency and structure to the beverage by the formation of complexes with the proteins of saliva. Their knowledge is very important to predict wine ageing attitude and attempting to solve problems about color stability, namely in the case of red wines that are destined to long ageing periods [17]. Their presence and structures are affected by several factors including grape variety, sun exposure, vinification techniques and ageing. The wine ageing also changes the phenolic composition, as these compounds can suffer diverse transformations, like oxidation processes, condensation and polymerization reactions and extraction from wood, usually associated to the changes in color and colloidal stability [18], flavor, bitterness and astringency [19]. The polyphenolic fingerprint can be useful for the classification of wines, since it can give us information about the variety, the geographic and winery origin and even the applied wine-making technology [2, 20–24].

The aim of the present work was to develop and optimize a rapid and simple methodology which could be used to separate, identify and quantify simultaneously 17 phenolic compounds (Fig. 1) in distinct types of Madeira and Canary wines (red, rosé and white wines). The method was carefully validated, evaluating the selectivity, linear range, detection and quantification limits, the accuracy and the repeatability. The studied compounds were determined by HPLC coupled to a photodiode array detector (DAD) using a binary gradient. Previously, total phenols were analyzed according to the Folin–Ciocalteu (FC) method and the results were given as gallic acid equivalents (GAEs).

To our knowledge, the nature and/or concentration of low-molecular-mass polyphenols compounds studied in the referred wines have not been reported yet. Since these compounds are usually involved in health-related properties, their characterization is a requirement.

2 Experimental

2.1 Reagents and standards

Methanol, purchased from Sigma-Aldrich (St Louis, MO, USA), was of HPLC grade. Ethanol was provided by Panreac Química SA (Barcelona, Spain), diethyl ether from Lab-Scan (Dublin, Ireland), hydrochloric acid (37%) from

Table 1. Identification of wine samples according to variety, harvest year and geographic origin

Wine samples	Varietal composition	Vintage	Origin
Red	VT1 Tinta Negra Mole	2005	Madeira
	VT2 Tinta Negra Mole , Cabernet Sauvignon, Merlot	2005	
	VT3 Tinta Negra Mole , Cabernet Sauvignon, Merlot, Complexa	2005	
	VT4 Cabernet Sauvignon , Merlot, Touriga Nacional, Touriga Barroca	2005	
	VT5 Touriga Nacional , Merlot, Cabernet Sauvignon	2005	
	CT4 Negramol	2004	Canary
	CT5 Negramol	2005	
Rosé	VR1 Tinta Negra Mole	2005	Madeira
White	VB1 Verdelho	2005	Madeira
	VB2 Verdelho	2005	
	VB3 Verdelho	2005	
	VB4 Verdelho , Arnburger,	2005	
	VB5 Malvasia	2005	
	VB6 Ansbürger	2005	
	VB7 Verdelho , Arnburger, Boal	2005	
	VB8 Verdelho , Ansbürger	2005	
	VB9 Verdelho , Ansbürger	2005	
	VB10 Ansbürger	2005	
	VB11 Ansbürger	2005	
	VB12 Verdelho	2005	
	CM4 Malvasia	2004	Canary
	CM5 Malvasia	2005	
	CB4 Malvasia , Gual	2004	
	CB5 Malvasia , Gual	2005	
	CG4 Gual	2004	
	CG5 Gual	2005	

Riedel-de-Häen (Seelze, Germany) and glacial acetic acid was supplied by Merck (Darmstadt, Germany). Tartaric acid (Riedel-de-Häen) and sodium hydroxide (Panreac Química SA) were used to prepare synthetic wine.

Polyphenols standards: gallic acid, gentisic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, *m*-coumaric acid, sinapic acid, *o*-coumaric acid, ellagic acid, cinnamic acid, (+)-catechin, (–)-epicatechin and myricetin, were supplied by Fluka Biochemika AG (Buchs, Switzerland), protocatechuic acid, vanillin, syringic acid, FC reagent and *trans*-resveratrol by Sigma-Aldrich, while syringaldehyde was acquired from Acros Organics (Geel, Belgium) and quercetin from Riedel-de Häen. HPLC grade water (Milli Q-System, Millipore, Bedford, MA, USA) was used during the preparation of mobile phases.

Stock solutions at concentration of 1000 mg/L for each polyphenol were prepared by dissolving the appropriate amount of the compounds in ethanol. These solutions were stored at 4°C and used to make working-strength solutions in synthetic wine, prepared with 12% v/v ethanol and 3.5 g/L tartaric acid and pH adjusted to 3.2 with HCl 1 M. The working standard solutions were prepared by dilution of the respective stock solutions and kept under similar conditions. After extraction with diethyl ether, these standards were injected to determine individual retention times (t_R) and for linearity range, precision, accuracy and detection limit tests. All prepared samples and extracts were filtered through a 0.45 µm

membranes (Acrodisc® CR PTFE, Ann Arbor, SOM, USA) and degassed in an ultrasonic bath (Sonorex Super RK102H, Berlin, Germany) before use.

2.2 Wine samples

All varieties used were *V. vinifera* L. species. The most important and representative commercial table wines produced in Madeira and Canary Islands were selected (Table 1), produced according to standard procedures. The grapes used for wines production were harvested at optimum maturity evaluated by indices of sugar and acid content. Grapes from different varieties were crushed, de-stemmed, racked and pressed. The musts were fermented in stainless-steel containers, with spontaneous yeast. Alcoholic fermentation was carried out at 18–20°C. The Madeira wine samples, produced in Adega de São Vicente (Northern of Madeira Island), were supplied by the Instituto do Vinho Bordado e Artesanato da Madeira while Canary wines were supplied by winery Viñatigo (Tenerife Island). The code of analyzed wines and the varietal composition of the different wine samples are presented in Table 1. All samples were taken from bottled wines (750 mL) ready for sale and were stored at –20°C until analysis. After sampling the wines were immediately stored at –20°C and each one was opened before the analysis. Each sample was analyzed with three replicates and the values were averaged.

2.3 Determination of total polyphenol content

The content of total phenolic compounds in wine samples was determined by the FC reagent [25] using gallic acid as standard. This method is based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products. For the preparation of calibration curve, 1 mL aliquots of 5, 10, 20, 25 and 50 mg/L aqueous gallic acid solutions were mixed with 0.025 mL FC reagent. One milliliter of wine sample (adequately diluted) was added to 0.25 mL of carbonate-tartarate solution (20 g of Na_2CO_3 and 1.2 g of $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$ in 100 mL of deionized water) and 0.025 mL of FC reagent. The absorbance of analytes was measured in a Perkin-Elmer Lambda 2 spectrophotometer (Perkin-Elmer, Germany) at 700 nm after 30 min of reaction at room temperature. The results were expressed as milligram of GAE/L. High reproducible results for standards ($r^2 > 0.9993$) and samples were obtained. All determinations were performed in triplicate.

2.4 Analytical procedure and RP HPLC-DAD analysis

The pH of the wine and standards was adjusted to 2.0 adding small amounts of a hydrochloric acid solution (1 M). Then, an aliquot of 5 mL of wine/standard was extracted twice with 2.5 mL of diethyl ether for 20 min. The organic layer was separated and evaporated to dryness using a slow nitrogen stream. The dry residue was re-dissolved in a methanol-water (1:1, v/v) mixture and filtered through a 0.45 μm membrane prior to injection into HPLC system.

A Waters High Performance Liquid Chromatography system (Ann Arbor) equipped with a Waters 1525 Binary HPLC Pump, a Waters 996 DAD and a Waters 717 Plus Autosampler was used for the chromatographic determination of the studied phenolics. A Millennium³² chromatography manager software, version 3.2, was used for analysis and data acquisition. Polyphenolic compounds were separated on a Nova-Pak C_{18} column (150 mm \times 3.9 mm id, 4 μm) from Waters.

Phenolic compounds were eluted using a binary elution system that constitute the mobile phase: eluent A (water-acetic acid-methanol, 88:2:10, v/v) and eluent B, prepared with the same solvents but with the following composition 8:2:90 v/v. The gradient elution began at 100% A to 85% A in 15 min, down to 50% A in 10 min, followed by a reduction to 30% A in 9 min and finally the column was regenerated in 12 min. The mobile phases used were previously filtered through a 0.45 μm membrane disc filter (Pall Corporation, Ann Arbor) and were degassed before use. The flow rate was 0.7 mL/min, the injection volume was set to 20 μL and column was at

room temperature. The spectrophotometric detection operated at a wavelength range of 240–390 nm and was performed with a resolution of 1.2 nm. Polyphenols were monitored and quantified at 270 nm (hydroxyl benzoic acids, flavan-3-ols and flavanones), 307 nm (hydroxycinnamic acids) and 360 nm (flavonols and flavones). Phenolic compounds were identified and quantified by comparison of their retention time with UV-Vis spectral data and the standards previously injected.

2.5 Method validation

The method was validated in terms of linearity, precision, accuracy, sensitivity and detection and quantification limits. Linearity was studied injecting mixtures of phenolic compounds at different concentration levels, in order to cover the working range. Calibration curves for every compound with the respective correlation coefficient were calculated by least-squares linear regression analysis of the peak area of each analyte. The polyphenols concentration was determined using the area response of each individual wine compound by interpolation in the corresponding calibration graphs. The calculation for the LODs were based on the SD of y-intercepts of regression analysis (σ) and the slope (S) using the following equation: $\text{LOD} = 3.3 \sigma/S$. LOQs were calculated by the equation $\text{LOQ} = 10 \sigma/S$. The precision of the method based on within-day repeatability was assessed by replicate ($n = 10$) measurements from samples. Accuracy was expressed as the recovery of analytes in comparison to the added amounts. Selectivity was assessed by the absence of interferences in the same retention time as examined polyphenols in the respective wine samples.

2.6 Statistical treatment

Significant differences between Madeira and Canary wine varieties for each of the phenolic constituents were determined by one-way analysis of variance using a SPSS Program, version 15.0 (SPSS, 2006). Principal component analysis and stepwise linear discriminant analysis were performed using the same SPSS program. These techniques were applied to the normalized relative amounts of the wine volatile compounds [26, 27].

3 Results and discussion

3.1 Total polyphenol content

The phenolic amount varies considerably in the different wine types, depending on the grape variety, environmental factors in the vineyard and the wine processing techniques. The results obtained for the wine samples tested confirm this variation and also are in agreement with those available in literature [28, 29]. The presence of high

Table 2. Total phenolics contents (TPC) determined in commercial Madeira and Canary wine samples (average \pm %RSD) ($n = 3$)

Madeira wine				Canary wines			
Red	TPC (mg/L) ^{a)}	White	TPC (mg/L) ^{a)}	Red	TPC (mg/L) ^{a)}	White	TPC (mg/L) ^{a)}
VT1	1827 \pm 8.1	VB1	370 \pm 5.8	CT4	1999 \pm 3.2	CM4	460 \pm 3.1
VT2	1871 \pm 8.2	VB2	372 \pm 5.8	CT5	1544 \pm 3.7	CM5	488 \pm 3.1
VT3	1853 \pm 8.1	VB3	282 \pm 10.7			CB4	229 \pm 5.1
VT4	1936 \pm 8.3	VB4	389 \pm 5.1			CB5	319 \pm 3.6
VT5	1724 \pm 7.9	VB5	434 \pm 3.5			CG4	231 \pm 5.1
		VB6	340 \pm 5.9			CG5	301 \pm 3.8
Rosé	665 \pm 2.1	VB7	309 \pm 7.0				
		VB8	442 \pm 3.8				
		VB9	461 \pm 3.1				
		VB10	344 \pm 5.8				
		VB11	718 \pm 2.7				
		VB12	770 \pm 3.2				

^{a)} Values expressed as mg of GAE/L.

concentrations of gallic acid in red wines was expected since this phenolic acid is principally formed by hydrolysis of flavonoid gallate esters, usually absent in white wines due to the lack of skin extraction. The content of phenolic compounds (free and total) determined by the FC method for the different analyzed wines is shown in Table 2. The total polyphenol content in red wine was significantly higher than in rosé and white wine ($p < 0.05$ for both cases). The relative values determined from regression equation of calibration curve ($y = 0.0493x - 0.0633$; $r^2 = 0.9993$) were expressed in GAE. Madeira red wines had the highest phenolic concentrations averaging 1842 mg/L and Canary red wines of about 1771 mg/L. The lowest values were found in CB4 (229 mg/L) and VB3 (282 mg/L) Canary and Madeira wines, respectively. The highest values were found in VT4 (1936 mg/L) and CT4 (1999 mg/L) (Table 2). For rosé wine was obtained 665 mg/L GAE.

3.2 Method validation

Calibration curves were obtained using pure standards at concentrations normally present in wines. The curves were constructed using five-point ($n = 3$) calibration of each compound. The responses *versus* nominal concentration fitted well to a straight line with R^2 values higher than 0.9943. Linear regression analysis using the least-squares method was used to evaluate the calibration curve of each analyte as a function of its concentration. Table 3 shows the results obtained for parameters which enabled to evaluate the method performance: linearity range, sensitivity (b), intercept (a), regression coefficient (R^2), LOD and LOQ and recovery values. For this purpose, the peak areas were measured in the chromatograms obtained at the wavelengths of maximum absorption for each compound quantified (as indicated in Table 3). The variation of the retention times shown in Table 3 was the

observation of ten successive standard injections. The highest slope (8.14 $\mu\text{g/mL}$) was shown for *trans*-resveratrol (307 nm) while (–)-epicatechin (270 nm) obtained the lowest sensitivity (0.13 $\mu\text{g/mL}$).

The obtained LODs, 0.03 to 11.5 $\mu\text{g/mL}$, and LOQ, 0.03 to 31.6 $\mu\text{g/mL}$, indicates that the proposed method is sensitive enough for the determination of phenolic compounds in wines. The variation coefficients of the analyses were comprised between 2.2 and 7.9%. Accuracy was evaluated from extraction efficiencies. Table 3 shows the extraction efficiencies for the phenolic structures studied.

3.3 Method application to the analysis of low molecular-mass polyphenols in wines

Once analytical conditions for separation and detection were optimized, the method was used to determine low molecular-mass polyphenols in 26 commercial Madeira and Canary Islands wines (Table 1). Seventeen compounds, including flavonoids and nonflavonoids, could be separated and quantified by the RP-HPLC method employed. The different wine types (red, rosé and white wines) were submitted to the above-mentioned sample preparation (Section 2.4.). As shown in Fig. 2, the separation of a standard mixture of the 17 phenolic compounds can be achieved in 46 min. HPLC determination of phenolic compounds has become one of dominant analytical procedures because of its advantages, *e.g.* simple sample treatment, possibility to pre-separate and to remove impurities, possibility to change the polarity of mobile phase during analysis, short analysis time and high reproducibility.

Figure 3 shows the chromatogram recorded at 270, 307 and 360 nm corresponding to CT5 red wine made with Negramol grapes. As can be noted, the three chromatographic profiles are quite different and their com-

Table 3. Parameters of the linear regression ($y = ax + b$) and experimental retention times (t_R)^{a)}, LOQ, reproducibility (RSD) and percentage of recovery for the studied compounds by RP-HPLC-DAD.

Peak No	Compounds	UV (nm)	t_R (min) \pm RSD(%)	Conc. Range ($\mu\text{g/mL}$)	a ($\times 10^5$) ^{b)}	b ($\times 10^4$) ^{c)}	R^2 ^{d)}	LOD ($\mu\text{g/L}$)	LOQ ($\mu\text{g/mL}$)	RSD (%)	Recovery (%)
1	Gallic acid	270	2.68 \pm 0.01	2.0–80.8	1.23	−0.32	0.9990	1.09	3.63	2.23	99.9
2	Protocatechuic acid	270	4.65 \pm 0.03	2.0–64.0	1.99	−4.59	0.9994	1.97	6.57	3.27	80.3
3	Gentisic acid	307	7.69 \pm 0.01	2.5–80.0	0.90	−3.54	0.9989	1.25	3.26	3.8	91.8
4	Vanillic acid	270	11.07 \pm 0.04	2.0–64.0	2.42	−4.90	0.9992	0.47	1.28	3.43	98.6
5	Caffeic acid	307	11.78 \pm 0.01	1.5–36.0	4.58	−0.20	0.9990	0.44	1.45	3.92	105.4
6	Syringic acid	270	13.83 \pm 0.07	2.0–64.3	2.07	5.38	0.9990	0.29	0.58	3.19	100.1
7	Vanillin	270	14.25 \pm 0.08	8.0–48.0	3.10	5.03	0.9990	0.87	1.48	4.46	96.7
8	(−)-Epicatechin	270	15.10 \pm 0.06	12.6–400.3	0.13	−1.59	0.9989	4.24	8.28	2.73	85.2
9	Syringaldehyde	307	17.19 \pm 0.11	2.0–48.0	2.92	−0.50	0.9990	0.29	0.43	3.33	58.9
10	<i>p</i> -Coumaric acid	307	18.52 \pm 0.04	0.6–25.0	7.19	15.15	0.9986	0.56	1.26	3.93	95.9
11	Ferulic acid	307	21.77 \pm 0.05	0.7–24.0	4.74	−6.43	0.9991	0.16	0.21	3.61	96.6
12	<i>o</i> -Coumaric acid	270	25.78 \pm 0.09	0.5–20.0	5.10	10.63	0.9991	0.06	0.59	4.3	85.5
13	<i>trans</i> -Resveratrol	307	26.88 \pm 0.05	0.5–8.2	8.14	2.52	0.9991	0.07	0.03	4.23	108.0
14	Ellagic acid	270	27.15 \pm 0.09	10.0–100.0	1.19	−34.41	0.9943	11.51	31.64	1.88	43.5
15	Myricetin	360	28.02 \pm 0.05	2.1–24.6	3.91	−41.38	0.9963	1.96	6.54	7.87	95.5
16	Cinnamic acid	270	30.16 \pm 0.53	0.4–12.0	7.04	−1.74	0.9992	0.03	0.04	3.75	105.3
17	Quercetin	360	30.82 \pm 0.05	5.0–60.5	2.85	−100.00	0.9980	2.88	9.59	4.91	97.3

a) The retention times (t_R) are the mean of ten replicates \pm SD.

b) slope

c) intercept

d) R^2 : regression coefficient.

plexity decreases as wavelength increases. At 270 nm (gallic, vanillic, syringic and (−)-epicatechin) and 307 nm (caffeic, *p*-coumaric and ferulic acids and *trans*-resveratrol) four phenolic acids were detected based on their retention times and UV-Vis spectra compared with standards. At 360 nm, only two compounds were identified: myricetin and quercetin.

Table 4 presents analytically the content of the individual polyphenols found in wines produced in different geographic regions – Madeira and Canary Islands. The highest content of polyphenols was found in red wines, as expected. The highest values were found in CT5 wine from Negramol grapes (835 mg/L) and VT4 wine from Cabernet Sauvignon, Merlot, Touriga Nacional and Touriga Barroca grapes (742 mg/L), and the lowest in wines from Verdelho (35 mg/L) and Arnsburger (41 mg/L) grapes.

In Madeira red wines, the polyphenol content varied from 519 mg/L (VT3 wine; Tinta Negra Mole, Cabernet Sauvignon, Merlot and Complexa grapes) to 742 mg/L (VT4 wine), being the average value of 608 mg/L, while in white wines the levels ranged between 34 mg/L (VB2 wine) and 175 mg/L (VB12 wine). In red wines, gallic acid (in average 397.1 mg/L) was by far the most predominant phenolic acid and accounted for 65–66% of all phenolics. (−)-Epicatechin was the second most abundant found (mean 105.6 mg/L) followed by quercetin (mean 55.7 mg/L). Vanillic, syringic and *p*-coumaric acids, were found in all red wine samples. On the other hand, either protocatechuic, gentisic, *o*-coumaric, ellagic and cinnamic acids, or vanillin and syringaldehyde were found at detectable levels in any wine sample. *Trans*-resveratrol usually

appears in low concentrations in studied wines, but VT4 and VT5 wines contained a considerable level, 57.7 ± 0.9 and 30.8 ± 1.2 mg/L, respectively. Baptista *et al.* [30] found *trans*-resveratrol amounts in Portuguese red wines between 0.63–5.21 mg/L, and in Italian red wines the levels of *trans*-resveratrol have been reported between 0.56–2.86 mg/L [31].

In Madeira white wines the predominant phenolic constituents were gallic acid (mean value 17.1 mg/L). Substantial amounts of caffeic (average value 13.5 mg/L) and *p*-coumaric acid (mean value 8.2 mg/L) were also found. (−)-Epicatechin average levels were higher in red wines, namely in VT2 wine (159.5 mg/L). These results show great similarity to those previously reported by Minussi *et al.* [25]. Gambuti *et al.* [32] found in Spanish red wines concentrations of (−)-epicatechin between 42.4 and 46.6 mg/L and in young red wines 2.02–3.02 mg/L.

In Canary red wines (Table 4) the polyphenol content was much higher than those determined in Madeira red wines (*i.e.* 819 mg/L (CT4 wine; Negramol grapes) and 835 mg/L (CT5 wine; Negramol grapes)). Gallic acid (376.5 ± 8.2 mg/L in CT5 wines) followed by caffeic acid (182.3 ± 4.7 mg/L in CT4 wines) and quercetin (96.9 ± 0.1 mg/L in CT4 wines) were the principal phenolic compounds found in these wines. Cinnamic acid was only measurable in CT4 wines and (−)-epicatechin just in CT5 wines.

In Canary white wines (Table 4) the levels of phenolics varied from 60 mg/L (CB4 wine; Malvasia and Gual grapes) to 150 mg/L (CM4 wine; Malvasia grapes). The most predominant compound was caffeic acid

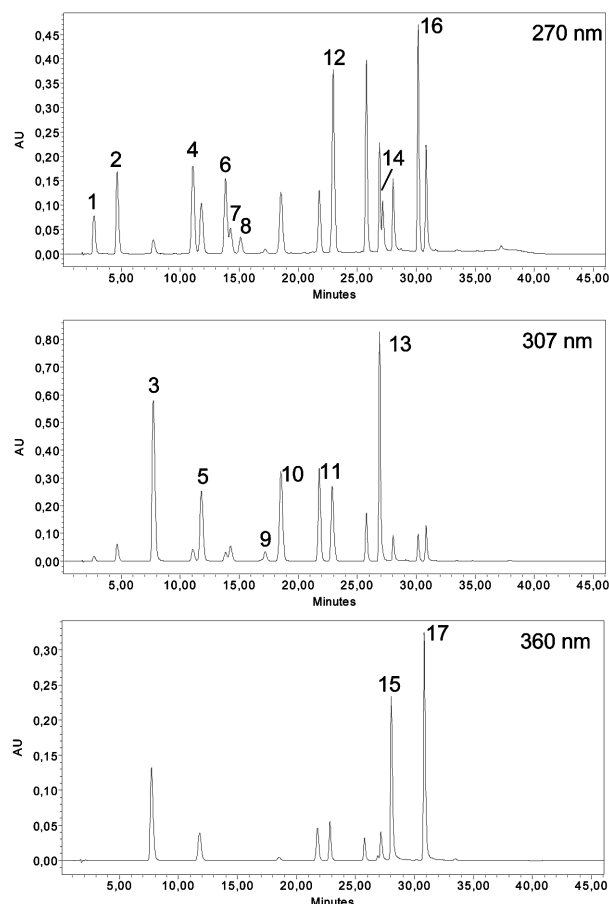


Figure 2. RP-HPLC chromatographic separation of a mixture of phenolic standards registered at three different wavelengths. For peak identification, see Table 3.

(114.9 ± 3.4 mg/L; CM5 wines), while good levels of quercetin (mean value 21.4 mg/L), gallic acid (mean value 20.7 mg/L), *p*-coumaric acid (mean value 15.2 mg/L) and ferulic acid (mean value 5.1 mg/L), were also found in Canary white wines. The total concentrations of low-molecular-mass polyphenols determined from the analysis of Madeira and Canary wines are depicted in Fig. 4

The results obtained confirm a variation in the phenolic content among wine samples tested. It is irrefutable that the amounts as well as the various species of phenolics that occur in wines depend on a wide range of factors, including cultural practices, local climate conditions and vinification techniques.

3.4 Principal component analysis

Principal components analysis was applied to the obtained data in order to achieve any differentiation based on their phenolic content. Figure 5 shows the distribution of wines according to the two first principal components (PC) which explained 91.9% of the total var-

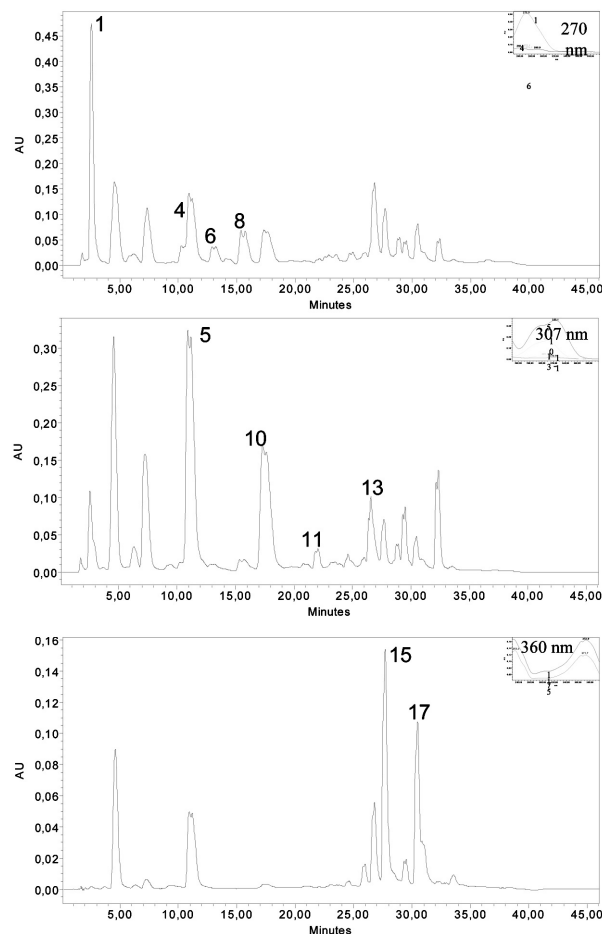


Figure 3. RP-HPLC chromatograms and representative on-line DAD/UV-Vis spectra of a CT5 red wine extract monitored at different wavelengths. Peak identification: 270 nm: (1) gallic acid, (4) vanillic acid, (6) syringic acid, (8) (–)-epicatechin; 307 nm: (5) caffeic acid; (10) *p*-coumaric acid, (11) ferulic acid, (13) *trans*-resveratrol; 360 nm: (15) myricetin, (17) quercetin.

iance. As shown in Fig. 5(a), some grouping could be observed in the space formed by the two first components, according to wine kind. The cluster corresponding to white wine is clearly separated from the other ones. In the case of red wine, all clusters are well separated. The red wines were located on the positive side of PC1 axis, characterized by vanillic (0.981), gallic (0.971) and syringic (0.892) acids. The white wines were mostly centred on negative side of PC2, while rosé wine is located on the negative side of PC1 and PC2 axis. Figure 5(b) shows that Madeira and Canary wines were grouped separately on the basis of their origin. The second extracted principal component, correlated with caffeic and *p*-coumaric acids, discriminates primarily between Canary and Madeira wines.

This measuring system permits us to classify the signals in separate clusters and to discriminate one set from

Table 4. Content (mg/L) of phenolic compounds identified in Madeira and Canary commercial table wine samples^{a), b)}

Compounds	Canary white wines ^{c)}					
	CM4	CM5	CB4	CB5	CG4	CG5
Gallic acid	nd	30.95 ± 0.12	nd	18.55 ± 0.17	11.79 ± 0.09	20.56 ± 0.74
Protocatechuic acid	92.33 ± 1.84	nd	nd	nd	nd	nd
Gentisic acid	nd	nd	nd	nd	nd	nd
Vanillic acid	3.85 ± 0.12	3.62 ± 0.03	1.91 ± 0.06	nd	1.94 ± 0.03	2.89 ± 0.01
Caffeic acid	65.08 ± 0.23	114.99 ± 3.40	27.47 ± 0.27	31.04 ± 0.08	15.76 ± 0.25	21.43 ± 0.41
Syringic acid	3.30 ± 0.11	2.09 ± 0.15	nd	nd	nd	nd
Vanillin	nd	nd	nd	nd	nd	nd
(-)-Epicatechin	nd	nd	nd	nd	nd	nd
Syringaldehyde	nd	nd	nd	nd	nd	nd
p-Coumaric acid	33.83 ± 0.09	26.76 ± 0.34	8.29 ± 0.11	3.52 ± 0.06	10.02 ± 0.13	7.58 ± 0.04
Ferulic acid	6.93 ± 0.08	5.25 ± 0.02	4.56 ± 0.14	2.65 ± 0.03	6.30 ± 0.13	5.95 ± 0.28
o-Coumaric acid	nd	nd	nd	nd	nd	nd
Trans-resveratrol	6.29 ± 0.07	3.78 ± 0.06	nd	1.55 ± 0.06	0.71 ± 0.05	5.85 ± 0.04
Ellagic acid	nd	nd	nd	nd	nd	nd
Myricetin	nd	nd	nd	nd	nd	nd
Cinnamic acid	nd	nd	nd	nd	nd	nd
Quercetin	30.53 ± 0.09	nd	18.24 ± 0.01	18.61 ± 0.01	19.27 ± 0.02	20.50 ± 0.07

	Madeira red wines ^{c)}					Canary red wines ^{c)}		Rosé wine ^{c)}
	VT1	VT2	VT3	VT4	VT5	CT4	CT5	VR1
Gallic acid	429.02 ± 1.54	392.17 ± 6.82	341.16 ± 8.44	416.40 ± 5.82	411.66 ± 8.41	347.49 ± 9.60	376.23 ± 8.15	86.69 ± 1.70
Protocatechuic acid	nd	nd	nd	nd	nd	nd	nd	nd
Gentisic acid	nd	nd	nd	nd	nd	nd	nd	nd
Vanillic acid	16.77 ± 0.23	28.02 ± 1.45	28.04 ± 0.78	31.22 ± 2.20	31.42 ± 2.13	18.91 ± 0.56	20.20 ± 0.27	7.87 ± 0.65
Caffeic acid	10.47 ± 0.81	nd	nd	12.87 ± 0.26	17.86 ± 1.27	182.32 ± 4.70	145.61 ± 7.13	19.18 ± 0.39
Syringic acid	4.80 ± 0.04	21.74 ± 0.92	28.59 ± 0.87	16.78 ± 0.14	21.11 ± 1.26	26.41 ± 0.09	27.67 ± 1.61	5.45 ± 0.12
Vanillin	nd	nd	nd	nd	nd	nd	nd	nd
(-)-Epicatechin	nd	159.50 ± 9.21	113.10 ± 1.10	98.06 ± 3.84	nd	nd	85.57 ± 2.58	20.55 ± 1.73
Syringaldehyde	nd	nd	nd	nd	nd	nd	nd	nd
p-Coumaric acid	1.48 ± 0.11	4.88 ± 0.41	4.49 ± 0.14	16.06 ± 1.21	6.79 ± 0.64	73.07 ± 1.96	61.17 ± 3.27	11.62 ± 0.16
Ferulic acid	nd	nd	nd	nd	nd	3.13 ± 0.22	1.97 ± 0.04	nd
o-Coumaric acid	nd	nd	nd	nd	nd	nd	nd	nd
Trans-resveratrol	nd	4.81 ± 0.19	4.50 ± 0.08	57.74 ± 0.95	30.82 ± 1.17	27.68 ± 0.09	6.42 ± 0.14	7.28 ± 0.04
Ellagic acid	nd	nd	nd	nd	nd	nd	nd	nd
Myricetin	14.83 ± 0.46	9.43 ± 0.30	nd	16.87 ± 0.79	17.39 ± 0.22	42.93 ± 0.43	49.12 ± 1.47	nd
Cinnamic acid	nd	nd	nd	nd	nd	8.01 ± 0.08	7.74 ± 0.45	nd
Quercetin	50.02 ± 0.85	35.95 ± 1.54	nd	79.15 ± 2.63	57.67 ± 2.14	96.95 ± 0.14	61.41 ± 1.22	nd

	Madeira white wines ^{c)}											
	VB1	VB2	VB3	VB4	VB5	VB6	VB7	VB8	VB9	VB10	VB11	VB12
Gallic acid	8.69 ± 0.30	8.89 ± 0.14	8.41 ± 1.70	21.06 ± 0.24	30.10 ± 0.24	19.43 ± 0.70	11.27 ± 0.69	9.46 ± 0.50	9.66 ± 0.18	8.55 ± 0.59	34.76 ± 1.62	35.17 ± 1.34
Protocatechuic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Gentisic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vanillic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Caffeic acid	14.62 ± 0.47	14.78 ± 0.38	12.28 ± 0.45	15.60 ± 0.67	14.30 ± 0.25	13.77 ± 0.99	11.86 ± 0.66	16.16 ± 0.88	12.87 ± 0.31	7.65 ± 0.33	13.38 ± 0.26	14.95 ± 0.17
Syringic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vanillin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
(-)-Epicatechin	nd	nd	nd	35.32 ± 1.97	nd	nd	nd	19.55 ± 0.99	19.03 ± 0.44	19.33 ± 0.22	78.33 ± 3.34	89.36 ± 2.52
Syringaldehyde	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
p-Coumaric acid	6.64 ± 0.40	6.81 ± 0.85	8.75 ± 0.40	9.34 ± 0.28	6.31 ± 0.11	5.27 ± 0.31	7.38 ± 0.32	9.36 ± 0.55	12.40 ± 0.18	5.19 ± 0.34	11.23 ± 0.20	9.17 ± 0.38
Ferulic acid	nd	3.33 ± 0.14	3.17 ± 0.15	nd	1.31 ± 0.11	1.48 ± 0.04	3.24 ± 0.14	nd	nd	nd	1.86 ± 0.14	4.38 ± 0.08
o-Coumaric acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Trans-resveratrol	nd	nd	2.15 ± 0.14	2.32 ± 0.03	1.18 ± 0.07	0.84 ± 0.07	3.91 ± 0.20	0.10 ± 0.03	2.30 ± 0.09	nd	2.94 ± 0.13	1.94 ± 0.13
Ellagic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Myricetin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cinnamic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Quercetin	20.18 ± 0.12	nd	nd	nd	nd	nd	nd	nd	nd	nd	19.14 ± 0.04	19.72 ± 0.18

^{a)} Values are means of triplicate determination ($n = 3$) ± SD.

^{b)} nd – not detectable and/or found in amount lower than quantification limit

^{c)} each value is the mean of three replicates ± RSD

the others (Fig. 6). It was also observed that red and white wines were grouped on the basis of their origin and kind of wine. The first two discriminant functions (roots) were effective in the discrimination between the wine types

(red, white and rose). The prediction capacity of the SLDA model was evaluated by the “leave-one-out” cross validation. Table 5 summarizes the results of the classification matrix of the LDA model obtained for all the samples

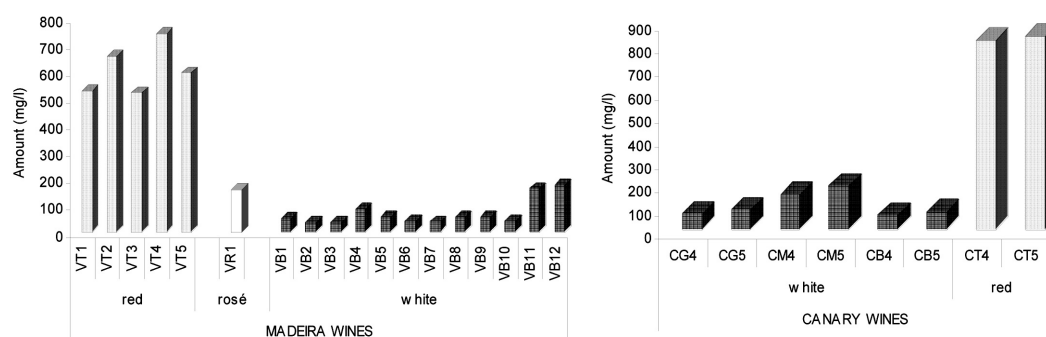


Figure 4. Total polyphenolic compounds in the studied wines.

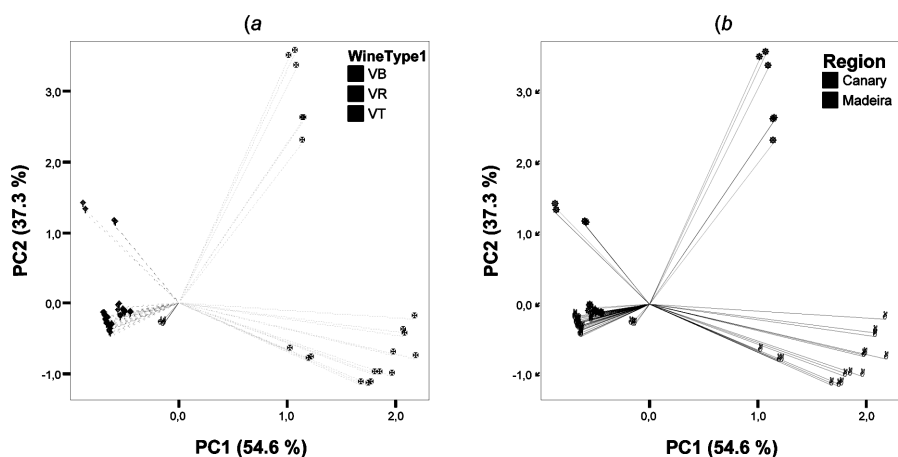


Figure 5. PC1 vs. PC2 scatter plot of the main sources of variability between different wines: (a) distinction according to kind of wine – VB: white wine; VR: rosé wine; VT: red wine; and (b) distinction according to geographic origin.

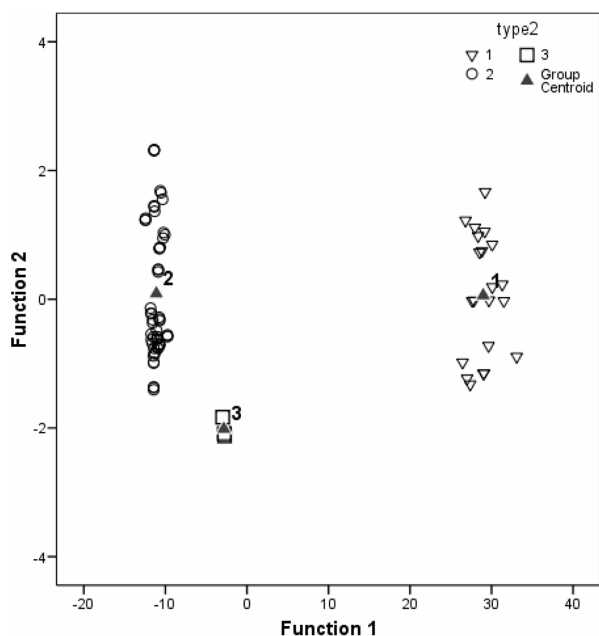


Figure 6. Projection of the wines obtained by linear discriminant analysis application. (1- red wines; 2- rose wines; 3- white wines)

according to wine kind, showing an average classification of 100%, which means that all the objects were correctly classified. Hence, the results can be considered sat-

isfactory and acceptable being the selected variables useful to classify and differentiate these wines according to wine type. This was a very interesting result since it means that origin had an influence on the phenolics analyzed.

4 Concluding remarks

In this study, we developed a chromatographic method for the identification and quantification of 17 low molecular-mass polyphenols compounds in different wine types. The proposed method showed enough separation to enable the quantification of these polyphenols although some peak overlapping was observed. The separation was achieved by RP-HPLC, coupled with UV-Vis detector.

The content of polyphenols determined in Canary red wines was, in average, 1.13 times higher than levels found in Madeira red wines. Since the phenolic content of wine, particularly catechins and proanthocyanidins, have been of interest due to their potential health benefits, this study should be of value to wine makers and consumers seeking high levels of these compounds. The validated method could be used as a quality control screening in different wine types.

Table 5. Prediction abilities using stepwise discriminant analysis (1- red wine; 2- white wine; 3- rose wine)

Classification results ^{a, b)}		Type 2	Predicted group membership			Total
			1.00	2.00	3.00	
Original	Count	1.00	21	0	0	21
		2.00	0	54	0	54
		3.00	0	0	3	3
	%	1.00	100	0	0	100
		2.00	0	100	0	100
		3.00	0	0	100	100
Cross-validated ^{c)}	Count	1.00	21	0	0	21
		2.00	0	54	0	54
		3.00	0	0	3	3
	%	1.00	100	0	0	100
		2.00	0	100	0	100
		3.00	0	0	100	100

a) 100.0% of original grouped cases correctly classified.

b) 100.0% of cross-validated grouped cases correctly classified.

c) Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

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