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Phenolic Content and Antioxidant Capacity of Cypriot Wines

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Abstract

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We characterised 12 wines (10 red, 1 sweet red, and 1 white) from different Cypriot cultivars in terms of their phenolic, anthocyanin composition, and antioxidant capacity as determined by two activities: radical scavenging and ferric reducing ability. Two different phenolic fractions (tannin-free and 1-kDa permeate) were also isolated from Cypriot wines with an ultimate goal of investigating their role in the overall antioxidant capacity of wines. The results indicated that Maratheftiko and Lefkada cultivars had higher concentrations of *o*-diphenols, hydroxycinnamic acid derivatives, flavonols and anthocyanins compared to other Cypriot cultivars like Ofthalmo, Mavro, and Giannoudi. The higher concentrations of phenols did not always correspond to a higher antioxidant capacity, probably due to the antagonistic action observed between hydroxycinnamic acid derivatives, flavonols, and anthocyanins. The latest interactions restricted the release of flavonols' advanced antiradical activity in wines.

Keywords: *o*-diphenols; hydroxycinnamic acid; flavonols; anthocyanins; antiradical activity

Phenols comprise a wide variety of compounds occurring in fruits and vegetables, whereas red grapes and wines are among the food products that contain an appreciable amount of them. In particular, wine phenolics belong to two main groups: nonflavonoids and flavonoids. The major nonflavonoid phenolic compounds are hydroxycinnamic acid derivatives of low molecular weight (0.15–0.35 kDa), while the most common flavonoids are flavan-3-ols (i.e. catechins), flavonols (i.e. other quercetin derivatives), and anthocyanins in red wines (Revilla *et al*. 2003; Ivanova *et al*. 2011). Anthocyanins represent basically monomers, but they can also exist as oligomers and polymers. Condensed flavanols are typically reported as proanthocyanidins or tannins (i.e. catechin–gallate polymers). Among the latest compounds found in grapes, procyanidins consist of (epi)catechin units (3',4' di-OH, R = H) and prodelphinidins are derived from (epi)gallocatechin moieties (3',4',5' tri-OH, R = OH) (Brossaud *et al*. 2001). Hydrolysable tannins such as gallotannin and ellagitannin can also be found in wines depending on the winemaking and ageing conditions (Obreque-Slíer *et al*. 2009). Besides, the phenolic profile of a wine has been shown to be influenced by different viticultural practices and enological techniques as well as the grapes variety, vintage, and region where they grow (Brossaud *et al*. 2001; Cliff *et al*. 2007). For instance, ripening and ageing conditions can affect hydroxycinnamic acids content (anthocyanin copigments) that plays an important role in colour stabilisation (Barrio-Galán *et al*. 2012; Giuffre 2013).

Phenols are considered as the main compounds responsible for the quality of grapes and, consequently, of the respective wines. For instance, it is well known that anthocyanins, flavonols, catechins, and other flavonoids contribute to the wine colour and astringency, while it has recently been demonstrated that they scavenge the excess radicals and mitigate oxidative stress. These properties contribute to the anticarcinogenic, antiatherogenic, antiinflammatory, antimicrobial, and antioxidant activities of wines (Llaudy *et al*. 2004; Chang *et al*. 2012; Xu *et al*. 2012). Red wines contain higher anthocyanin and phenolic contents compared to white ones and consequently possess a higher antiradi-

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cal efficiency (Tsai *et al.* 2004). The molecular size of phenolic compounds is another parameter affecting their corresponding bitterness and astringency (Brossaud *et al.* 2001). Thereby, monomers (flavan-3-ols and low molecular weight proanthocyanidins) are generally rather bitter than astringent, whereas the reverse is true for the higher molecular weight proanthocyanidins (Budić-Leto *et al.* 2008).

On the other hand, the impact of particular phenol classes and relative molecular fractions on the antioxidant properties of wines has not yet been adequately investigated. The relationship between the phenolic content and antioxidant properties of wine could be an emerging objective in current enological and functional foods research. In particular, the aim would be to understand which phenolic classes influence different antioxidant activities of wines and to what extent they affect them. This information is very useful not only in view of the health effects, wine ageing or preservation process, but also in regard to the recovery of target bioactive compounds and development of tailor-made applications as food preservatives (Galanakis 2012, 2013; Galanakis & Schieber 2014).

In our earlier research, we investigated the recovery of phenolic compounds from related sources (i.e. ethanolic extracts from winery sludge) and their fractionation into different classes by ultrafiltration (Galanakis *et al.* 2013; Galanakis 2015). The objectives of the current study were: (*i*) to analyse wines from different Cypriot cultivars and create a database in relation to their phenolic, anthocyanin content and antioxidant capacity, (*ii*) to isolate two different phenolic fractions (tannin-free and 1-kDa permeate) from Cypriot wines and investigate the extent to which their presence affects the overall antioxidant capacity of wines, and (*iii*) to find correlations between the wine compositional and antioxidant properties of different varieties. Correlation monitoring was conducted by determining total and particular (hydroxycinnamic acid derivatives, flavonols, and anthocyanins) phenol classes, while antioxidant capacity was detected by determining radical scavenging and ferric reducing ability of the samples.

MATERIAL AND METHODS

Materials. The reagents used were of analytical grade. Twelve wine samples (10 dry red, 1 sweet red, and 1 dry white) were purchased in triplicates from different producers as shown in Table 1.

Preparation of wine fractions

Tannin-free fraction. "Tannin-free" fraction of wines was collected according to the protocol described by Makkar (2003). Aliquots (1 ml) of each wine sample were placed together with 1 ml distilled water and 100 mg polyvinylpolypyrrolidone (PVPP) into a 100 × 12 mm test tube. If total phenolic content of wine was higher than 10% on a dry matter basis, the wine was appropriately diluted. The tubes were then vortex shaken and kept at 4°C for 15 minutes. Thereafter, the mixtures were centrifuged at 3000 *g* for 10 min and the supernatants (tannin-free fractions) were collected.

1 kDa-permeate fraction. Aliquots (600 ml) of each wine sample were diluted up to 3 l with distilled wa-

Table 1. Samples of wines from different Cypriot cultivars assayed in the current study

Wine sample	Brand name	Cultivar	Harvest season	Type
1	Stroumpeli	Lefkada	2008	dry red
2	Chatziantonas	Maratheftiko	2010	dry red
3	Zambartas	Maratheftiko	2011	dry red
4	Tychikos	Maratheftiko	2005	dry red
5	Stroumpeli	Maratheftiko	2008	dry red
6	Cypriot Department of Agriculture	Maratheftiko	2011	dry red
7	Nelion	Ofthalmo	2010	dry red
8	Cypriot Department of Agriculture	Ofthalmo	2011	dry red
9	Cypriot Department of Agriculture	Mavro	2011	dry red
10	Cypriot Department of Agriculture	Giannoudi	2011	dry red
11	Komandaria	Xinisteri-Mavro	2007	sweet red
12	Kamantarena	Xinisteri	2010	dry white

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ter. Thereafter, the diluted samples were processed in a cross-flow ultrafiltration module (DSS Labstak M10; Alfa Laval, Nakskov, Denmark). A composite fluoro polymer membrane (ETNA01PP; Alfa Laval, Nakskov, Denmark) of 1 kDa molecular weight cut off was used. The membranes were placed into the cross-flow module and pre-treated with de-ionised water as feed liquid (3 l) in order to avoid membrane compaction during UF experiments and wash out preservatives. The membrane were pressurised at 1, 2, 3, 4, 5, 4, 3, 2, and 1 bar in two sequential rounds of 15 minutes. The samples were processed in the membrane apparatus and operated at 5 bar for 60 min prior to the samples collection. Aliquots (50 ml) of feed and permeate samples were kept in the freezer (–20°C) until analysis. After the completion of each experiment, feed solutions were replaced with 10 g NaOH/l solution and treated with de-ionised water treated for 30 min at 5 bar in two sequential clean-up rounds. The temperature was kept constant at 25°C during the pretreatment, processing and cleaning.

Chemical analysis. The determination of phenolic compounds in the samples was conducted by using three colorimetric methods. Total phenols were determined using the Folin-Ciocalteau reagent and the protocol reported by Tsakona *et al.* (2012). An appropriately diluted sample was mixed with 0.25 ml Folin-Ciocalteau reagent. One ml of saturated sodium carbonate solution (35 g/100 ml) was added after 3 min stirring and the final solution was left in the dark for 1 hour. The absorbance of the solution was measured at 725 nm. A standard curve was prepared using 0–50 mg/l solutions of tannic acid in methanol/water. The standard solutions were prepared by several dilutions of the initial stock solution in water. The stock solution was prepared as follows: 1 g of tannic acid was solubilised in 100 ml of methanol and then 1 ml of the resulted solution was make up to 100 ml with water. Total phenol values were expressed in tannic acid equivalents (mg/l).

Phenols were classified in total, hydroxycinnamic acid derivatives, flavonoids, total anthocyanins, and *o*-diphenols were determined by adopting the protocols recorted by Galanakis *et al.* (2010b). One ml of the diluted ethanolic extract (1 : 10 in water) was mixed with 1 ml of HCl-ethanol solution (0.1 ml HCl/100 ml in 95 ml ethanol/100 ml) in a 10 ml volumetric flask and the volume was made up to 10 ml with 2 ml HCl/100 ml. After mixing, the absorbance was measured at 280, 320, 360, and 520 nm to determine total phenols, hydroxycinnamic acid derivatives, flavonols, and anthocyanins, respectively. The blank was prepared by mixing the HCl-ethanol solution with 2 ml HCl/100 ml. The corresponding standard curves to the above determinations were prepared using solutions (10 ml ethanol/100 ml water) of gallic acid (0–200 mg/l), caffeic acid (0–100 mg/l), quercetin (0–150 mg/l), and cyanidine chloride (0–100 mg/l), respectively.

Anthocyanins were classified in percentages of copigmented, monomeric and polymeric fractions according to the method described by Mazza *et al.* (1999). Initially, the pH of all wines was adjusted to 3.6 and then the wines were filtered with a 0.45 µm syringe filter. Then, 20 µl of 10 ml acetaldehyde/100 ml solution was added to 2 ml of the prepared wine. The samples were allowed to rest at room temperature for 45 min, and thereafter the absorbance at 520 nm was measured (A_{acet}). To another 2 ml of prepared wines, 260 µl of 5 g SO_2/100 ml solution was added, and A_{520} was measured (A_{SO_2}). The absorbance at 520 nm was measured of the prepared wine using a 1-mm cuvette and was multiplied by 10 to provide the value of A_{wine}. Different forms of anthocyanins were expressed in percentages as follows: % copigmented anthocyanins = $[(A_{acet} - A_{wine})/A_{acet}] \times 100$, % monomeric anthocyanins = $[(A_{wine} - A_{SO_2})/A_{wine}] \times 100$, and % polymeric anthocyanins = $[A_{SO_2}/A_{acet}] \times 100$. The concentrations of the anthocyanins fractions were calculated from the total content and the corresponding percentage of each of them.

Determination of antioxidant capacity. Antioxidant capacity was determined by following two different activities: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, and ferric reducing-antioxidant power (FRAP). DPPH radical scavenging activity of the wines and the corresponding fractions was performed according to the method described by Kulisic *et al.* (2004) and the protocol given by Galanakis *et al.* (2010a). 100 µl of several dilutions of each extract (1 : 5, 1 : 10, 1 : 20, 1 : 30, and 1 : 40) were vigorously mixed with 1.5 ml methanolic solution of DPPH radical (32 mg/l) in 2-ml plastic tubes. After 1 h, the absorbance at 517 nm of the resulting mixtures was measured against methanol, which was used to zero the absorbance. A blank solution of the DPPH radical without antioxidant was utilised as the control sample. The percentage inhibition of the DPPH radical by the samples was calculated according to the equation: % inhibition = $((A_{C(0)} - A_{A(t)})/A_{C(0)}) \times 100$, where: $A_{C(0)}$ – absorbance of the control at $t = 0$ min; $A_{A(t)}$ (mg DPPH/ml wine) – absorbance of the antioxidant

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at $t = 1$ hour. The results were expressed in EC_{50} values (mg DPPH/ml wine). The latest is the effective sample volume that resulted in 50% scavenging of DPPH radical. FRAP assay was conducted using the method described by Pantelidis *et al*. (2007). FRAP solution was freshly prepared by adding 10 mmol TPTZ/l and 40 mmol $FeCl_3 \cdot 10H_2O)$/l in 0.3 mol/l acetate buffer of pH = 3.6. 100 µl of the appropriately diluted sample was mixed with 3 ml of FRAP solution and incubated at 37°C for 4 minutes. Thereafter, the absorbance was measured at 593 nm. The results were expressed in µg TROLOX/ml wine using the standard curve.

Statistical analysis. All the wine preparations were carried out in duplicates and the mean ± standard deviation was calculated using the two corresponding variable values obtained with each of the conducted preparation. The data were statistically processed using students t-test (pair wise comparisons, Office Excel 2007) and significant differences between different samples were detected when the acceptable level of probability was ≤ 0.05 for all the comparisons.

RESULTS

Phenolic classes, anthocyanins, and antioxidant capacity of wines from several Cypriot cultivars. Table 2 represents the phenolic and anthocyanins characteristics as well as the corresponding antioxidant activities of Cypriot wines. As expected, Koumandaria (sweet red) and Kamantarena (white) wines showed values smaller by a factor 3 and 10, respectively, compared to the red wines. Maratheftiko and Lefkada wines of different seasons and producers showed in general the significantly highest values of total phenols (measured both at 725 and 280 nm), *o*-diphenols, hydroxycinnamic acid derivatives, and flavonols in comparison to the other cultivars (Mavro, Giannoudi, and Ofthalmo). An exception could be observed with Maratheftiko 2010, where the corresponding values were rather low. On the other hand, total anthocyanins content varied between the different red wine samples, i.e. the highest value was observed for cv. Maratheftiko wines harvested in 2011 (sample 6 and 3: 559 and 398 mg/l, respectively), while the lowest value was obtained for cv. Maratheftiko 2010 (sample 2: 75 mg/l). Expect for the Komandaria wine, all the other samples did not contain monomeric anthocyanins (less than 3% for red wines). The antioxidant capacity followed the tendency of total and particular phenol classes concentrations (the highest values observed for Maratheftiko and Lefkada wines), although the very low content of anthocyaninis in sample 3 resulted in relatively low values of scavenging activity (28 mg DPPH/ml) and ferric reducing-antioxidant power (466 µg TROLOX/ml).

The samples obtained from Cyprus Department of Agriculture (samples 6, 8, 9, and 10) were harvested in the same year (2011) and thereby they were herein monitored in order to observe the differences between the cvs Maratheftiko, Ofthalmo, Mavro, and Giannoudi, respectively. Thereby, cv. Maratheftiko (sample 6) showed the highest concentration of total phenols (both at 725 and 280 nm) and specific phenol classes, whereas cv. Ofthalo (sample 8) showed the lowest values. Likewise, the concentrations of flavonols and hydroxycinnamic acid derivatives decreased significantly in to the following order: Martheftiko (sample 6) > Giannoudi (sample 10) > Mavro (sample 9) > Ofthalmo (sample 8). Nevertheless, the scavenging and ferric reducing-antioxidant activities of cv. Maratheftiko (sample 6) were significantly higher only compared to cvs Giannoudi (sample 10) and Ofthalmo (sample 8), but were equal compared to cv. Mavro (sample 9). Indeed, the latter sample contained the lowest concentration (80 mg/l) of total anthocyanins compared to all the other samples of the season (6, 8, and 10).

Phenolic classes and respective antioxidant capacities of "tannins free" wine fractions. The concentrations of several phenolic classes and the corresponding antioxidant activities of Cypriot wines after the removal of the contained tannins are shown in Table 3. The removal of total phenols (both at 725 and 280 nm), hydroxycinnamic acid derivatives, flavonols, and anthocyanins was massive and almost quantitative (> 97% per wine) for all the assayed red wines. This loss of phenolic compounds resulted in more than 98% reduction of fractions processing the scavenging activity, while the reduction was a little lower in the case of ferric reducing-antioxidant power. For instance, the residual FRAP values of wines obtained from Cyprus Department of Agriculture in 2011 ranged between 6 and 11% of the initial activity. The loss of phenolic compounds and the respective antioxidant activities were even lower in the case of sample 12 since white wine is known not to contain tannins and anthocyanins.

Phenolic classes and respective antioxidant capacity of "1 kDa-permeate" wine fractions. Table 3 shows the concentrations of phenolic classes of wine fractions containing compounds passing through

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Table 2. Total phenolic content, anthocyanins composition and antioxidant efficacy of Cypriot wines

Sample No.	Phenolic content[1]						Anthocyanin composition[1]			Antioxidant capacity[1]	
	total[2] (725 nm)	*o*-diphenols[3] (517 nm)	total[4] (280 nm)	hydroxycin. acids[5] (320 nm)	flavonols[6] (360 nm)	anthocyanins[7] (520 nm)	monomeric (520 nm)	copigmented (520 nm)	polymeric (520 nm)	scavenging activity (mg DPPH/ml)	FRAP (µg TROLOX/ml)
	mg/l						%				
1	3804 ± 70^{a}	1843 ± 51^{a}	1766 ± 24^{ab}	368 ± 6^{a}	246 ± 4^{a}	113 ± 2^{a}	1 ± 1^{a}	74 ± 1^{a}	25 ± 1^{a}	61 ± 11^{ab}	744 ± 33^{a}
2	1930 ± 38^{b}	1061 ± 62^{b}	973 ± 13^{c}	305 ± 29^{b}	164 ± 8^{b}	75 ± 4^{b}	nd^{a}	10 ± 1^{b}	90 ± 1^{b}	28 ± 5^{c}	466 ± 21^{b}
3	2665 ± 23^{c}	1663 ± 40^{c}	1632 ± 64^{d}	351 ± 14^{a}	282 ± 5^{c}	398 ± 3^{c}	3 ± 2^{a}	68 ± 2^{c}	29 ± 2^{c}	54 ± 11^{abd}	719 ± 62^{acd}
4	4413 ± 85^{d}	2751 ± 147^{d}	1979 ± 254^{a}	405 ± 20^{c}	300 ± 11^{d}	126 ± 4^{d}	nd^{a}	26 ± 2^{d}	74 ± 2^{d}	63 ± 4^{a}	810 ± 47^{c}
5	3565 ± 82^{e}	1564 ± 167^{c}	1580 ± 83^{d}	265 ± 1^{d}	198 ± 10^{e}	94 ± 4^{e}	nd^{a}	14 ± 1^{e}	86 ± 1^{e}	50 ± 2^{b}	798 ± 33^{ac}
6	3437 ± 29^{e}	2186 ± 60^{e}	1861 ± 44^{b}	444 ± 12^{e}	369 ± 10^{f}	559 ± 12^{f}	nd^{a}	14 ± 1^{e}	86 ± 1^{e}	44 ± 3^{d}	786 ± 15^{c}
7	2033 ± 35^{f}	1109 ± 28^{b}	1000 ± 37^{ce}	247 ± 15^{f}	183 ± 7^{e}	100 ± 4^{e}	nd^{a}	33 ± 1^{f}	67 ± 1^{f}	31 ± 3^{c}	538 ± 30^{e}
8	2302 ± 110^{g}	1047 ± 99^{b}	1034 ± 31^{e}	121 ± 2^{g}	118 ± 3^{g}	163 ± 1^{g}	nd^{a}	40 ± 3^{g}	60 ± 3^{g}	38 ± 3^{e}	474 ± 11^{b}
9	2577 ± 121^{c}	1235 ± 145^{b}	1099 ± 44^{e,f}	184 ± 9^{h}	154 ± 6^{b}	80 ± 2^{b}	nd^{a}	50 ± ?^{h}	50 ± 1^{h}	40 ± 4de	806 ± 27^{c}
10	2212 ± 19^{g}	1053 ± 57^{b}	1155 ± 49^{f}	200 ± 9^{h}	166 ± 6^{b}	292 ± 10^{h}	nd^{a}	60 ± 1^{i}	40 ± 1^{i}	32 ± 2^{c}	647 ± 51^{d}
11	730 ± 49^{h}	413 ± 26^{f}	1008 ± 34^{ce}	87 ± 4^{i}	81 ± 3^{h}	19 ± 1^{i}	38 ± 2^{b}	13 ± 2^{j}	50 ± 2^{h}	6 ± 1^{f}	98 ± 26^{e}
12	224 ± 3^{i}	184 ± 13^{g}	208 ± 10^{g}	19 ± 4^{j}	30 ± 2^{i}	15 ± 1^{j}	6 ± 1^{c}	50 ± 1^{h}	43 ± 1^{j}	4 ± 1^{f}	95 ± 7^{e}

[1]values represent mean ± standard deviation (n = 3); values these possessing the same superscripted letters (at least one) within a column block are not significantly different ($P \leq 0.05$). [2]expressed as mg tannic acid/l of wine; [3]expressed as mg caffeic acid/l of wine; [4]expressed as mg gallic acid/l of wine; [5]hydroxycin. acids – hydroxycinnamic acid derivatives, results expressed as mg caffeic acid/l of wine; [6]expressed as mg quercetin/l of wine; [7]expressed as mg cyanidine chloride/l of wine

FRAP – Ferric Reducing Antioxidant Power; DPPH – 2, 2-diphenyl-1-picrylhydrazyl; TROLOX – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid nd – not detected

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Table 3. Total phenolic content and antioxidant efficacy of "tannins free"and "1 kDa-permeate" wine fractions

Sample No.	Phenolic content[1]										Antioxidant capacity[1]			
	total phenols[2] (725 nm)		total phenols[2] (280 nm)		hydroxycin. acids[5] (320 nm)		flavonols[5] (360 nm)		anthocyanins[7] (520 nm)		scavenging activity		FRAP	
	(mg/l)	(%)	(mg/l)	(%)	(mg/l)	(%)	(mg/l)	(%)	(mg/l)	(%)	(mg DPPH/ml)	(%)	(μg TROLOX/ml)	(%)
Tannins free														
1	39 ± 13^{ab}	1 ± 1^{a}	6 ± 1^{a}	nd^{a}	nd^{a}	nd^{a}	2 ± 1^{a}	nd^{a}	3 ± 1^{a}	1 ± 1^{a}	0.8 ± 0.2^{a}	1 ± 1^{a}	32 ± 1^{a}	4 ± 1^{a}
2	23 ± 4^{ac}	1 ± 1^{a}	6 ± 1^{a}	1 ± 1^{ab}	nd^{a}	nd^{a}	2 ± 1^{a}	1 ± 1^{ab}	3 ± 1^{a}	2 ± 1^{ab}	0.6 ± 0.1^{a}	2 ± 1^{a}	14 ± 3^{b}	3 ± 1^{a}
3	31 ± 6^{ad}	1 ± 1^{a}	11 ± 1^{b}	1 ± 1^{ab}	nd^{a}	nd^{a}	3 ± 1^{ab}	1 ± 1^{ab}	3 ± 1^{a}	2 ± 1^{ab}	0.8 ± 0.1^{a}	1 ± 1^{a}	52 ± 6^{c}	7 ± 1^{b}
4	47 ± 3^{b}	1 ± 1^{a}	29 ± 3^{c}	2 ± 1^{b}	1 ± 1^{ab}	nd^{a}	7 ± 2^{c}	2 ± 1^{b}	7 ± 1^{b}	3 ± 1^{b}	0.4 ± 0.1^{b}	1 ± 1^{a}	13 ± 1^{b}	2 ± 0^{c}
5	17 ± 6^{ce}	nd^{a}	11 ± 1^{b}	1 ± 1^{ab}	nd^{a}	nd^{a}	4 ± 1^{b}	2 ± 1^{b}	4 ± 1^{a}	2 ± 1^{ab}	0.6 ± 0.1^{a}	1 ± 1^{a}	31 ± 1^{a}	4 ± 1^{a}
6	51 ± 11^{b}	1 ± 1^{a}	17 ± 1^{d}	1 ± 1^{ab}	nd^{a}	nd^{a}	3 ± 1^{ab}	1 ± 1^{ab}	4 ± 1^{a}	nd^{a}	0.6 ± 0.1^{a}	1 ± 1^{a}	74 ± 2^{d}	9 ± 1^{d}
7	26 ± 7^{a}	1 ± 1^{a}	17 ± 1^{d}	2 ± 1^{b}	2 ± 1^{b}	1 ± 1^{a}	6 ± 1^{c}	3 ± 1^{b}	5 ± 1^{a}	3 ± 1^{b}	0.4 ± 0.1^{b}	1 ± 1^{a}	53 ± 4^{c}	10 ± 1^{d}
8	nd^{f}	nd^{a}	7 ± 1^{a}	1 ± 1^{ab}	nd^{a}	nd^{a}	2 ± 1^{a}	2 ± 1^{b}	3 ± 1^{a}	1 ± 1^{a}	0.4 ± 0.2^{ab}	1 ± 1^{a}	50 ± 7^{ce}	11 ± 1^{d}
9	43 ± 7^{bd}	2 ± 1^{a}	11 ± 1^{b}	1 ± 1^{ab}	nd^{a}	nd^{a}	3 ± 1^{ab}	2 ± 1^{b}	3 ± 1^{a}	2 ± 1^{ab}	0.1 ± 0.1^{c}	nd^{a}	44 ± 1^{e}	6 ± 1^{b}
10	22 ± 1^{c}	1 ± 1^{a}	17 ± 1^{d}	1 ± 1^{ab}	nd^{a}	nd^{a}	4 ± 1^{b}	2 ± 1^{b}	4 ± 1^{a}	1 ± 1^{a}	0.2 ± 0.1^{c}	1 ± 1^{a}	70 ± 2^{d}	11 ± 1^{d}
11	29 ± 1^{a}	4 ± 1^{b}	6 ± 1^{a}	1 ± 1^{ab}	nd^{a}	nd^{a}	3 ± 1^{ab}	2 ± 2^{ab}	3 ± 1^{a}	7 ± 2^{c}	0.5 ± 0.1^{ab}	9 ± 2^{b}	9 ± 3^{f}	9 ± 1^{d}
12	13 ± 5^{e}	nd^{a}	6 ± 1^{a}	3 ± 1^{b}	nd^{a}	nd^{a}	2 ± 1^{a}	10 ± 1^{c}	3 ± 1^{a}	11 ± 1^{d}	0.5 ± 0.1^{ab}	13 ± 2^{c}	15 ± 2^{b}	16 ± 1^{e}
1 kDa-permeate														
1	505 ± 10^{ab}	13 ± 1^{a}	1002 ± 12^{a}	57 ± 1^{a}	208 ± 4^{a}	56 ± 2^{a}	188 ± 7^{a}	77 ± 4^{a}	80 ± 5^{a}	71 ± 6^{ab}	29 ± 5^{a}	47 ± 4^{ab}	174 ± 2^{a}	23 ± 1^{ab}
2	592 ± 9^{c}	31 ± 1^{b}	509 ± 6^{b}	52 ± 2^{b}	116 ± 9^{b}	38 ± 3^{b}	112 ± 5^{b}	68 ± 4^{b}	57 ± 2^{b}	77 ± 2^{a}	23 ± 4^{a}	83 ± 5^{c}	155 ± 1^{b}	33 ± 1^{c}
3	477 ± 7^{d}	18 ± 1^{c}	854 ± 19^{c}	52 ± 3^{b}	205 ± 8^{a}	58 ± 3^{a}	222 ± 7^{c}	79 ± 3^{a}	116 ± 1^{c}	29 ± 1^{b}	29 ± 2^{a}	54 ± 7^{a}	139 ± 4^{c}	19 ± 3^{d}
4	511 ± 14^{b}	12 ± 1^{a}	1002 ± 17^{a}	51 ± 8^{abc}	190 ± 10^{a}	47 ± 2^{c}	181 ± 11^{a}	60 ± 3^{c}	72 ± 3^{d}	57 ± 2^{c}	27 ± 2^{a}	43 ± 2^{b}	194 ± 10^{d}	24 ± 1^{b}
5	818 ± 26^{e}	23 ± 1^{d}	719 ± 11^{d}	46 ± 2^{c}	139 ± 8^{c}	53 ± 3^{a}	129 ± 6^{d}	65 ± 3^{c}	74 ± 1^{d}	79 ± 3^{a}	37 ± 3^{b}	76 ± 8^{c}	273 ± 5^{e}	34 ± 2^{c}
6	787 ± 11^{e}	23 ± 1^{d}	1358 ± 100^{e}	73 ± 2^{d}	253 ± 2^{d}	57 ± 2^{a}	337 ± 4^{e}	91 ± 3^{d}	376 ± 4^{e}	67 ± 2^{b}	35 ± 3^{b}	80 ± 7^{c}	195 ± 3^{d}	25 ± 1^{b}
7	298 ± 7^{g}	15 ± 1^{e}	387 ± 52^{f}	39 ± 5^{e}	131 ± 3^{e}	53 ± 3^{a}	126 ± 4^{d}	69 ± 5^{b}	64 ± 1^{f}	64 ± 2^{b}	18 ± 4^{c}	58 ± 9^{a}	83 ± 1^{f}	15 ± 1^{e}
8	239 ± 16^{g}	10 ± 1^{f}	364 ± 4^{f}	35 ± 1^{f}	57 ± 3^{f}	47 ± 2^{c}	104 ± 2^{f}	88 ± 4^{d}	74 ± 2^{d}	45 ± 1^{d}	20 ± 1^{c}	53 ± 7^{a}	103 ± 1^{g}	22 ± 1^{a}
9	666 ± 26^{h}	26 ± 2^{g}	527 ± 14^{b}	48 ± 2^{c}	91 ± 9^{g}	49 ± 2^{c}	118 ± 24^{bdf}	77 ± 3^{a}	51 ± 2^{g}	65 ± 2^{b}	36 ± 6^{b}	89 ± 10^{c}	165 ± 19^{ab}	20 ± 2^{a}
10	490 ± 7^{a}	22 ± 1^{d}	847 ± 22^{c}	73 ± 5^{d}	106 ± 5^{b}	53 ± 5^{a}	137 ± 3^{g}	83 ± 4^{ad}	168 ± 1^{h}	58 ± 2^{c}	24 ± 4^{a}	77 ± 12^{c}	145 ± 1^{c}	23 ± 2^{ab}
11	253 ± 19^{i}	35 ± 3^{h}	428 ± 60^{f}	42 ± 7^{c}	29 ± 7^{h}	33 ± 7^{b}	61 ± 2^{h}	75 ± 3^{a}	14 ± 1^{i}	74 ± 8^{ab}	4 ± 1^{d}	69 ± 13^{c}	38 ± 3^{h}	40 ± 8^{ce}
12	206 ± 6^{j}	77 ± 3^{i}	600 ± 68^{g}	53 ± 6^{ab}	122 ± 20^{be}	37 ± 7^{a}	107 ± 21^{bdf}	51 ± 9^{e}	79 ± 4^{a}	72 ± 6^{ab}	3 ± 1^{d}	81 ± 16^{c}	41 ± 1^{h}	3 ± 4^{e}

[1]values represent mean ± standard deviation (n = 3); values these possessing the same superscripted letters (at least one) within a column block are not significantly different ($P \leq 0.05$); [2]expressed as mg tannic acid/l of wine; [3]expressed as mg gallic acid/l of wine; [4]hydroxycin. acids – hydroxycinnamic acid derivatives, results expressed as mg caffeic acid/l of wine; [5]expressed as mg quercetin/l of wine; [6]expressed as mg cyanidine chloride/l of wine

FRAP – Ferric Reducing Antioxidant Power; DPPH – 2, 2-diphenyl-1-picrylhydrazyl (results expressed as mg DPPH/l of wine); TROLOX – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; nd – not detected

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the 1 kDa-membrane as well as the corresponding antioxidant properties. The removal of total phenols (determined at 725 nm) was rather high (10–31%) for all the red wines assayed. On the other hand, the removal of total phenols (determined at 280 nm), hydroxycinnamic acid derivatives and anthocyanins was moderate (35–73, 38–53, and 45–77%, respectively). An exception was observed with cv. Zambartas wine (sample 3) that retained importantly corresponding anthocyanins (29%) in the concentrate. Besides, the removal of flavonols from the red samples was rather low (69–88%) and in some cases (i.e. cv. Marathertiko 2011, sample 6) almost negligible (91%). Sweet red (sample 11) and white (sample 12) wines showed gradual lower removal of total phenols (35 and 77%, respectively), but a higher retention of hydroxycinnamic acid derivatives (33–37%).

Scavenging activity of red wines could be categorised into two groups. Half of the red wines (samples 1, 3, 4, 7, and 8) followed the tendency of total phenols (determined at 280 nm), hydroxycinnamic acid derivatives, and anthocyanins by showing moderate retention in the permeate stream (43–58%). The second group of red wines (samples 2, 6, 7, 9, and 10) followed the tendency of flavonols and showed a lower removal (76–89%) of the scavenging activity. The last values were not significantly different between one another. Nevertheless, the corresponding FRAP loss was relatively high (15–33%) with all the assayed samples, following the tendency of total phenols at 725 nm. Again, the antioxidant capacity (for both parameters tested) showed gradually higher loss for sweet red and white wines.

With regard to the different cultivars of Agriculture Cyprus Department wines, cv. Maratheftiko (sample 6) showed again the highest concentration of total phenols (for both parameters) and particular phenol classes in the 1-kDa permeates, whereas cv. Ofthalmo (sample 8) showed the lowest one. The same exception was observed as in the case of whole wine samples since the respective permeate of sample 9 showed again the lowest concentration of total anthocyanins. Besides, total phenols (determined at 280 nm), hydroxycinnamic acid derivatives and flavonols concentrations of the permeates decreased significantly in the already observed order: Martheftiko (sample 6) > Giannoudi (sample 10) > Mavro (sample 9) > Ofthalmo (sample 8). Nevertheless, the antioxidant capacity value (both parameters) of cv. Maratheftiko (sample 6) permeate was generally higher compared to those of cvs Giannoudi, Ofthalmo, and Mavro (samples 10, 8 and 9, respectively). An exception was observed for the latter sample that showed a higher but not significantly different scavenging activity compared to Maratheftiko wine. Finally, the determination of *o*-diphenols and anthocyanins concentrations was not possible for both "tannins free" and "1 kDa-permeate" wine fractions due to their negligible concentration in the first case, and the relatively low concentration of the 6-fold diluted and ultrafiltrated samples in the second.

DISCUSSION

The overall antioxidant status of extracts or substrates like wine should be based on the estimation of both total phenols and particular fractions, since the overall efficacy may be defined by the contribution of the most active compounds and their respective amounts (Makris *et al.* 2007; Tsakona *et al.* 2012). Different phenolic compounds are able to donate hydrogen atoms, scavenge hydroxyl radicals and contribute to the redox reactions (Arnous *et al.* 2002). For instance, monomeric and polymeric anthocyanins are specifically associated with FRAP and DPPH scavenging activities, respectively (Tsai *et al.* 2004). Copigmentation of anthocyanins (i.e. malvidin-3-glucoside) with hydroxycinnimic acid derivatives (i.e. coumaric acid) and *o*-diphenols (i.e. caffeic acid) is known to increase both activities (Azevedo *et al.* 2011). On the other hand, *o*-diphenols or respective structures of flavonoids are known to possess advanced radical scavenging properties compared to the rest of phenolic classes (Chen *et al.* 1997), while flavanols have been reported to contribute more to the wine reducing ability compared to anthocyanins (Arnous *et al.* 2002).

The results of the current study verified that the highest concentrations of *o*-diphenols, hydroxycinnamic acid derivatives, flavonols, and anthocyanins in wine samples (i.e. Maratheftiko and Lefkada) corresponded in general to the higher antioxidant capacity with both activities assayed, although the latter values were not always significantly different. Indeed, the wines showed a balance between FRAP and radical scavenging activities, despite the fact that they contained only polymeric and copigmented anthocyanins (except for sample 11). This result implies that the rest of phenolic classes (copigmented or not with anthocyanins) contributed to the FRAP activity of the wines. Moreover, the composition of monomeric,

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copigmented, and polymeric anthocyanins seems not to play any important role in the overall antioxidant capacity of wine, as their concentrations were either very low or negligible compared to the rest of phenolic classes. A similar balance between FRAP and radical scavenging activities has been reported to exist in Moravian and Austrian wines (Soyollkham *et al*. 2011). Likewise, the assayed white wine (sample 12) possessed about one 5th to one 15th of the antiradical and reducing activities present in red wines (samples 1–10). This result is in accordance with a previous study reporting that the average FRAP value of red wines was almost 10-fold higher compared to those of white wines (Katalinić *et al*. 2004).

As already mentioned, Martheftiko wine was shown to contain the highest concentrations of both particular and total phenols among the different varieties of Cypriot Department of Agriculture (2011), whereas cv. Ofthalmo possessed the lowest one. Nevertheless, the higher concentrations of phenols did not always reflect significantly higher antioxidant capacity. In other words, not only the quantity, but also the final phenolic composition of wines is important, since phenols such as flavonoids can have either synergistic or antagonistic effect on the overall antioxidant capacity (Hidalgo *et al*. 2010). For instance, Maratheftiko wine (sample 6) possessed about 2-fold higher concentration of *o*-diphenols, hydroxycinnamic acid derivatives, and flavonols, 5-fold higher concentration of anthocyanins, but not significantly different radical scavenging and FRAP activities compared to Mavro wine (sample 9). This outcome could be attributed to an antagonistic impact of anthocyanins in Maratheftiko wine, as in the case of cv. Mavro their very low concentration (80 mg/l) resulted in a higher antioxidant capacity. The same conclusion can be also obtained by comparing the phenolic profiles of samples 3 and 9. On the other hand, although sample 9 did not possess significantly different concentrations of phenolic classes as compared to sample 2 (Table 2), it showed almost 2-fold higher FRAP activity. This result could be related to the higher concentration of total phenols, determined by Folin-Ciocalteau method. Besides, the latest method is known to determine not only phenols, but also other low molecular weight-reducing compounds of the samples (Galanakis *et al*. 2010a,b).

In addition, a higher concentration of *o*-diphenols seems to provide advanced radical scavenging activity, as seen by comparing phenolic profiles of samples 9 and 5. This hypothesis is stengthened by the fact that the wines containing a higher concentration of *o*-diphenols in combination with a low concentration of anthocyanins (samples 1 and 4) showed the highest radical scavenging activity (61 ± 1 mg DPPH/ml) among all the wine samples. However, this was not observed in the case of sample 6 (44 mg DPPH/ml), probably due to the simultaneous occurrence of high *o*-diphenols, flavonols, and anthocyanins contents (2186, 369, and 559 mg/l, respectively). The antagonistic effect of other phenolic compounds against anthocyanins in terms of radical scavenging activity has been also referred by other researchers (Rivero-Pérez *et al*. 2008). The antagonistic effect of phenolic antioxidants could occur as a result of synergism in autoxidation, i.e. a phenolic compound with a lower oxidation potential acts as a hydrogen donor in the regeneration of the former phenol from its phenoxy radical (Ohkatsu & Suzuki 2011).

The impact of different phenolic classes on the overall antioxidant capacity of wines can be verified by observing the activities of the recovered fractions (Table 3). PVPP is a fining agent used in winemaking technology to control browning (Baron *et al*. 2000), while its addition has been reported to reduce importantly both non-flavonoid and flavonoid concentrations of wines (Castillo-Sánchez *et al*. 2008; Cosme *et al*. 2012). Particularly, it binds phenols by hydrogen bonding between the PVPP-carbonyl groups with the phenolic-hydroxyl groups of both fractions (Laborde *et al*. 2006). In analytical chemistry, PVPP is generally used as an index to identify the proportion of tannin-bound anthocyanins after passing wine extracts through a column filled with PVPP (Pérez-Lamela *et al*. 2007). In the current study, PVPP was used to remove tannins according to Makkar (2003). In practice, it removed dramatically (> 97%) both total (determined at 280 nm) and particular phenolic classes (including flavonols) of red wines. This process resulted in a quantitative loss of radical scavenging activity (> 98%) and a bit lower reducting of FRAP activity (89–98%). The residual antioxidant efficacy of the "tannin-free" fractions could be attributed to any remaining glucosides of the flavonoid fraction, as it is known that the sugar moiety removes the driving forces and linkage with PVPP, i.e. contrarily to phenolic aglycons (Laborde *et al*. 2006).

The contribution of phenolic compounds to wines antioxidant capacity as a function of their molecular weight was estimated using a membrane of 1 kDa

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molecular weight cut off. This barrier has recently been reported by our group to partially separate hydroxycinnamic acid derivatives from anthocyanins and flavonols in diluted and concentrated extracts derived from winery sludge (Galanakis *et al.* 2013). The diluted extract contained ~351 mg total phenols/l, while the concentrated one contained 1446 mg/l, both determined with Folin-Ciocalteau reagent. Since the wine samples of the current study were diluted 6-fold prior to the membrane treatment, the corresponding initial feed solutions contained total phenol amount between 300 and 700 mg/l (determined at 725 nm). This concentration variation is close to the diluted extract of the previous study and thus hydroxycinnamic acid derivatives were herein expected to be partially removed (twice as much compared to anthocyanins) from the respective 1 kDa-permeates. However, this was not the case since only two samples (2 and 11) followed the above tendency. Indeed, other samples (2, 8, 10, and 11) followed the tendency of the more concentrated sample of the previous study by removing hydroxycinnamic acid derivatives in contrast to flavonols.

The above tendencies generated a completely different phenolic profile of 1 kDa-permeates compared to the initial wine samples. Nevertheless, the concentration of flavonols in the permeate samples was generally enhanced since they were retained in rather high percentages (60–91%). This fact seems to contribute to the relatively high retention of radical scavenging activity observed in the permeate samples, which in some cases (samples 2, 6, and 9) reached up to 80–91% of the initial activity of the wine samples. The latest observation is rather impressive taking into count the rather high removal of total phenols (determined at 725 nm), which was followed by an important removal of hydroxycinnamic acid derivatives. This result implies that the low molecular weight flavonols of red wines possess advanced antiradical properties. Moreover, it verifies that other phenols (i.e. hydroxycinnamic acid derivatives or anthocyanins) act antagonistically against flavonols in spite of the overall wine radical scavenging ability. The result of the current study is in contrast with the results of Brahmi *et al.* (2012), as these reported a synergistic action of flavonoids with other phenolics, however, their study concerned interactions in olive leaf extracts.

With regard to the FRAP activities, the permeates retained them in low percentages (15–34%) as compared to the initial values in red wine samples. In addition, FRAP activities followed the removal of total phenols (10–31% per red wine) in contrast to particular phenol classes (29–91%). This fact indicates that other non-phenolic compounds (measured by Folin-Ciocalteau method) may contribute to the overall reducing potential of wines. On the other hand, the reduction of FRAP activity could be eventually related to the removal of *o*-diphenols, whose concentration in diluted permeate stream was too low to be determined. Finally, as concerns the different wines of Cypriot Department of Agriculture (2011), the 1 kDa-permeates of cvs Maratheftiko and Ofthalmo showed again the highest and lowest concentrations, respectively, of both total and particular phenols, and this time the antioxidant capacity followed the same tendency. This result implies the absence of antagonistic interactions between different phenol classes, probably due to the fact that permeates contained low concentrations of particular phenols compared to the initial wine samples. Another explanation could be the lower molecular size of phenols that simplifies their structure while thus restricts the numerous interactions and complexes occur between different phenolics during winemaking and ageing (Zafrila *et al.* 2003; Ivanova *et al.* 2011).

CONCLUSION AND PERSPECTIVES

The outcomes of the current study can be summarised in the following remarks:

– Cvs Maratheftiko and Lefkada were shown to generate wines with generally higher concentrations of *o*-diphenols, hydroxycinnamic acid derivatives, flavonols, and anthocyanins compared to other Cypriot cultivars like Ofthalmo, Mavro, and Giannoudi.
– The higher concentrations of phenols did not always reflect higher antioxidant capacity of wines, probably due to the observed antagonistic effect between hydroxycinnamic acid derivatives, flavonols, and anthocyanins.
– Other non-phenolic, low molecular weight, reducing compounds (determined by Folin-Ciocalteau method) may also contribute to the antioxidant capacity of wines.
– Low molecular weight (< 1 kDa) flavonols seem to have advanced radical scavenging activity, which is restricted by the antagonistic action of the rest of phenolic classes present in wines.

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Finally, the results of our two latest studies (including Galanakis *et al*. 2013) allow the valorisation of grape derivatives and by-products as substrates for the recovery of phenolic mixtures in crude extracts, possessing antioxidant capacity as high as in wine. In addition, the proposed recovery methodology is based on the fractionation of hydroxycinnamic acid derivatives, flavonols, and anthocyanins using a fluoro polymer membrane (1 kDa) in the edge of ultrafiltration with nanofiltration. The latest membrane allows a selective enhancement of the concentrate with hydroxycinnamic acid derivatives and increases the content of flavonols and anthocyanins mixtures in the permeate. The latest process removes the antagonistic impact of different phenolic classes as regards their antiradical properties and subsequently releases the bioactivity of low molecular weight flavonols. Ultimately, the enriched mixtures of flavonols and anthocyanins could be utilised as flavorings and colorants with a high antioxidant potency.

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