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Bioactive non-coloured polyphenols content of grapes, wines and vinification by-products: Evaluation of the antioxidant activities of their extracts

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ABSTRACT

The comparative quantization of main non-coloured polyphenols, the assessment of total polyphenolic content (TPC) and the detailed evaluation of the antioxidant activities of various grape-based products – from the harvest stage up to the production of the corresponding wines – are presented. The material studied consisted of grape tissues (berries, seeds and skins) of native Greek *Vitis vinifera* cultivars, which provided polyphenol-rich extracts via an optimized ultrasound extraction procedure, while the respective wine samples were condensed by a novel extraction procedure using XAD-4 adsorption resin column. The extraction methods accuracies were thoroughly validated and the polyphenolic content of extracts was assessed by HPLC–DAD and photometric methods. Their antioxidant properties were evaluated by following assays, modified to fit into a high throughput approach: DPPH· radical scavenging, FRAP, inhibition of $CuSO_4$-induced LDL oxidation and the reduction of intracellular reactive oxygen species (ROS) in smooth muscle cell cultures. Seed samples exhibited the highest TPC values, which are well correlated with their significant antioxidant properties in all assays performed. Of special interest is the significant capability of the tested extracts to prevent the LDL oxidation at very low concentrations. Furthermore, the good correlation between the antioxidant activities assessed for the LDL oxidation inhibition and the intracellular ROS assays is indicative of the possible *in vivo* antioxidant properties of the extracts. Results herein reveal the considerable antioxidant potential of the Greek grapevine production and exploits their vinification by-products as a potential inexpensive source of high added value antioxidants.

1. Introduction

Grapevine is a thermophilic plant cultivated in the temperate zone practically all over the world. According to the "Food and Agriculture Organization (FAO)", global grape production in 2004 was 65.4 million tones of which approximately 71% was used for winemaking (www.FAO.org). The larger production occurs in Europe, accounting annually more than 44 million tones.

Grapes contain large amounts of phytochemicals which accounts for most of the sensory characteristics of the respective wines, such as colour, aroma, browning and astringency (Waterhouse, 2002) and/or their health beneficial properties (German & Walzem, 2003). The most studied group of grape phytochemicals are polyphenols, a group of secondary metabolites with diverse chemical structures–functions, which are being produced during the physiological plant growth process and/or as a response to various forms of environmental stress (Naczk & Shahidi, 2004). Their biological activities have been extensively studied during the last decades, providing strong evidences of their potential health benefits. The latter are mainly attributed to their strong antioxidant properties, since they can act as free-radical scavengers, electron or hydrogen donors and strong metal chelators, thus preventing the lipid peroxidation, DNA damage, etc. (Afanasev, Dorozhko, Brodskii, Kostyuk, & Potapovitch, 1989; Blokhina, Virolainen, & Fagerstedt, 2003). Moreover, as antioxidants, polyphenols protect cell constituents against oxidative damage and, therefore, limit the risk of developing degenerative diseases associated to oxidative stress, such as the Alzheimer disease. Finally, several studies on animal models have shown that, the incorporation of polyphenols into the diet, limits the possibility to develop cancer, cardiovascular diseases, neurodegenerative diseases, diabetes, and osteoporosis (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005).

Grape and wine polyphenols are mainly flavonoids (anthocyanins, flavonols and flavanols), stilbenes and phenolic acids, all well

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known for their strong biological actions (Monagas, Bartolome, & Gomez-Cordoves, 2005). Among them, the non-coloured polyphenols have attracted considerable research attention due to their ease of application by pharmaceutical, cosmetic and food industries and their strong *in vitro* and *in vivo* biological properties. Among them, *trans*-resveratrol constitutes the most studied compound (Hiroyuki et al., 2001; Jang et al., 1997; King, Bomser, & Min, 2006; Russo et al., 2003), while during the last decade various clinical trials have indicated that several non-coloured polyphenols such as phenolic acids (both hydroxybenzoic and hydroxicinnamic acids), flavonoids (catechin, quercetin, myricetin, kaempferol) and other polyphenols (epigallocatechins) are potent inducers of apoptosis in cancer cells. (Knekt et al., 1997; Soleas, Grassc, Josephy, Goldberg, & Diamandis, 2002; Weyant, Carothers, Dannenberg, & Bertagnolli, 2001). Thus, a vigorous interest has been initiated towards the exploitation of non-coloured polyphenol-rich extracts.

In this work we present the recovery and study of the principal non-coloured bioactive polyphenols of four native Greek varieties of *Vitis vinifera*. In this respect, we have used two approaches; one concerning an optimized ultrasound procedure combined with liquid–liquid extraction for the production of rich extracts of non-coloured polyphenols from the solid parts of the plant (berries, skins, seeds, pomace and stems) and a novel extraction procedure using XAD-4 adsorption resin column for their recovery from wines. This resin was selected because its chemical structure favors the adsorption – through weak interactions – of molecules bearing high electron density groups, such as aromatic rings, while sugars or polar lipids cannot establish similar interactions. Thus, the latter are being eluted with the water flow during the rinsing phase. The adsorbed polyphenolic compounds are recovered via EtOH elution to produce a polyphenol enriched extract.

All the grape varieties studied are cultivated in the island of Santorini, an isolated geographical area which is characterized by a unique volcano peculiar clima producing famous wines with appellation of origin names. Although there are several studies on the polyphenolic content of Greek wines, there are only limited reports on Santorini wines, concerning mainly the Mandilaria red variety, and no reports for the Aidani white variety. Final goal is to contribute to the vineyard products knowledge through the comparable-comprehensive study of different grape berries tissues (whole berry, skin and seeds) with their corresponding wines. The antioxidant activities of all samples were assessed by four different *in vitro* assays and the results obtained were correlated with their total polyphenolic content.

2. Materials and methods

2.1. Grapes and wines

The samples studied were wines and their respective grape berries and seeds, obtained from the most representative native red (Mandilaria and Voidomatis) and white (Asyrtiko and Aidani) varieties of *V. vinifera* species, cultivated in the restricted area of the Greek island complex Cyclades. All samples were collected during the 2006 harvest at their commercial maturity from the Santorini island and the local cooperative winery. Grape berries and skins were manually destemmed and unseeded, weighed, freeze dried, mill-powdered, and stored in freezer. Seeds were directly obtained by manual separation. These samples were air dried, mill-powdered and stored at room temperature.

2.2. Reagents and standards

All solvents were purchased from Baker as analytical (polyphenol extraction) or HPLC (chromatographic analyses) grades. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, while all HPLC-solvents were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size.

Calibration curves were constructed for the following polyphenols: gallic acid, (+)-catechin, (−)-epicatechin, procyanidin B2, epicatechin gallate, *p*-coumaric acid, ferulic acid, caffeic acid, syringic acid, vanillic acid, kaempferol, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, and *trans*-resveratrol (all obtained from Sigma–Aldrich). Procyanidin B3, *trans*-caftaric acid and ε-viniferin, were isolated with preparative HPLC.

The following reagents were used for the antioxidant assays: Diphenyl-1-picrylhydrazyl (DPPH) radical, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), $FeCl_3{\cdot}6H_2O$, CH_3COONa, HCl 37% ACS reagent, lipoprotein low density from human plasma (LDL), 2-thiobarbituric acid (TBA), phosphate buffered saline (PBS) pH 7.4, 2,2(thienylmethylene)malonaldehyde (MDA), $CuSO_4$ anydrous powder, dimethyl sulfoxide (DMSO) anhydrous, Na_2CO_3 anhydrous powder (Sigma–Aldrich), $FeSO_4$ (Riedel de Haén), CH_3COOH (Baker), Trichloroacetic acid (TCA) Biochemica Ultra, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Folin & Ciocalteu's phenol reagent (Fluka).

All isolated compounds were unambiguously characterized based on their physical and spectral data, as previously described (Anastasiadi, Chorianopoulos, Nychas, & Haroutounian, 2009a).

The XAD-4 resin used for the production of wine extracts was purchased from Rhom & Hass Co.

2.3. Sample preparation and extraction

Wine samples were prepared using an ion exchange resin according to the following process: 150 mL of wine were diluted with water to 6% alcoholic volume and the solution was eluted through a XAD-4 resin column, which has the ability to retain selectively the polyphenols. Then, the column was washed with deionised water (to remove sugars and water soluble antocyanines), dried under a N_2 stream and the polyphenols were recovered by eluting the column with 60 mL of ethanol and evaporation of the eluted solvent under vacuum. The remaining solid was weighed and dissolved in Methanol (MeOH) to 1 mg/mL, membrane filtered (0.45 μm) and subjected to HPLC analysis.

The solid samples were prepared from dried material (powdered grape berries, skins, seeds) as follows: 10 g of dried sample were poured into 50 mL mixture of $MeOH/H_2O/HCl$ 1.0 N (90:9.5:0.5 v/v) and sonicated in an ultrasonic bath for 10 min. The solvent was separated by filtration and the remaining solid was re-extracted for three additional times. The combined extracts were evaporated under vacuum and redissolved in 30 mL of $MeOH/H_2O$ (1:1) and centrifuged for 10 min (7000 rpm). The supernatant liquid was extracted with Petroleum Ether (3×30 mL) to remove the lipids and concentrated under vacuum. The remaining residue was poured into 30 mL of brine and extracted repetitively with Ethyl Acetate (EtOAc, 4×30 mL). The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under vacuum. The remaining solid was weighed and dissolved in MeOH to 1 mg/mL, membrane filtered (0.45 μm) and subjected to HPLC analysis.

To avoid the polyphenols degradation, all the aforementioned activities were performed in the absence of direct sunlight and temperatures below 35 °C.

2.4. HPLC analysis

All HPLC analyses were carried out on a Thermo Finnigan 3000 system equipped with quaternary pump, auto-sampler, degasser and diode array detector (DAD). The column used was a

Lichrosphere C_{18} column (250 mm × 4.1 mm, particle size 5 μm) with a guard column of the same material (8 × 4 mm). Injection was by means of a Rheodyne injection valve (model 7725I) with a 20 μL fixed loop. Chromatographic data were acquired and processed using the ChromQuest Version 4.1 software.

The HPLC analysis was performed using the modified chromatographic method reported previously in our laboratory (Anastasiadi et al., 2009a). Briefly, polyphenols were separated on a Lichrosphere C18 column (250 mm × 4.1 mm, particle size 5 μm) and a C_{18} guard column of the same type. Mobile phase consisted of solvent A (2 mM sodium acetate aqueous solution with 3% v.v acetic acid) and solvent B (ACN). Run time was 70 min and the flow was 1.0 mL/min. The injection volume was 20 μL and polyphenols were eluted using a gradient system. The analysis was monitored at 280, 320 and 360 nm simultaneously. Peaks were identified by comparing their retention time and UV–Vis spectra with reference compounds and data were quantified using the corresponding curves of the reference compounds as standards. All standards were dissolved in synthetic wine matrix consisting of a H_2O/EtOH (85:15) solution with 0.3% w.v. tartaric acid. Results were expressed in mg/L of wine.

Peaks were identified by comparing their retention time and UV–Vis spectra with the reference compounds and data were quantitated using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol. Results were expressed as mg/100 g fresh grape berries, mg/L for wine samples and mg/100 dry matter for vinification by-products.

2.5. Total phenolic content (TPC)

The concentration of total soluble phenolics in the sample extracts were determined via a modified version of the Folin–Ciocalteu method (Singleton & Rossi, 1965), applied in 96-well microplates. Stock solutions (10 mg/mL) of the sample extracts were prepared in MeOH and further dilutions were performed with H_2O/EtOH (85:15). Fifty microlitre of the diluted solution were pipetted in the wells in triplicate and 100 μL of 20% (w/v) Sodium Carbonate (Na_2CO_3) were added in each well, followed by 50 μL of Folin–Ciocalteu reagent (2-fold diluted). The plates were placed in dark for 1 h in room temperature and the absorbance was measured at 650 nm. The results were expressed as milligram of gallic acid per gram of extract.

2.6. DPPH· radical scavenging assay

The radical-scavenging activity of the extracts was evaluated by a modified version of the method proposed by Brand-Williams, Cuvelier, and Berset (1995), converted into a micromethod. More specifically, a stock methanolic solution (10 mg/mL) of each extract was diluted with H_2O/EtOH (85:15) to prepare samples ranging from 500 to 0.8 μg/mL. Then, 50 μL of each sample were pipetted into 96-well plates in triplicate and was added in every well 50 μL of DPPH· solution (0.5 mM in absolute ethanol). Plates were placed in dark for 30 min at room temperature and then the absorbance was measured at 510 nm. The results were plotted as the percentage of remaining DPPH· (%I DPPH·) against the concentration (μg/mL) of the samples added.

$$\%\mathrm{I}(\mathrm{DPPH}^{\cdot}) = \left(\frac{A_{\mathrm{sample}}}{A_{\mathrm{blank}}}\right) \times 100 \quad (1)$$

A_{sample} = absorbance of the sample, A_{blank} = absorbance of the blank.

Results are expressed as Inhibitory Concentration (IC_{50}), which corresponds to extract concentration (μg/mL) required to quench 50% of the initial DPPH· radicals under the given experimental conditions.

2.7. FRAP assay

The typical FRAP assay (Benzie & Strain, 1996) was modified to fit in 96-well microplates. Specifically, the FRAP reagent was prepared just before the analysis by mixing 10 mL of acetate buffer (pH 3.6) with 1 mL of ferric chloride hexahydrate (20 mM in distilled water) and 1 mL of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM in HCl 0.04 N) and placed in a water bath at 37 °C. The stock methanolic solutions (10 mg/mL) of the extracts were diluted with H_2O/EtOH (85:15) to a final concentration of 50 μg/mL. The standard curves were constructed by preparing different concentrations of $FeSO_4$ (125–20 μM). Then, 25 μL of each sample were pipetted into the wells in triplicates and 175 μL of FRAP reagent were added in each well. Microplates were incubated in darkness at 37 °C for 30 min and the absorbance was measured using a Fluostar Galaxy microplate reader (BMG Lab Technologies) at 595 nm. The results were corrected for dilution and expressed in μM $FeSO_4$ (FRAP value), which corresponds to the reducing ability of 100 μg/mL for each tested extract.

2.8. Copper induced LDL oxidation assay

Stock solutions (10 mg/mL) of the sample extracts were prepared in DMSO and successively diluted in PBS to 100, 10 and 1 μg/mL concentrations. A commercial LDL solution was adjusted in a final concentration of 1.12 mg/mL protein by adding phosphate buffered saline (PBS, pH = 7.4). The reaction mixture was prepared as follows: 36 μL of LDL solution were transferred in Eppendorf tubes with 20 μL of sample solution (100, 10 and 1 μg/mL) for each extract and 100 μL $CuSO_4$ (0.2 mM in distilled water). The final volume was set at 200 μL with PBS. A tube with $CuSO_4$ served as the positive control and a tube without extract served as the negative control. The Eppendorf tubes were incubated in the absence of light at room temperature for 24 h. Then, 100 μL of TBA (1% in 50 mM NaOH) were added in tubes, followed by 100 μL of TCA (2.8% v/v). The tubes were placed at 95 °C for 1 h and then allowed to reach the room temperature. After the extraction of a pink chromogen by centrifugation at 12,000 rpm for 10 min, the supernatant obtained was placed in 96-well plates. The thiobarbituric acid-reactive species were detected by measuring the absorbance at 550 nm. Data were expressed as the percentage of oxidized LDL (LDL_{ox})

$$\%\mathrm{LDL_{ox}} = \left(\frac{A_{\mathrm{sample}} - A_{\mathrm{positive}}}{A_{\mathrm{negative}} - A_{\mathrm{positive}}}\right) \times 100 \quad (2)$$

A_{sample} = absorbance of the sample, A_{positive} = absorbance of the positive control, A_{negative} = Absorbance of the negative control.

The assay results are expressed as Inhibitory Concentration (IC_{50}), which corresponds to extract concentration (μg/mL) required to inhibit the oxidation of 50% of initial LDL. The IC_{50} was calculated using the statistical program OriginPro 7.0.

2.9. Intracellular reactive oxygen species content (intracellular ROS assay)

The capability of the extracts to reduce the intracellular reactive oxygen species (ROS) content was evaluated on a human vascular smooth muscle cell line (HVTs-SM1) (Kletsas & Pratsinis, 2005), developed in our laboratory (Hsieh et al., 2000). The cells were routinely cultured at 36 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with neomycin (G418 200 μg/mL, Gibco-Invitrogen, Paisley, UK), as well as, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% Fetal Bovine Serum. HVTs-SM1 cells when being at approx. 80–90% confluency were subcultured with trypsin–citrate (0.25–0.3%, respectively).

The cytotoxicity of the extracts was evaluated using a modification of the MTT-assay (Kasiotis et al., 2001). Briefly, cells were plated in 96-well, flat-bottomed microplates at a density of approx. 7000 cells/well, in medium containing 10% FBS; 24 h after the plating, serial dilutions of the extracts were added ranging from 100 μg/mL to 1 μg/mL. Blank solutions were prepared by adding DMSO in culture medium in order to achieve the same % DMSO content as in each of the five concentration levels. After a 48-h incubation, the medium was replaced with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) dissolved at a final concentration of 1 mg/mL in serum-free, phenol red-free DMEM (Biochrom KG), for a further 4-h incubation. Then, the MTT-formazan was solubilised in isopropanol, and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm. The cytotoxicity of the extracts was estimated by calculating the ratio of the absorbance for each concentration level, against the absorbance of the corresponding blank solution. When the calculated value was ≈1.0, the corresponding concentration level was considered to be non toxic; hence it was used for further experiments with cell cultures.

For the intracellular ROS assay (Athanasas et al., 2004), the cells were plated at a density of approximately 10,000 cells/cm^2 in medium supplemented with 10% FBS; 18 h after the plating, the extracts were added (diluted in the culture medium at the range of concentrations found to be not cytotoxic using the above MTT-assay). Blank solutions were prepared for each concentration level as mentioned before 24 h later the medium was replaced with a 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) solution in PBS. After 3 h of incubation at 37 °C in a humidified chamber, fluorescence was determined using a Fluostar Galaxy microplate reader (BMG Labtechologies); excitation was at 485 nm, and emission was read at 520 nm. The effect on the concentration of intracellular ROS, was estimated by calculating the ratio:

$$\%\mathrm{ROS} = \left(\frac{F_{\mathrm{sample}}}{F_{\mathrm{control}}}\right) \times 100 \quad (3)$$

F_{sample} = fluorescence of the sample, F_{blank} = fluorescence of the blank.

Results are expressed as Inhibitory Concentration (IC_{75}), corresponding to extract concentration (μg/mL) required to reduce initial ROS by 25%. The IC_{75} was determined using the statistical program OriginPro 7.0.

2.10. Statistical analysis

Folin–Ciocalteu method and all photometric experiments of antioxidant assays were conducted in triplicate and data were expressed as mean ± SEM (standard error of the mean).

3. Results and discussion

3.1. HPLC and extraction methods validation

The standard curves and method validation data are presented in Table 1. The detection and quantitation limits were calculated using the following equations:

$$\mathrm{DL} = \frac{3.3\ \mathrm{SD}}{b} \quad \text{and} \quad (4)$$

$$\mathrm{QL} = \frac{10\ \mathrm{SD}}{b} \quad (5)$$

where SD is the standard deviation of the intercept and b the slope of the curve.

The accuracy and repeatability of the method was determined by calculating the retention time and area precisions for seven successive injections. The retention time precisions were within 0.02–0.9% relative standard deviation (RSD), while the area precisions were within 0.28–2.74% RSD.

The extraction method accuracy was validated as follows: the grape samples were divided into three equal parts of 100 g each. One part was extracted and assayed according to the procedure described above. The remaining two samples were spiked with

Table 1
Calibration curves and validation data of the HPLC method used for the separation and quantification of the polyphenols studied.

Reference compound	Wavelength (nm)	Concentration range (mg/L)	Equation	R^2	DL (mg/L)	QL (mg/L)	RSD %RT (n = 7)	RSD %area (n = 7)
Gallic acid	280	30–0.47	y = 249828.7(±785.6) − 13898(±845.7)	0.9999	0.019	0.059	0.08	0.51
(+)-Catechin	280	50–0.78	y = 61799.7(±517.4) − 13619(±270.5)	0.9999	0.025	0.076	0.26	0.77
(−)-Epicatechin	280	40–0.62	y = 65704.3(±153.3) − 11164.2(±1808.2)	0.9999	0.159	0.477	0.22	2.48
Procyanidin B2	280	10–0.156	y = 75454.3(±2385.8) − 10741.3(±420.58)	0.9981	0.032	0.097	0.90	4.96
Epicatechin gallate	280	10–0.156	y = 357965.7(±3577.9) − 13301(±337)	0.9997	0.0054	0.016	0.66	2.53
trans-Resveratrol	320	5–0.078	y = 639485.3(±1739.5) − 8938.3(±212.1)	0.9999	0.002	0.006	0.06	1.02
Quercetin	360	5–0.078	y = 242052.7(±2031.3) − 23628(±436.6)	0.9959	0.01	0.031	0.03	2.74
Kaempferol	360	5–0.078	y = 298613(±2097) − 9211(±819.4)	0.9997	0.016	0.048	0.02	1.35
Quercetin 3-*O*-galactoside	360	10–0.156	y = 252815(±534.3) − 10003.5(±535.5)	0.9991	0.012	0.037	0.42	0.50
Quercetin 3-*O*-glucoside	360	10–0.156	y = 156465.3(±219.2) − 7696.7(±377)	0.9993	0.014	0.042	0.33	0.45
Quercetin 3-*O*-rhamnoside	360	10–0.156	y = 150606.7(±542.7) − 8547.7(±627.8)	1	0.024	0.072	0.18	0.87
Caffeic acid	320	10–0.156	y = 460098.3(±847.7) − 15122.8(±1303.1)	0.9999	0.016	0.049	0.26	0.91
p-Coumaric acid	320	40–0.62	y = 723836.7(±1583.7) − 65441.7(±2540)	1	0.02	0.061	0.19	0.52
Ferulic acid	320	10–0.312	y = 452688.3(±114.7) − 4922(±2326.2)	1	0.03	0.089	0.15	0.38

Table 2
Validation of the wine extraction method by calculating the recovery of five polyphenols through a spiking process at two different concentration levels.

	Gallic acid	(+)-Catechin	(−)-Epicatechin	Quercetin	*trans*-Resveratrol
Initial concentration (mg/100 g)	1.24	27.47	8.97	0.04	0.05
Spiking level 1[a] % recovery	108.2	101.1	110.2	105.7	109.0
Spiking level 2[b] % recovery	105.6	96.2	93.3	115.4	111.4

[a] An equal amount of pure compound was added to achieve a 2-fold concentration of the original sample (spiking level 1).
[b] Twice the initial amount of pure compound was added to achieve a 3-fold concentration (spiking level 2).

Table 3
Polyphenolic composition of extracts from grape berries, skins and seeds of the varieties studied.

	Gallic acid	(+)-Catechin	(−)-Epicatechin	Procyanidin B3	Procyanidin B2	Epicatechin gallate	*trans*-Caftaric acid	*trans*-Resveratrol	ε-Viniferin	[a]Q 3-*O*-galactoside	[a]Q 3-*O*-glucoside	[a]Q 3-*O*-rhamnoside	TPC[b]
Grape berries (mg/100 g fb[c])													
Mandilaria	0.72	18.37	8.30	8.15	10.59	0.80	0.56	0.02	ND[d]	3.50	2.78	ND	100.45
Voidomatis	0.38	11.38	13.53	2.10	5.12	1.93	0.06	0.02	0.21	1.00	1.24	0.47	92.89
Asyrtiko	0.88	22.40	13.33	4.33	2.06	3.49	0.96	0.03	0.06	4.08	0.50	0.53	141.72
Aidani	0.68	9.58	3.94	2.05	5.10	1.28	0.03	ND	ND	0.67	1.78	1.49	50.79
Grape seeds (mg/100 g dm[e])													
Mandilaria	161.74	661.22	203.25	129.24	275.30	7.20	ND	ND	ND	ND	ND	ND	2612.5
Voidomatis	19.00	478.64	371.65	67.73	101.69	304.59	ND	ND	ND	ND	ND	ND	2732.4
Asyrtiko	7.22	1067.0	220.53	172.41	74.79	284.18	ND	ND	ND	ND	ND	ND	3313.5
Aidani	9.35	143.63	89.21	44.37	127.86	7.39	ND	ND	ND	ND	ND	ND	825.80
Grape skins (mg/100 g dm)													
Mandilaria	0.17	ND	ND	ND	ND	ND	1.20	0.93	6.53	49.94	21.28	6.24	351.97
Voidomatis	0.05	ND	ND	ND	ND	ND	0.15	0.01	0.54	8.84	3.12	3.80	64.50
Asyrtiko	0.18	ND	ND	ND	ND	ND	5.86	1.18	1.71	50.07	10.72	1.95	119.31
Aidani	0.02	ND	ND	ND	ND	ND	0.16	ND	0.51	6.53	12.84	3.47	90.42

[a] Quercetin.
[b] Total polyphenolic content in gallic acid equivalents (GAE).
[c] Fresh berries.
[d] Not detected.
[e] Dry matter.

known amounts of standards up to 2-fold and 3-fold total concentrations of the original sample. Then, their recoveries were determined as 93.3–115.4% RSD. All results are summarized in Table 2.

The selectivity of XAD-4 for low molecular weight non-coloured polyphenols has already been examined thoroughly by ^{1}H NMR, revealing its suitability for application on polyphenols isolation from wines. Their ability to produce enriched polyphenolic extracts has been delineated even for white wines, which display a substantially low polyphenolic concentration, as compared to red wines (Anastasiadi et al., 2009b). Furthermore, XAD-4 resins have also been used successfully for the recovery of polyphenolic compounds from other plant materials (Grougnet et al., 2006).

3.2. Polyphenolic composition

The total polyphenolic content (TPC) and the bioactive polyphenol quantization of all assayed samples are presented in Tables 3 and 4. The concentration of each polyphenol is expressed as mg/100 g of fresh grape berries, mg/100 g of dry matter for grape skins and by-products and mg/L for wine samples. Finally, TPC was determined as mg/g of gallic acid equivalents.

3.2.1. Grape berries

Flavan-3-ol monomers and dimmers and 3-*O*-flavonol glycosides, constitute the bulk of non-coloured polyphenols detected in the grape berries extracts. The monomers (+)-catechin and (−)-epicatechin were the most abundant, assayed in concentrations ranging from 9.58 to 22.40 mg/100 g and 3.94 to 13.53 mg/100 g, respectively. These results correlate well with previous findings on different Greek *V. vinifera* varieties (Sakkiadi, Georgiou, & Haroutounian, 2007). The flavonol aglycons quercetin and kaempferol were detected only in trace amounts and their quantification was not possible. On the contrary, 3-*O*-flavonol glycosides were present in considerable amounts, with quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside being the most abundant, while quercetin-3-*O*-rhamnoside was detected in small amounts. The presence of quercetin-3-*O*-galactoside has been previously reported in the grape varieties Concord, Chanauc (Oszmianski & Lee, 1990) and Cabernet Sauvignon (Pena-Neira et al., 2004), in comparable amounts, while quercetin-3-*O*-glucoside was detected in grapes in amounts between 3.30 and 8.40 mg/100 g (Andrade, Mendes, Falco, Valentao, & Seabra, 2001) Gallic and caffeoyltartaric acids (*trans*-caftaric, a caffeic acid derivative) were the only detected phenolic acids, while stilbenes *trans*-resveratrol and ε-viniferin were present in relatively small amounts averaging from 0.03 mg/100 g (*trans*-resveratrol) to 0.14 mg/100 g (ε-viniferin). Since stilbenes are phytoalexins and their existence in grapes is directly related to environmental stress, such as botrytis infections and UV-irradiation (Jeandet, Bessis, & Gautheron, 1991), healthy plants contain small amounts. In any case, resveratrol was mainly detected, as glycoside and/or as oligomer, in grape skins and stems (Pussa, Floren, Kuldkepp, & Raal, 2006; Waterhouse & Lamuela-Raventos, 1994).

The comparison among the polyphenolic profile of the four grape varieties studied reveals that the varieties Mandilaria, Voidomatis and Asyrtiko exhibit higher polyphenolic content, as compared to the white variety Aidani.

3.2.2. Skins and seeds

Direct comparison of polyphenols assayed in grape skins and seeds, with those detected in their respective berries (Table 3), reveals that they exhibit a very different qualitative and quantitative profile. Seeds are particularly rich in monomeric flavan-3-ols (+)-catechin, (−)-epicatechin, epicatechin gallate and the dimeric procyanidins B2, B3. They also display a high TPC, ranging between 3313.5 and 825.80 mg/100 g gallic acid equivalents. Notably, seeds of the white variety Asyrtiko exhibited the highest TPC value among the varieties tested. These results are in accordance with previous studies on polyphenolic composition involving seeds of Greek cultivars (Guendez, Kallithraka, Makris, & Kefalas, 2005a, 2005b). On the contrary, skin extracts contain mostly flavanol glycosides. This result can be rationalized considering the combine effects of variety and environmental conditions to flavanol biosynthesis, which is directly linked to UV-irradiation (Flint, Jorda, & Caldwell, 1985), since the pronounced sunshine of the Santorini island is responsible for the large amounts of flavonols detected. Among glycosides, quercetin-3-*O*-galactoside was the most abundant, while quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside were detected in smaller amounts. The stilbenes *trans*-resveratrol and ε-viniferin, were present in skin extracts in relatively small amounts. Finally, the TPC values for grape skins ranged between 64.50 and 351.97 mg/100 g of gallic acid equivalents.

Table 4
Polyphenolic composition of the varieties studied wines.

	Gallic acid	(+)-Catechin	(−)-Epicatechin	Procyanidin B3	Procyanidin B2	Epicatechin gallate	*trans*-Caftaric acid	*trans*-Resveratrol	ε-Viniferin	[a]Q 3-O-galactoside	[a]Q 3-O-glucoside	[a]Q 3-O-rhamnoside	Quercetin	Kaempferol	Caffeic acid	Syrigic acid	p-Coumaric acid	Ferulic acid	TPC[b]
Wines (mg/L)																			
Mandilaria	134.12	83.86	62.61	41.00	6.15	ND[c]	57.30	1.48	0.23	11.20	6.88	1.90	3.30	0.34	6.99	5.07	3.15	ND	3956.1
Voidomatis	119.52	56.62	39.37	59.18	ND	ND	44.23	0.88	0.63	10.02	6.24	1.73	3.73	0.68	9.82	2.38	3.90	0.62	2712.0
Asyrtiko	3.71	21.27	5.22	34.86	ND	ND	48.42	0.71	ND	5.01	3.24	0.61	1.93	0.40	2.69	0.25	1.62	ND	430.02
Aidani	4.98	7.29	4.80	1.21	ND	ND	36.34	0.34	0.34	1.30	1.77	0.74	0.86	0.37	2.53	0.31	0.82	0.36	436.32

[a] Quercetin.
[b] Total polyphenolic content as gallic acid equivalents (GAE).
[c] Not detected.

3.2.3. Wines

The polyphenolic composition of wines is more complex as compared to their corresponding grape berries, because of the numerous reactions involving phenolic compounds that occur during the wine making and maturation processes (enzymatic and chemical oxidation reactions, condensation reactions, hydrolysis, etc.) (Monagas et al., 2005). Mandilaria displayed the highest TPC value (3956.1 mg/L) among red wines and Asyrtiko among the white ones (430.02 mg/L). Previous studies on the polyphenolic composition of varietal Greek wines have also focused on the exceptional polyphenolic profile of Asyrtiko cultivar, which produces polyphenol rich white (Makris, Kallithraka, & Kefalas, 2003; Psarra, Makris, Kallithraka, & Kefalas, 2002). On the other hand, there are no data concerning the polyphenolic profile of Aidani cultivar and only one concerning the Mandilaria and Voidomatis wines (Kallithraka, Tsoutsouras, Tzourou, & Lanaridis, 2006). It must be noted, however, that this report concerns wines obtained from Paros island (Mandilaria) and the wine institute of Athens (Voidomatis) that displayed considerably lower TPC values and different polyphenolic profile. For example, the TPC for Mandilaria wine of Paros was reported 2918.0 mg/L, while the respective wine of Santorini contained 3956.1 mg/L. These differences are attributed to the unique soil (volcano origin) and clima (hot, sunny and windy) conditions of the Santorini island and are indicative of the cultivation area influence on grape variety's polyphenolic profile.

The major polyphenols detected in wines by direct HPLC injection were:

Flavan-3-ols: the monomer (+)-catechin was present in considerable amounts in red wines especially of Mandilaria variety (83.86 mg/L), while its isomeric (−)-epicatechin was detected in lower concentrations. The dimeric procyanidin B3 was detected in large amounts only in red and Asyrtiko wines, while the quantification of procyanidin B2 was not always possible because its co-elution with caffeic acid. Finally, the presence of epicatechin gallate (a condensation product of flavanol with gallic acid) was also revealed.

Flavonols: the wine flavonols are mainly consisted of aglycones, produced from their respective grape berries glycosides by hydrolysis during the vinification, maturation, and/or ageing processes (Monagas et al., 2005). Quercetin was the prevailing flavonol, followed by kaempferol, while quercetin-3-*O*-galactoside was the most abundant detected glycoside (Table 4).

Phenolic acids: in contrast to grape berries, wines contain a variety of free phenolic acids. In all investigated wine samples, gallic acid constitutes the most abundant phenolic acid detected. Same class acids (hydroxybenzoates) detected were syringic (present in low concentrations) and vanillic acids (detected in traces and its quantification was not possible). In respect to the hydroxycinnamates, caffeic and caffeoyltartaric (*trans*-caftaric) acids were assayed in considerable amounts in all wine samples, while *p*-coumaric and ferulic acids were found in lower concentrations.

Table 5
Antioxidant activity of selected reference compounds assessed with the DPPH radical scavenging and FRAP assays.

Compound	FRAP assay[a]	DPPH IC_{50} (μM)
Quercetin	1197 ± 13	23.1 ± 0.6
(−)-Epicatechin	968 ± 14	23.9 ± 0.9
(+)-Catechin	928 ± 14	24.9 ± 0.0
Gallic acid	972 ± 6	23.3 ± 0.7
Syringic acid	820 ± 7	34.5 ± 0.0
Caffeic acid	708 ± 5	38.1 ± 1.2
Feroulic acid	466 ± 2	67.7 ± 2.3
p-Coumaric acid	71.4 ± 3.8	>500
Trolox	492 ± 7	54.5 ± 0.6

[a] Reducing capacity of 1 mM reference compound solution.

Stilbenes: trans-resveratrol was the principal stilbene assayed in wines with Mandilaria displaying the largest quantity (1.48 mg/L) for red and asyrtiko (0.71 mg/L) for white wine. These values are higher as compared to previously reported resveratrol content in other Greek wines (Arnous, Makris, & Kefalas, 2001; Kallithraka et al., 2001; Sakkiadi, Stavrakakis, & Haroutounian, 2001). On the contrary, ε-viniferin was found only in traces and its quantification was not possible.

3.3. Antioxidant activity

The antioxidant properties of the most abundant grape polyphenols (as 15% ethanolic solutions) were determined by the DPPH· and FRAP assays and compared with the activity of reference antioxidant compound Trolox [**(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid**]. The results are presented in Table 5. For both assays, the phenolic compounds tested exhibited more intense antioxidant activity than Trolox. Their activity order was: quercetin > gallic acid > (−)-epicatechin>(+)-catechin > syringic acid > caffeic acid > Trolox > feroulic acid > p-coumaric acid and is rationalized considering their molecular structure–antioxidant activity relation (Cook & Samman, 1996; Rice-Evans, Miller, & Paganda, 1996). On the other hand, the intracellular ROS assay was determined only for the most abundant polypehnols (+)-catechin, quercetin and the reference antioxidant Trolox. The results, presented in Table 6, indicate that (+)-catechin and quercetin possess relatively good ability to reduce the intracellular ROS, in contrast to Trolox which was ineffective in the concentration-range tests.

Table 7 summarizes the results of the antioxidant activity assays for all extracts studied and their respective TPC values. It should be pointed out that most of the extracts tested were found to display an extreme capability on preventing the oxidation of LDL at very low concentrations. This is of great importance since, according to the proposed mechanism, polyphenols prevent the atherosclerosis *in vivo* by inhibiting the oxidative modification of LDL and acting as free-radical scavengers, metal chelators or reducing the oxidative stress (Aviram, 1996).

Seed extracts displayed the best antioxidant properties, regardless the assay method. Their activities are in direct relation with their high TPC, which ranged between 428 and 628 mg GAE/$g_{extract}$. Grape extracts were also found to possess considerable antioxidant abilities, in line with previous literature reports on other varieties (Yang, Liu, & Martinson, 2009). The low antioxidant activity of skin extracts is attributed on their low TPC and their high content of quercetin glycosides which possess reduced antioxidant activities as compared to their aglycon (Rice-Evans et al., 1996).

Table 6
Ability of three reference compounds to reduce the intracellular ROS concentration.

Compound	Intracellular ROS IC_{75} (mM)
(+)-Catechin	12.5 ± 1.3
Quercetin	8.8 ± 0.8
Trolox	>250

Table 7
Total polyphenolic content and antioxidant activity of the extracts prepared from grape berries, wines and vinification by-products.

	Folin–Ciocalteu (mg/g extract)	FRAP value (μM $FeSO_4$)	DPPH IC_{50} (μg/g extract)	LDL IC_{50} (μg/g extract)	Intracellular ROS IC_{75} (μg/g extract)
Grape berries					
Mandilaria	418.5 ± 3.0	108.0 ± 1.1	10.9 ± 0.1	1.91 ± 0.4	11.7 ± 0.9
Voidomatis	365.2 ± 8.8	81.2 ± 1.9	14.4 ± 0.3	3.60 ± 0.2	4.9 ± 1.1
Asyrtiko	414.3 ± 15.6	108.8 ± 6.0	13.0 ± 0.6	2.50 ± 0.1	8.2 ± 2.5
Aidani	322.2 ± 6.3	90.3 ± 0.8	10.6 ± 1.0	NM[a]	4.0 ± 0.1
Grape seeds					
Mandilaria	628.0 ± 12.5	129.0 ± 1.0	6.7 ± 0.2	2.38 ± 0.18	4.6 ± 1.8
Voidomatis	575.0 ± 6.9	150.0 ± 3.5	18.1 ± 1.4	6.00 ± 0.23	8.1 ± 2.0
Asyrtiko	428.2 ± 9.9	111.4 ± 4.2	14.8 ± 2.5	2.11 ± 0.01	7.8 ± 1.2
Aidani	540.6 ± 9.4	126.0 ± 1.9	13.4 ± 2.2	NM	11.3 ± 3.5
Grape skins					
Mandilaria	144.3 ± 3.3	26.3 ± 1.9	55.7 ± 0.4	31.6 ± 1.2	50.0 ± 9.6
Voidomatis	43.0 ± 5.8	9.3 ± 2.4	177.5 ± 2.0	NM	>100
Asyrtiko	47.5 ± 2.2	10.6 ± 0.2	117.0 ± 3.1	57.7 ± 8.3	74.0 ± 0.8
Aidani	32.3 ± 2.3	3.1 ± 0.1	274.2 ± 2.1	NM	>100
Wine extracts (mg/g extract)					
Mandilaria	382.3 ± 10.7	87.6 ± 1.7	14.1 ± 3.0	8.3 ± 2.0	5.4 ± 0.2
Voidomatis	300.0 ± 1.2	77.0 ± 2.7	15.6 ± 1.3.0	5.9 ± 0.2	15.8 ± 3.9
Asyrtiko	169.3 ± 4.7	30.0 ± 2.2	40.9 ± 3.8	20.2 ± 0.8	23.7 ± 0.4

[a] Not measured.

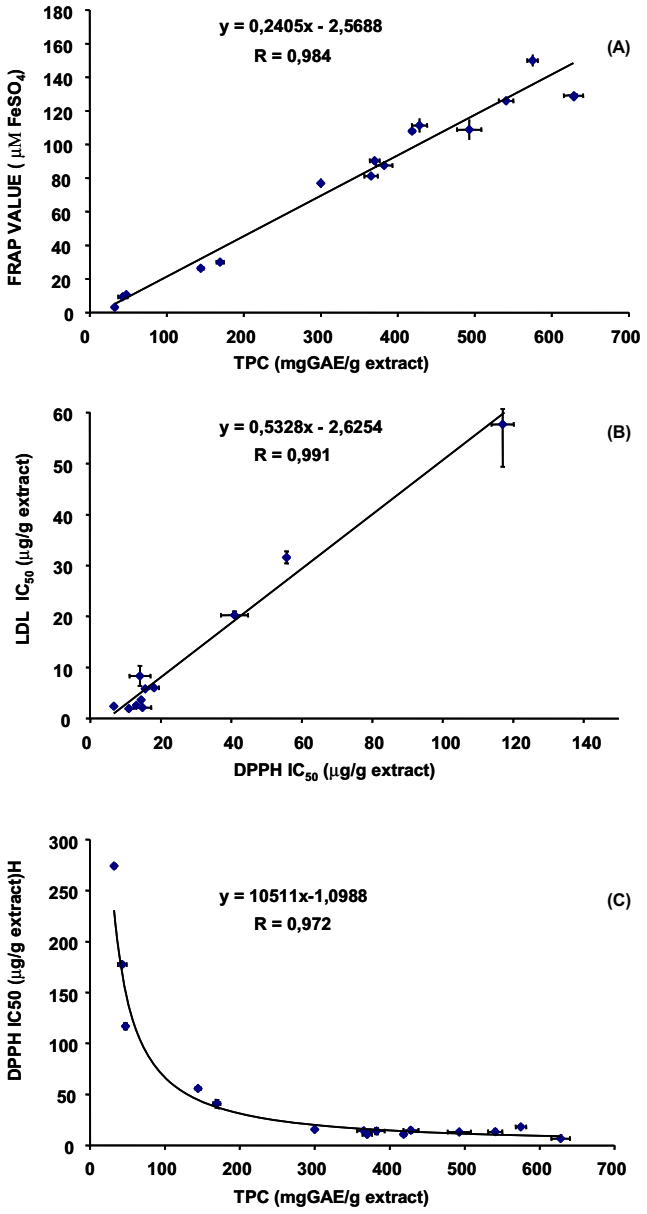

Fig. 1. Linear regression analysis between: (A) total polyphenolic content (TPC) and FRAP assays; (B) DPPH· radical scavenging and LDL oxidation assays and (C) TPC and DPPH· radical scavenging assays.

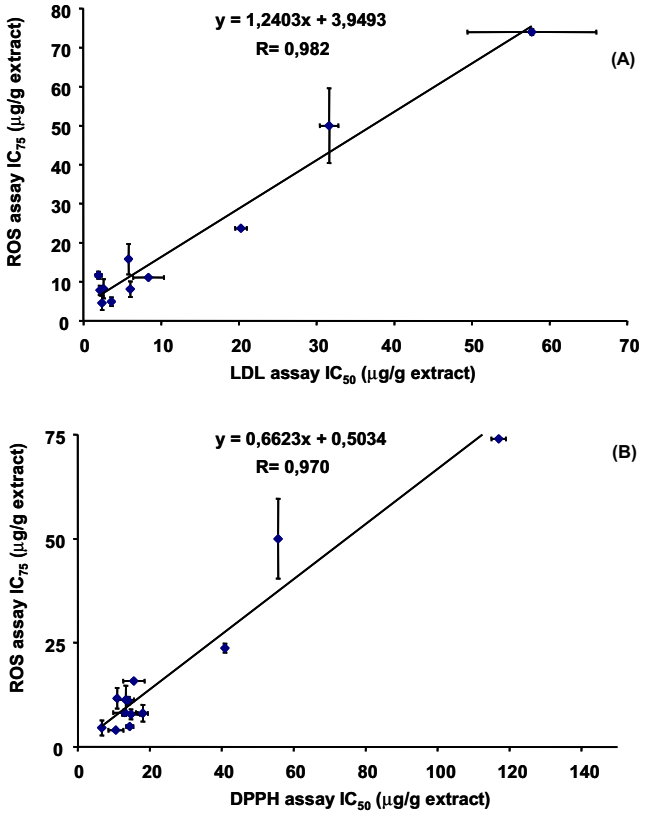

Fig. 2. Linear regression analysis between: (A) LDL oxidation and intracellular ROS assays and (B) DPPH· radical scavenging and intracellular ROS assays.

3.3.1. Correlation among antioxidant methods

A regression analysis was used to correlate the results of the five assays. High correlation coefficients were found between the FRAP and Folin–Ciocalteu assays ($R = 0.984$), as well as between LDL oxidation and DPPH· assays ($R = 0.991$) (Fig. 1A and B). The good correlation between the FRAP and Folin–Ciocalteu assays can be rationalized considering that both assays rely on the same reaction mechanism, which involves electron transfer (Foti, Daquino, & Geraci, 2004). On the other hand, the significant correlation between LDL oxidation and DPPH assays is indicative of previous suggestion (Katsube et al., 2004) that the LDL oxidation preventing mechanism mainly depends on the radical-scavenging activity of polyphenols. High correlation coefficients were also observed between Folin–Ciocalteu and DPPH· assays ($R = 0.972$, Fig. 1C), as well as between FRAP and LDL oxidation assays ($R = 0.911$), both realized as hyperbolic equations, revealing that the extracts antioxidant properties may be predicted accurately by considering their respective TPC values.

A rather significant result is the observed good correlation between the reduction of intracellular reactive oxygen species (ROS) in smooth muscle cell cultures and LDL oxidation inhibition assays ($R = 0.982$) (Fig. 1D). Since these assays display a high biological relevance, their assessed antioxidant activity might account as a possible index of the *in vivo* antioxidant activities of the extracts. Interestingly, a rather good correlation was observed between the DPPH· and ROS assays ($R = 0.970$, Fig. 2B), and FRAP and ROS assays ($R = 0.864$), especially for extracts exhibiting lower antioxidant activity. Thus, DPPH· and FRAP assay results may constitute a first indication of the potential intracellular activity of an antioxidant, though both DPPH· and FRAP assay were performed in a cell-free system. It must be noted however, that this approach does not consider cellular mechanisms which influence the net response to a substance such as the possibility that the cell membrane can impede the endocytosis of a potent free-radical scavenger (Athanasas et al., 2004). Since DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) is a nonpolar molecule capable to diffuse into the cell, where via hydrolysis by cellular esterases produces dichlorodihydrofluorescein, which, upon reaction with a broad range of oxidizing species, yields the highly fluorescent dichlorofluorescein. Thus, the ROS assay provides a better insight into the interactions on cell level and thus provides a better indication of the potential antioxidant activity of the polyphenolic extracts.

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