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# Determination of low molecular weight polyphenolic constituents in grape (*Vitis vinifera* sp.) seed extracts: Correlation with antiradical activity

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**Abstract**

Ethyl acetate extracts of seeds originating from nine Hellenic native and international *Vitis vinifera* varieties cultivated in Greece were screened for their contents of characteristic polyphenols. The compounds determined were principal constituents of low molecular weight, including gallic acid (GA), catechin (CT), epicatechin (ECT), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and the procyanidins $B_1$ and $B_2$ (dimers). Total content varied from 55.1 to 964 mg per 100 g of seeds, the average being 380 mg per 100 g. The most abundant polyphenol was CT, accounting for 49.8% of the total content, followed by ECT (26.0%), ECG (9.3%), procyanidin $B_1$ (5.8%), and procyanidin $B_2$ (5.1%), whereas EGC and GA were minor constituents. The assessment of the in vitro antiradical activity ($A_{AR}$), employing the stable radical DPPH·, showed that there is a significant correlation with total polyphenol content ($r^2 = 0.6499$, $P < 0.01$). The correlations with the individual compounds, however, revealed that procyanidin $B_1$ may be one of the most important radical scavengers in grape seed extracts ($r^2 = 0.7934$, $P < 0.002$), despite its low contribution to the overall polyphenol content.


*Keywords:* Antiradical activity; DPPH; Grape seed extracts; Flavanols; HPLC; Polyphenols; Procyanidins; *V. vinifera*; Value-added products

## 1. Introduction

Epidemiological data support the association of high intakes of vegetables and fruit with low risk of various diseases, while there have been several plausible explanations why consumption of vegetables and fruit might delay or even prevent the onset of cardiovascular disorders, certain types of cancer, and other chronic dysfunctions (Lampe, 1999). Plant foods and products are rich sources of a variety of biologically active compounds, and these phytochemicals have been found to possess hypolipidemic, antiplatelet, antitumor, antioxidant, and immuno-stimulating properties (Craig, 1999). In recent years particular attention has been given to a specific class of antioxidant phytochemicals, the polyphenols, which are comprised basically of phenolic acids, including benzoate and hydroxycinnamate derivatives, and flavonoids. Polyphenolic substances are naturally present in essentially all plant material, and are prominently ubiquitous in vegetables, cereals, fruits, nuts, but also in plant products, such as wine, cider, beer, tea and cocoa (Bravo, 1998).

Flavanols (flavan-3-ols) and flavanol oligomers and polymers (proanthocyanidins) are flavonoids of profound significance, because they have been proven to possess powerful antioxidant properties (Pannala, Chan, O'Brien, & Rice-Evans, 2001; Rice-Evans & Miller, 1996; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995; Terao, Piskula, & Yao, 1994; Yang, Kotani, Arai, & Kusu, 2001)

*Abbreviations:* $A_{AR}$, antiradical activity; CT, catechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate; ECT, epicatechin; ECG, epicatechin gallate; $B_1$, procyanidin $B_1$; $B_2$, procyanidin $B_2$; GA, gallic acid; S.D., standard deviation.

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and other beneficial biological activities (Bomser, Singletary, Wallig, & Smith, 1999; Rapport & Lockwood, 2001; Simonetti, Ciappellano, Gardana, Bramati, & Pietta, 2002; Sugihara, Ohnishi, Imamura, & Furuno, 2001). The evidence from animal and human studies, accumulated so far, suggests that flavanol monomers, including CT, ECT, ECT, EGC, and EGCG, may be potent anticarcinogens (Ahmad & Mukhtar, 1999; Wang, Provan, & Helliwell, 2000) and antiatherogenic agents (Rapport & Lockwood, 2001), and therefore they have attracted prominent attention as very promising chemopreventive phytochemicals. There is thus a constant need for isolation, examination and implementation of natural antioxidants. However, the high cost of the exploitation of naturally occurring, biologically active agents constitutes a major limitation of financially viable exploitation and, as a result, efforts have been focussed on inexpensive plant sources but also on agricultural wastes rich in polyphenols.

Recent investigations have stressed the importance of vinification by-products as plant materials particularly rich in a wide range of polyphenols (Alonso, Guillén, Barroso, Puertas, & García, 2002; Bonilla, Mayen, Merida, & Medina, 1999; Torres et al., 2002; Torres & Bobet, 2001). Marcs, stems and dregs (sludgy residual deposits at the bottom of fermentation vats) represent sources of antioxidants that have been relatively unexploited to date, but are of increasing industrial interest. If stalks are stripped from grapes prior to crushing, winery marc consists of approximately 30% seeds and 70% skin and pulp. However, studies on grape seeds are rather limited, despite their richness in polyphenolic substances, mainly monomeric and oligomeric flavanols.

The Hellenic vineyard is composed principally of native *Vitis vinifera* species, many of them being occasionally studied, and there have been no reports so far of the polyphenols in the grape seeds. The present study was undertaken to generate analytical data on the polyphenolic composition of grape seeds originating from certain major red varieties cultivated in Greece and to provide information related to their antioxidant characteristics, which may be of both technological and nutritional interest.

## 2. Materials and methods

### 2.1. Chemicals

Perchloric acid (60%) and Folin–Ciocalteau reagent were from Merck (Germany). Methanol (MeOH) and ethyl acetate (EtOAc) were from Readel-de Haën (Germany). 2,2-Diphenyl-picrylhydrazyl (DPPH·) stable radical, gallic acid, (−)-epicatechin and (+)-catechin were from Sigma Chemical Co (St. Louis, MO). (−)-Epicatechin gallate, (−)-epigallocatechin gallate, (−)-epigallocatechin, and procyanidins $B_1$ and $B_2$ were from Extrasynthese (France).

Table 1
Origin and location of the varieties examined

| Cultivar | Origin | Location |
|---|---|---|
| Cabernet Sauvignon | Volos | Thessaly (C) |
| Grenache Rouge | Attica | Sterea Ellada (C) |
| Merlot | Chalkidiki | Macedonia (N) |
| Mandilaria | Rhodes | Aegean Isles (S) |
| Agiorgitiko | Nemea | Peloponnese (S) |
| Negoska | Goumenissa | Macedonia (N) |
| Xinomavro | Naousa | Macedonia (N) |
| Mavrodafni | Patra | Peloponnese (S) |
| Limnio | Chalkidiki | Macedonia (N) |

*Letters N, C, and S denote Northern, Central, and Southern Greece, respectively.

### 2.2. Plant material

All seed samples studied were from varieties selected to cover major parts of the Hellenic vineyard. Varieties were Hellenic native but also international ones (*V. vinifera* sp.) cultivated in Greece. Analytical information about the origin and vineyard location is given in Table 1. The grapes used were harvested at optimum technological maturity, as judged by indices of sugar and acid content, established by the Institute of Wine. Grape berries were manually deseeded and the seeds were frozen in liquid nitrogen immediately afterwards, and stored in the freezer (−20 °C) until analysed.

### 2.3. Polyphenol extraction

A lot of 2 g of seeds was ground with a pestle and a mortar with 8 ml of EtOAc. The extract was centrifuged at 6000 rpm for 5 min, at −4 C, and this process was repeated twice more. The clear extracts were then pooled and taken to dryness in a rotary vacuum evaporator ($T \leqslant 40$ °C), and the resulting residue was dissolved in 8 ml of MeOH, containing 5% (v/v) perchloric acid. The solution was filtered through Gelman GHP Acrodisc 13 syringe filters (0.45 μm) prior to analyses.

### 2.4. Total polyphenol determination

Total polyphenols (TP) were determined using the Folin–Ciocalteau reagent, with the microscale protocol previously developed (Arnous, Makris, & Kefalas, 2001, 2002). Gallic acid was employed as a calibration standard and results were expressed as gallic acid equivalents (mg GAE/100 g of seeds).

### 2.5. Determination of individual polyphenols by HPLC

Chromatographic analyses were carried out on an HP 1090, series II, liquid chromatography apparatus, coupled to a HP 1090 diode array detector. Eluent (A) was 0.6‰ aqueous perchloric acid, and eluent (B) MeOH, and the flow rate was kept constant throughout the

analysis at 1 ml min$^{-1}$. Injections were accomplished with a 20-μl fixed loop. The elution programme used was as follows: from 100% A to 78% A in 55 min, from 78% A to 0% A in 10 min, and then isocratic for a further 10 min. Chromatograms were monitored at 280 nm, and identification was based on retention times and on-line spectral data in comparison with authentic standards. Quantification was performed by establishing calibration curves for each compound determined, using the standards.

### 2.6. Measurement of antiradical activity ($A_{AR}$)

Measurements were performed using the stable radical DPPH·, as described previously (Arnous et al., 2001; Brand-Williams, Cuvelier, & Berset, 1995). Five different dilutions of each extract were prepared and 0.025 ml of the diluted extract was mixed with 0.975 ml of DPPH· solution (0.6 mM in MeOH). The absorbance was read immediately at 515 nm ($A_{515(0)}$), and afterwords the mixture was left for 120 min in the dark ($A_{515(120)}$), using an HP 8452 diode array spectrophotometer. The DPPH· concentration in the reaction medium was calculated from the following equation:

$$A_{515} = 0.0262 \times [\text{DPPH}^{\cdot}] + 0.0068, \quad (1)$$

as determined after linear regression. The % remaining DPPH· was calculated as:

$$\%\text{DPPH}^{\cdot}_{\text{rem}} = [\text{DPPH}^{\cdot}_{(120)}]/[\text{DPPH}^{\cdot}_{(0)}] \times 100, \quad (2)$$

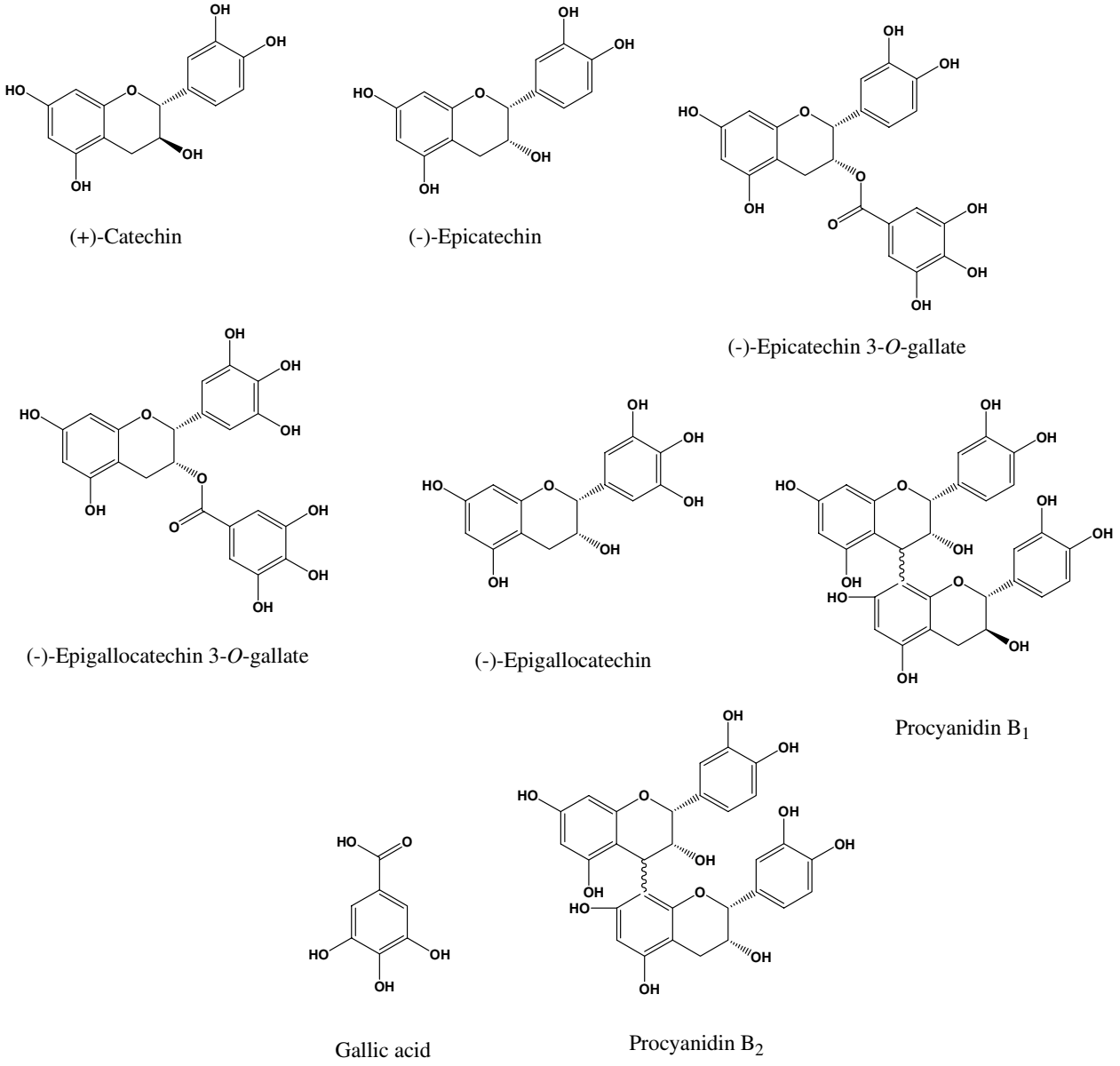


Fig. 1. Chemical structures of the polyphenolic compounds determined in this study.

where $[DPPH^{\cdot}_{(0)}]$ and $[DPPH^{\cdot}_{(120)}]$ are the DPPH· concentrations initially and after 120 min. The ratio [polyphenols]/[DPPH·] was plotted against % $DPPH^{\cdot}_{rem}$, and from the resulting exponential equation the amount of polyphenols to decrease the initial DPPH concentration by 50% ($EC_{50}$ in μg/μg DPPH·) was determined. Antiradical activity ($A_{AR}$) was defined as $1/EC_{50}$.

### 2.7. Statistics

All measurements were performed at least in triplicate ($n = 3$) and values were averaged and reported along with the standard deviation (±S.D.). Differences in polyphenol content and antiradical activity among varieties were compared by employing student's *t*-test. For all statistics, Microsoft Excel™ 2000 was used.

## 3. Results

For the examination of grape seed extracts, eight representative polyphenols (Fig. 1) were chosen, and their contents were determined by reversed-phase HPLC, coupled to diode-array detection. Under the experimental conditions established, the peaks corresponding to GA, the flavanol monomers CT, ECT, ECG, EGCG, and EGC, and the dimers $B_1$ and $B_2$, could be very satisfactorily separated, identified and quantified. As can be seen in a typical chromatogram recorded at 280 nm (Fig. 2), several minor peaks were also detected, especially in the richer extracts, which are presumed to correspond to other oligomers encountered in grape seeds (De Freitas, Glories, Bourgeois, & Vitry, 1998; Fuleki & Ricardo da Silva, 1997; Kallithraka, Garcia-Viguera, Bridle, & Bakker, 1995; Peng, Hayasaka, Iland, Sefton, Hoj, & Waters, 2001; Yang & Chien, 2000). The unresolved peaks, that appeared after 60 min, are likely to correspond to proanthocyanidins with higher degrees of polymerisation, which may reach up to 14.7 (Labarbe, Cheynier, Brossaud, Souquet, & Moutounet, 1999) or even higher (Kennedy & Jones, 2001).

The analytical polyphenolic composition of seeds is illustrated in Table 2. The most abundant polyphenol was CT (189 mg/100 g), accounting for 49.8% of the total polyphenol content, followed by ECT (98.6 mg/100 g, 26.0%), and ECG (35.5 mg/100 g, 9.3%). By contrast, GA, EGC and EGCG were minor constituents, their contributions to the total content being 1.3, 0.8, and 1.9%, respectively. Procyanidin $B_1$ was found only in five extracts, and its average content was 22.1 mg/100 g, whereas $B_2$ was of wider occurrence, although its level (19.3 mg/100 g) was comparable to that of $B_1$. Overall, the order of abundance was CT > ECT > ECG > $B_1$ > $B_2$ > EGCG > GA > EGC (Fig. 3). Fuleki and Ricardo da Silva (1997) reported similar values for CT, ECT, and procyanidins $B_1$ and $B_2$ corresponding to 76.7, 94.7, 21.9, and 53.3 mg/100 g. By contrast, the contents found in other studies (De Freitas & Glories, 1999) were significantly lower, being 25.2, 4.60, 1.20,

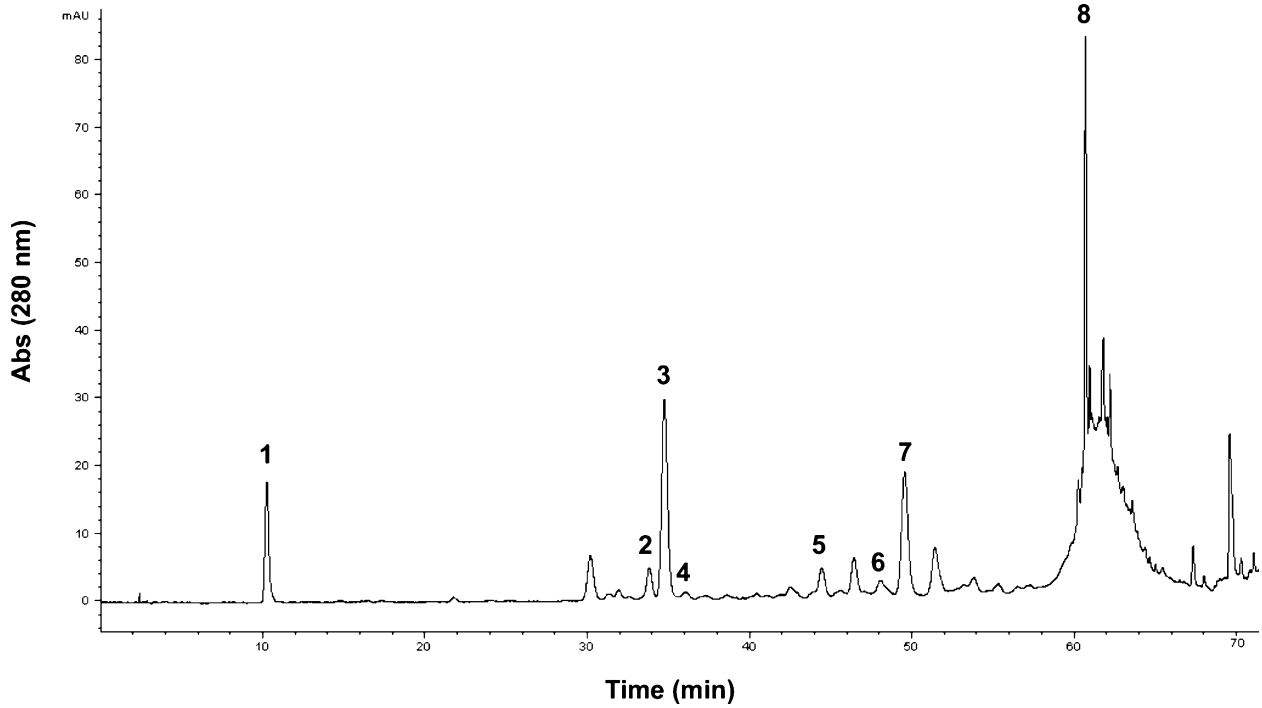


Fig. 2. Typical HPLC trace of a grape seed extract (*V. vinifera* var. Agiorgitiko), recorded at 280 nm. Peak assignment: 1, gallic acid; 2, procyanidin $B_1$; 3, catechin; 4, epigallocatechin; 5, procyanidin $B_2$; 6, epigallocatechin gallate; 7, epicatechin; 8, epicatechin gallate.

Table 2
Analytical polyphenolic composition of seed extracts examined

| Varieties | GA[a] | CT[b] | ECT[c] | ECG[d] | EGCG[e] | EGC[f] | $B_1^g$ | $B_2^h$ | Total |
|---|---|---|---|---|---|---|---|---|---|
| Cabernet Sauvignon | 2.79 ± 0.00 | 215 ± 0.15 | 89.3 ± 0.42 | 27.9 ± 1.35 | 6.46 ± 0.23 | 0.00 | 14.8 ± 0.49 | 11.3 ± 3.27 | 368 |
| Grenache Rouge | 3.43 ± 0.18 | 203 ± 3.26 | 86.8 ± 0.20 | 18.6 ± 1.04 | 9.52 ± 1.41 | 5.95 ± 0.85 | 10.6 ± 0.02 | 6.07 ± 0.06 | 344 |
| Merlot | 2.72 ± 0.01 | 183 ± 3.59 | 83.4 ± 1.20 | 58.0 ± 0.08 | 13.5 ± 2.08 | 12.9 ± 0.06 | 13.5 ± 1.17 | 17.6 ± 0.36 | 384 |
| Mandilaria | 10.5 ± 0.44 | 454 ± 0.40 | 249 ± 6.94 | 64.4 ± 0.83 | 15.6 ± 3.44 | 0.00 | 102 ± 6.32 | 69.2 ± 5.59 | 964[α] |
| Agiorgitiko | 17.9 ± 1.63 | 245 ± 4.08 | 172 ± 5.30 | 41.3 ± 2.24 | 10.9 ± 3.27 | 5.25 ± 4.28 | 31.9 ± 1.18 | 36.1 ± 3.18 | 560 |
| Negoska | 1.24 ± 0.12 | 186 ± 0.13 | 72.9 ± 0.80 | 46.7 ± 0.48 | 6.66 ± 0.01 | 2.50 ± 0.94 | 9.11 ± 0.31 | 12.5 ± 0.26 | 337 |
| Xinomavro | 0.65 ± 0.03 | 36.7 ± 0.10 | 17.5 ± 0.80 | 0.14 ± 0.00 | 0.05 ± 0.01 | 0.00 | 0.00 | 0.12 ± 0.02 | 55.1[β] |
| Mavrodafni | 2.87 ± 0.02 | 130 ± 0.16 | 97.8 ± 0.49 | 48.7 ± 0.65 | 0.65 ± 0.13 | 0.00 | 17.2 ± 0.34 | 21.2 ± 1.57 | 318 |
| Limnio | 1.15 ± 0.04 | 51.3 ± 0.61 | 20.1 ± 0.12 | 13.8 ± 0.20 | 0.25 ± 0.02 | 0.00 | 0.00 | 0.08 ± 0.03 | 86.6[γ] |
| Average | 4.80 (1.3) | 189 (49.8) | 98.6 (26.0) | 35.5 (9.3) | 7.06 (1.9) | 2.96 (0.8) | 22.1 (5.8) | 19.3 (5.1) | 380 |

Results reported are expressed as mg per 100 g of seeds and represent average values of triplicate determination ($n = 3$) ± S.D. a, gallic acid; b, catechin; c, epicatechin; d, epicatechin gallate; e, epigallocatechin gallate; f, epigallocatechin; g and h, procyanidins $B_1$ and $B_2$, respectively. Values in parentheses indicate the percent contribution of each polyphenol to the total amount.

Values with different superscripted Greek letters are statistically different: α, $P < 0.001$; β, $P < 0.01$; γ, $P < 0.05$.

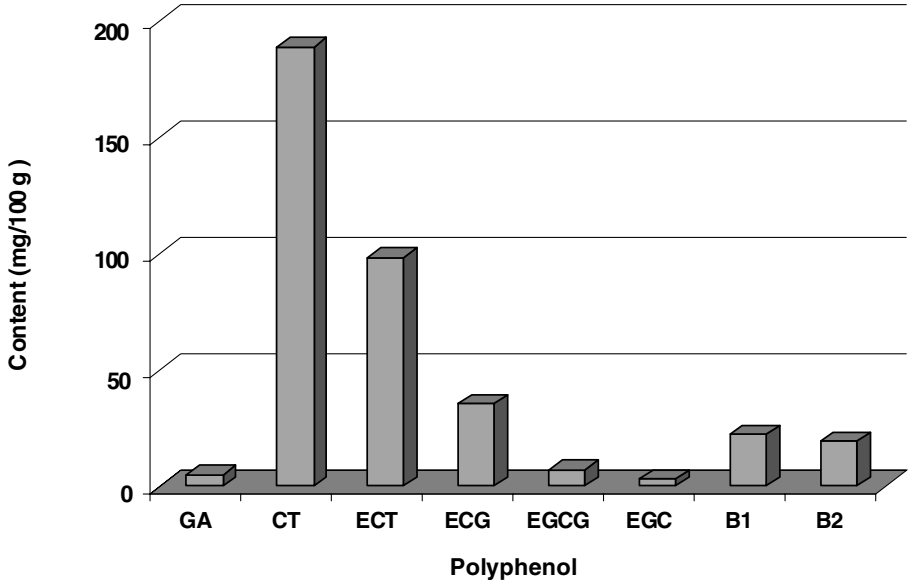


Fig. 3. Comparative diagram showing average values of individual polyphenols in grape seed extracts. Assignments: GA, gallic acid; $B_1$, procyanidin $B_1$; CT, catechin; EGC, epigallocatechin; $B_2$, procyanidin $B_2$; EGCG, epigallocatechin gallate; ECT, epicatechin; ECG, epicatechin gallate.

and 3.98 mg/100 g, for CT, ECT, and procyanidins $B_1$ and $B_2$, respectively, but calculations were based on a dry matter basis. In seeds from Muscadine grapes (*Vitis rotundifolia*), the content reported for gallic acid (6.91 mg/100 g) was of comparable magnitude, but levels of CT and ECT were much higher, reaching 558 and 1299 mg/100 g, respectively (Pastrana-Bonilla, Akoh, Sellapan, & Krewer, 2003). A point worth mentioning is that Mandilaria had significantly high total polyphenol content ($P < 0.001$), as opposed to Xinomavro and Limnio, which were found to be particularly poor ($P < 0.01$ and 0.05, respectively) (Table 2). These results are in agreement with data on anthocyanins (Kallithraka, Mohdaly, Makris, & Kefalas, 2004), providing further evidence for the polyphenolic potential of these varieties.

The antiradical activity ($A_{AR}$) of extracts varied from 1.09 to 4.17, the average being 1.94 (Table 3). The highest value found was for Mandilaria, which was also the richest in polyphenols, whereas Mavrodafni exhibited the weakest activity (Fig. 4). It is characteristic that Limnio, which had the lowest total polyphenol content had an important $A_{AR}$ value, but extracts from Agiorgitiko, Merlot and Cabernet Sauvignon, with much higher polyphenolic contents, had $A_{AR}$ values below average. This finding is evidence that certain constituents are particularly responsible for strong antiradical effects. In fact, regression analysis showed that total polyphenol content, determined by HPLC ($TP_{HPLC}$), was significantly correlated with $A_{AR}$ ($r^2 = 0.6282$, $P < 0.05$), but the correlation between $A_{AR}$ values and total polyphenol content, determined with the

Table 3
Total polyphenol (TP) content and antiradical activity ($A_{AR}$) of the grape seed extracts studied

| Variety | $TP_{FC}$ (mg/100 g of seeds)[a] | $TP_{HPLC}$ (mg/100 g of seeds)[b] | $EC_{50}$ (μg/μg DPPH·) | $A_{AR}$ ($1/EC_{50}$) |
|---|---|---|---|---|
| Cabernet Sauvignon | $869 \pm 9$ | 368 | $0.55 \pm 0.02$ | $1.82 \pm 0.00$ |
| Grenache Rouge | $982 \pm 21$ | 344 | $0.51 \pm 0.03$ | $1.96 \pm 0.00$ |
| Merlot | $1689 \pm 168$ | 384 | $0.54 \pm 0.07$ | $1.85 \pm 0.09$ |
| Mandilaria | $2228 \pm 26$ | 964 | $0.24 \pm 0.05$ | $4.17 \pm 0.01^{\alpha}$ |
| Agiorgitiko | $1126 \pm 8$ | 560 | $0.58 \pm 0.05$ | $1.72 \pm 0.01$ |
| Negoska | $1181 \pm 118$ | 337 | $0.66 \pm 0.00$ | $1.52 \pm 0.01$ |
| Xinomavro | $143 \pm 16$ | 55 | $0.73 \pm 0.00$ | $1.37 \pm 0.02$ |
| Mavrodafni | $397 \pm 9$ | 318 | $0.92 \pm 0.11$ | $1.09 \pm 0.09^{\beta}$ |
| Limnio | $1412 \pm 141$ | 86.6 | $0.50 \pm 0.02$ | $2.00 \pm 0.01$ |
| Average | 1114 | 380 | 0.58 | 1.94 |

Values reported are means of triplicate determinations ($n = 3$) ± S.D.
Values with superscripted Greek letters are statistically different: $\alpha$, $P < 0.001$; $\beta$, $P < 0.02$.
[a] Total polyphenol content, as determined by the Folin–Ciocalteau method. Results are expressed as gallic acid equivalents (GAE).
[b] Total polyphenol content determined by HPLC.

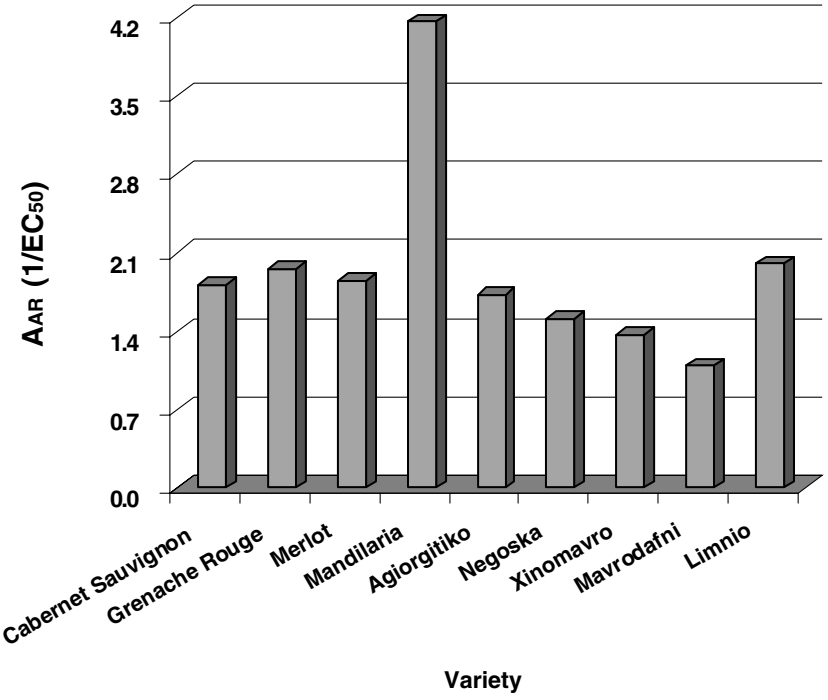


Fig. 4. Comparative diagram showing average values of antiradical activity ($A_{AR}$) of grape seed extracts.

Table 4
Statistical parameters calculated after regression analysis of antiradical activity ($A_{AR}$) versus content of individual and total polyphenols

| Polyphenol | Square correlation coefficient ($r^2$) | Equation | $P$ |
|---|---|---|---|
| GA | 0.1463 | $y = 0.0593x + 1.6591$ | 0.3096* |
| CT | 0.6630 | $y = 0.0059x + 0.8295$ | 0.0070 |
| ECT | 0.5344 | $y = 0.0090x + 1.0594$ | 0.0250 |
| ECG | 0.1778 | $y = 0.0172x + 1.3316$ | 0.2583* |
| EGCG | 0.4281 | $y = 0.0993x + 1.2424$ | 0.0550* |
| EGC | 0.0111 | $y = -0.0210x + 2.0059$ | 0.7872* |
| $B_1$ | 0.7934 | $y = 0.0250x + 1.3901$ | 0.0012 |
| $B_2$ | 0.5980 | $y = 0.0314x + 1.3362$ | 0.0145 |
| $TP^{a}_{HPLC}$ | 0.6282 | $y = 0.0026x + 0.9481$ | 0.0109 |
| $TP^{b}_{FC}$ | 0.6499 | $y = 0.0011x + 0.6870$ | 0.0086 |

[a,b] Total polyphenols determined using HPLC and Folin–Ciocalteau reagent, respectively.
* Values statistically insignificant.

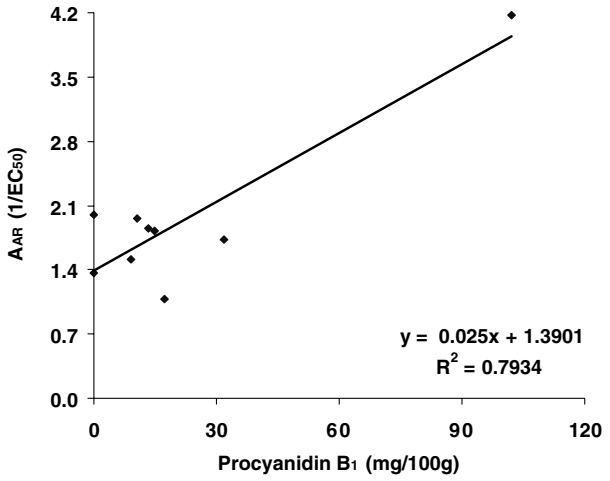


Fig. 5. Linear regression analysis of $A_{AR}$ values versus content of procyanidin $B_1$.

Folin–Ciocalteau methodology ($TP_{FC}$), was slightly higher ($r^2 = 0.6499$, $P < 0.01$), although $TP_{FC}$ value was, on average, 2.9-fold that of $TP_{HPLC}$ (Table 3), suggesting that other flavanols that occur in extracts have a rather low impact on the overall antiradical potential. When $A_{AR}$ was individually correlated with the contents of the polyphenolic components, it was clear that $A_{AR}$ was highly correlated with procyanidin $B_1$ (Table 4, Fig. 5), whereas correlations with GA, ECG, EGCG, and EGC were particularly low and statistically insignificant (Table 4). Furthermore, CT and ECT, the two principal substances in seed extracts, exhibited weaker correlations than that found for $B_1$, despite the fact that their contents were 8.5- and 4.5-fold higher, respectively.

## 4. Discussion

The wine industry would benefit from a more detailed evaluation of best-practice re-use methods and a set of guidelines that outline how to carry out by-product re-use activities with minimal environmental impact. In this context, there is a need for a more efficient exploitation of winery waste material, such as marcs, and the characterisation of grape seed composition may very well be regarded as a step toward a better estimation of vinification by-products as a low-cost source of value-added phytochemicals. The survey carried out aimed at analysing certain major, red varieties cultivated in Greece, in order to obtain a detailed and analytical picture of the profile of principal low molecular weight polyphenols that occur in seeds. For this reason, determinations were mainly focussed on flavanol monomers, but also on two major dimers, namely procyanidins $B_1$ and $B_2$, and gallic acid.

Total polyphenol levels ranged from 55.1 to 964 mg/100 g, suggesting that various factors may affect polyphenol content in seeds. One such factor may be the genetic potential of individual species for polyphenol biosynthesis, as shown by the case of Mandilaria, where capability of increased polyphenol formation was indicated by the examination of anthocyanin content in grape berries (Kallithraka et al., 2004). Apart from the genetic (varietal) background, maturation stage may also be critical in this respect. For example, it has been demonstrated that individual flavanol monomers and dimers behave differently during maturation of Sémillon and Ugni Blanc varieties, and the total content may differ significantly from season to season (De Freitas & Glories, 1999). Beginning at véraison, it was observed that amounts of all seed polyphenols in Shiraz grapes decline considerably, and this fact was attributed to an initiation of oxidative phenomena, which appeared to follow second order kinetics (Kennedy et al., 2000b). Similar results were found for Cabernet Sauvignon, where monomers decreased more rapidly than did proanthocyanidins (Kennedy, Matthews, & Waterhouse, 2000a). Moreover, it was clearly noted that changes in vine water status are able to impact polyphenol levels in seeds, indicating that cultural practices can be used to modify composition.

The assessment of the antiradical activity ($A_{AR}$) showed that total polyphenols, estimated by employing the Folin–Ciocalteau methodology, exerted a statistically important effect ($r^2 = 0.6499$, $P < 0.01$) which, however, was comparable to that seen by the components determined by HPLC ($r^2 = 0.6282$, $P < 0.02$). This finding indicates that there is a rather weak contribution of the extract constituents not quantified by HPLC. The correlations of individual substances with $A_{AR}$ revealed that CT and ECT make more profound contributions than all the other flavanol monomers and gallic acid, most probably because of their higher content. Also, from a mechanistic point of view, this seems reasonable because, according to the mechanism proposed by Kondo, Ohnishi, and Kawaguchi (1999), the compound produced from ECT, and presumably CT, by radical oxidation, can also function as an antioxidant, and thus ECT has a longer inhibition period with respect to lipid peroxidation. On the other hand, EGC is transformed into a quinone-like compound, and as a consequence, superoxide anion radicals may be produced. Similar considerations were claimed by Bors, Michel, and Stettmeier (2000), who reported that flavanols were excellent radical scavengers, yet their quinones were potential prooxidants, due to the formation of reactive oxygen species by redox cycling. It should be emphasised, however, that EGC, ECG, and EGCG were shown to possess stronger antioxidant ability (Saint-Cricq de Gaulejac, Provost, & Vivas, 1999; Salah, Miller, Paganga, Tijburg, Bolwell, & Rice-Evans, 1995), when comparisons were made on a molar basis, employing the TEAC assay (Table 5). These results are consistent with data obtained from studies on the prevention of lipid peroxidation,

Table 5
Comparative bibliographic data on the relative antioxidant capacity of flavanols

| Compound | $EC_{50}$ (μM)[a] | TEAC[b] | $IC_{50}$ (μM)[c] | $IC_{50}$ (μM)[d] | TEAC (mM)[e] |
|---|---|---|---|---|---|
| GA | – | 1.24 | – | – | $3.01 \pm 0.05$ |
| CT | $251 \pm 7$ | 1.40 | 51 | – | $2.40 \pm 0.05$ |
| ECT | $210 \pm 7$ | 1.44 | 30.0 | 25.7 | $2.50 \pm 0.02$ |
| EGC | – | 1.88 | 16.0 | 24.9 | $3.82 \pm 0.06$ |
| ECG | – | 2.02 | 10.0 | 24.6 | $4.93 \pm 0.02$ |
| EGCG | – | 2.24 | 11.0 | 19.0 | $4.75 \pm 0.06$ |
| $B_1$ | $85 \pm 3$ | – | – | – | – |
| $B_2$ | $202 \pm 7$ | – | – | – | – |

[a] Concentration required for decreasing $O_2^{\cdot-}$ by 50% (after Saint-Cricq de Gaulejac et al., 1999).
[b] Trolox equivalent antioxidant activities calculated by fast reaction kinetics (after Pannala et al., 2001).
[c] Concentration required to inhibit lipid peroxidation by 50% (after Yang et al., 2001).
[d] Concentration required to inhibit $Fe^{2+}$-induced lipid peroxidation by 50% (after Sugihara et al., 2001).
[e] Trolox equivalent antioxidant activities (Salah et al., 1995).

suggesting EGC, ECG, and EGCG to be more efficient than CT and ECT (Yang et al., 2001; Sugihara et al., 2001). These discrepancies are probably due to the fact that flavanols may behave differently in mixture than when they occur individually, due to interactions. In fact, the antioxidant activity of monomeric flavanols, such as catechin, has been shown to depend on the presence of other antioxidant agents (Saucier & Waterhouse, 1999). Additive effects were observed in mixtures containing catechin and ascorbic acid or α-tocopherol whereas, in the presence of sulphur dioxide, an important synergistic phenomenon was revealed.

The dimers $B_1$ and to a lesser extent $B_2$ appeared to play a pronounced role in the expression of $A_{AR}$, as illustrated by the correlation coefficients and significance level (Table 4). This observation is particularly important, considering that procyanidins $B_1$ and $B_2$ represent 5.8% and 5.1%, respectively, of the total amount of polyphenols determined by HPLC. Both dimers were demonstrated to be more efficient quenchers of superoxide anion radical that were CT and ECT (Saint-Cricq de Gaulejac et al., 1999), but the limited data available do not allow for a more profound insight into the factors that differentiate the antioxidant properties of proanthocyanidins from those of the component monomers. It has been claimed that proanthocyanidin quinones, formed from the initial semiquinone radicals after hydrogen abstraction, are capable of producing oligomeric compounds by various pathways. These coupling reactions (or nucleophilic additions) retain the number of hydroxyl groups and the commensurate higher number of radical target sites is primarily responsible for their enhanced antioxidant capacity (Bors et al., 2000). The differences that were observed in the superoxide radical scavenging potency between $C_4$–$C_6$ and $C_4$–$C_8$ dimers, suggested that intermolecular linkages might be of great importance in this respect (Ricardo da Silva, Darmon, Fernandez, & Mitjavila, 1991). This theory was further corroborated by findings providing evidence for the influence of oligomer chain length on the antioxidant activity of proanthocyanidins (Lotito et al., 2000). More specifically, it was observed that monomers, dimers and trimers were more effective antioxidants when liposome oxidation was initiated in the aqueous phase. However, when oxidation was initiated in the lipid domains, higher molecular weight proanthocyanidins were the most effective.

## 5. Conclusions

From the results reported herein, it is shown that utilisation of one kilogramme of grape seeds may afford, on average, almost 3.8 grammes of polyphenols, consisting mainly of flavanol monomers and dimers, which have appreciable antiradical activity. It should be kept in mind, however, that the actual amount of polyphenols that can be recovered is somewhat higher, due to the presence of several other dimers and, trimers, as well as oligomers and polymers. These data, along with the research showing the direct implication of monomeric flavanols in suppression of degenerative diseases, such as cancer and arteriosclerosis, make grape seeds an ideal candidate for a cost-effective and readily-exploitable source of natural, high value-added polyphenolic phytochemicals.

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