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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$^{\cdot}$) 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