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Characterization of Polyphenolic Phytochemicals in Red Grape Pomace

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Abstract

Polyphenolic phytochemicals are of particular importance to the food, pharmaceutical and cosmetics industry, because of their unique antioxidant properties. Red grape pomace is a solid waste of the wine manufacturing process and possesses a very high polyphenolic load, hence its significance as a rich and abundant residua source. However, in many instances there is a lack of analytical data regarding its polyphenolic composition. In this study, red grape pomace originating from the Greek native cultivar *Vitis vinifera var*. Agiorgitiko was efficiently extracted with 57% aqueous ethanol, which is a non-toxic and environmentally benign solvent, with the aim of obtaining a polyphenol-enriched extract. The extract was subsequently analyzed by liquid chromatography-diode array-mass spectrometry analysis, in order to tentatively characterize the major phytochemicals recovered. The compounds identified were a *p*-coumaric acid derivative, three flavonol conjugates (two glucosides and a glucuronide), along with three anthocyanin pigments that occur in grape berries. Three other major phenolics detected could not be assigned to a tentative formula and their structural elucidation merits further investigation. The data generated from this study could be used in assessing the overall polyphenolic profile of the pomace from this particular Greek, native variety, which could be of value in producing commercial formulations with high antioxidant activity.

Keywords: Antioxidants; Liquid chromatography-mass spectrometry; Polyphenols; Red grape pomace

Abbreviations: AAR, Antiradical Activity (mM TRE g^{-1} dpw); dpw: Dry Pomace Weight (g); GAE: Gallic Acid Equivalents; RGP: Red Grape Pomace; Rt: Retention Time; TRE: Trolox Equivalents; WISW: Wine Industry Solid Wastes; Y_{TP}: Total Polyphenol Yield (mg GAE g^{-1} dpw)

Introduction

Solid wastes in wine industry mainly consist of solid by-products, such as pomace and stems. The waste material may account on an average 30% (w/w) of the grapes used for wine production. Vinification wastes contain a relatively high content of polyphenolic phytochemicals [1,2], which depends on the type of grape (white or red), the part of the tissue (skins, seeds etc), as well as the processing conditions (e.g. pomace contact).

Solid wastes have attracted considerable attention as potential sources of bioactive phenolic compounds, which can be used in the pharmaceutical, cosmetics and food industry. Studies regarding WISW are mainly focused on the polyphenolic composition of seeds, which are very rich in flavanols [3-5], but red grape pomace (RGP), which is composed of seeds and skins, has also been evaluated as potential source of antioxidant polyphenols [6-9]. However, although several methods of extraction have been developed for the efficient recovery of pomace polyphenols [10], there is still a significant lack of analytical data on the polyphenolic profile of RGP originating from different cultivars. The composition of RGP is defined by the polyphenols occurring in both seeds and skins, which are mainly flavanols [11,12] anthocyanins and flavonols [13,14], although other minor constituents, such as stilbenes [15] and dihydroflavonols [16] have been reported. All these components are considered nutritionally important, since they may possess a variety of bioactivities [17]. Therefore, the investigation of the analytical polyphenolic composition of RGP is of undisputed significance in the development of tools and methodologies for extraction and final product formulation.

Because of the lack of analytical data regarding the polyphenolic composition, the scope of the present study was an examination on the polyphenolic composition of grape pomace from the native Greek variety Agiorgitiko (*Vitis vinifera* sp.). This variety is widely cultivated in the region of Peloponnese and it is the most important native species, in terms of quality wine production. The approach attempted was the examination of a polyphenol- rich pomace extract, obtained with a hydroalcoholic solution, employing liquid chromatography-diode array-mass spectrometry (LC-DAD-MS) analysis.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent and gallic acid were from Fluka (Steinheim, Germany). Trolox™, gallic acid, and 2, 2-diphenyl-picrylhydrazyl (DPPH•) stable radical were from Merck (Darmstad, Germany). p-Coumaric acid was from Sigma (St. Louis, MO, U.S.A.).

Vinification solid waste

RGP was from Agiorgitiko cultivar (*Vitis vinifera* sp.), obtained from a winery located in Nemea (Peloponnese). The pomace was left in contact with the fermenting must for 7 days. The material was obtained immediately after pressing the pomace, transferred to the laboratory within a few hours and stored at -40°C until used.

Extraction procedure

A suitable quantity of RGP (approx 4.5 g) was chopped into small

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pieces with a sharp, stainless steel cutter to facilitate extraction. The chopped material was ground with sea sand and a small portion of the extraction solvent, with a pestle and a mortar, and then left to macerate for 30 min in the dark. The paste formed was placed in a 100 mL conical flask with 25 mL of solvent (solvent-to-solid ratio 5.5) and extraction was performed under stirring at 700 rpm on a magnetic stirrer for 15 min. The extract was filtered through paper filter and this procedure was repeated twice more. The extracts were then combined in a 100 mL volumetric flask and made to the volume. All extracts were centrifuged at 4,500 rpm and filtered through 0.45 μm syringe filters prior to analyses.

Determination of total polyphenol yield (Y_{TP})

Analysis was carried out employing the Folin-Ciocalteu methodology [18]. In a 1.5-mL Eppendorf tube, 0.78 mL of distilled water, 0.02 mL of sample appropriately diluted, and 0.05 mL of Folin-Ciocalteu reagent were added and vortexed. After exactly 1 min, 0.15 mL of aqueous sodium carbonate 20% was added, and the mixture was vortexes and allowed to stand at room temperature in the dark, for 60 min. The absorbance was read at 750 nm (A_{750}), and the total polyphenol concentration was calculated from a calibration curve, using gallic acid as a standard. Yield in total polyphenols (Y_{TP}) was expressed as mg gallic acid equivalents (GAE) per g of dry weight, using the following equation:

$$Y_{Tp}(mgGAEg^{-1}) = \frac{(951 \times A_{750} - 1.49) \times V}{m} \quad (1)$$

Where, V is the volume of the extraction medium (mL) and *m* the dry weight of RGP (g).

Measurement of the antiradical activity (A_{AR})

Sample (0.025 mL), appropriately diluted, was added to 0.975 mL DPPH• solution (100 μM in methanol), and the absorbance at 515 nm was read at t=0 (A_{515}^{t0}) and t=30 min (A_{515}^{t30}). Results were expressed as Trolox equivalents (mM TRE) per g of dry weight, using the following equation:

$$A_{AR}\left(mMTREg^{-1}dw\right) = \left(\frac{0.018 \times \%\Delta A_{515} + 0.017}{m}\right) \times F_D \quad (2)$$

As determined from linear regression, after plotting % ΔA_{515} of known solutions of Trolox against concentration; where $\%\Delta A_{515} = \frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}} \times 100$, *m* the weight of dry material (g), and F_D the dilution factor.

Liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

A Finnigan MAT Spectra System P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18, 125×2 mm, 4 μm, column (Macherey-Nagel, Germany), protected by a guard column packed with the same material, and maintained at 40°C. Analyses were carried out employing electrospray ionization (ESI) at the positive ion mode, with acquisition set at collision energies of 12 and 70 eV, capillary voltage 4 kV, source voltage 45 V, detector voltage 650 V and probe temperature 400°C. Eluent (A) a nd MeOH, respectively. The flow rate was 0.33 mL min^{-1}, and the elution programme used was as follows: 0-2 min, 0% B; 2-52 min, 100% B; 52-60 min, 100% B.

Statistical analysis

All determinations were carried out at least in triplicate and values were averaged and given along the standard deviation (S. D.). For all statistics, SigmaPlot™ 12.0 and Microsoft Excel™ 2010 were used.

Results and Discussion

Effect of solvent composition

Ethanol percentage in the solvents used varied from 28.5 to 85.5, a range that has been previously shown to provide high yield for grape seed extraction [3,19], but also grape pomace [9] and other plant material, including olive leaves (59%) [20], black currants [21], onion peels [22] and white grape seeds, peels and stems (57%) [23]. A hydroalcoholic solution of 57% was found to be the most effective for high polyphenol recovery, as this was manifested by estimating both Y_{TP} and A_{AR} (Table 1). Thus the extract obtained with 57% ethanol was chosen for the examination of the analytical polyphenolic composition.

Tentative identification of major phytochemicals

The principal compounds detected in the extract analyzed (Figure 1) belonged to flavonol and anthocyanin classes. In particular, three flavonol and three anthocyanin conjugates were tentatively identified, along with a *p*-coumaric acid derivative (Figure 2). Three other substances could not be assigned to any known grape constituent and their identification merits further investigation.

Compound (2) (Table 2) showed an ion at m/z=327, which was assigned to its molecular ion, after considering a Na^+ adduct (m/z=349) and a dehydration ion at m/z=309. The UV-vis spectrum was identical to the original *p*-coumaric acid standard. These data concurred for the identification of this compound as *p*-coumaroyl glucoside. Compound (4) displayed a molecular ion at m/z=479. Since anthocyanins are positively ionised at acidic pH, this represents the actual molecular mass [24]. Characteristic fragment indicating the aglycone (m/z=301) were also observed. These findings were consistent with the anthocyanin petunidin 3-*O*-glucoside [25]. Likewise, peak 5 gave a molecular ion at m/z=493, a diagnostic fragment of the loss of two methyl units (m/z=463), the aglycon ion (m/z=315), and the demethylated aglycone (m/z=301). This peak was assigned to malvidin 3-*O*-glucoside. Similarly, peak 10 that displayed a molecular ion at m/z=639 and the ion corresponding to the aglycone (m/z=331), was assigned to malvidin 3-*O*-p-coumaroyl glucoside [26]. Compounds (6) and (7) with corresponding molecular ions at m/z=479 and 465 were found to yield the same daughter ion (m/z=303), and corresponding Na^+ adducts at m/z=501 and 487. Compound (6) gave also a characteristic fragment at m/z=561, indicating the formation of a double adduct with CH_3COOH and Na^+. These compounds were identified as quercetin 3-*O*-glucuronide and quercetin 3-*O*-glucoside, respectively [27]. In a similar fashion, compound (8) displayed a molecular ion at m/z=479 and adducts with both Na^+ and CH_3COOH at m/z=561. Ions at m/z=509 and 501 were also assigned to adducts with MeOH and Na^+, respectively. The m/z=317 also yielded a MeOH adduct at m/z=347. Based on these data, this compound was tentatively identified as isorhamnetin 3-*O*-glucoside.

Conclusions

Red grape pomace is an industrial by-product with a wide

Solvent (% v/v EtOH)	Y_{TP} (mg GAE g^{-1} dpw)	A_{AR} (mM TRE g^{-1} dpw)
28.5	25.46 ± 2.40	2.15 ± 0.09
57.0	72.59 ± 2.21	2.67 ± 0.10
85.5	48.17 ± 4.07	1.89 ± 0.04

Table 1: Values determined for Y_{TP} and A_{AR} of the extracts obtained using aqueous solvents with varying amounts of EtOH.

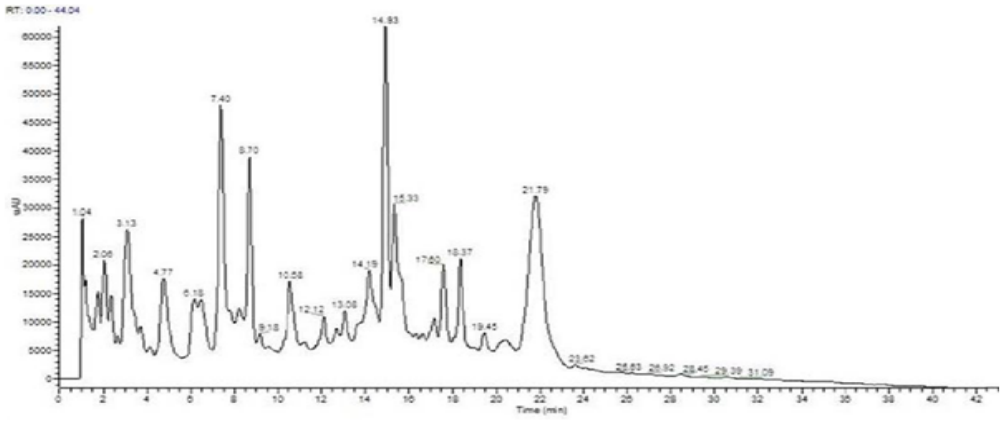

Figure 1: Chromatogram of the RGP extract monitored at 320 nm. Peak assignment according to retention time (Rt) is given in Table 2.

Peak No.	Rt (min)	λmax	$[M]^+$ (m/z)	$[M+H]^+$ (m/z)	Fragment ions (m/z)	Tentative identity
1	3.13	280, 296, 328(s)		-	385, 329, 203	Unknown
2	7.40	310		327	349, 309	*p*-Coumaroyl glycoside
3	8.70	277		675	409, 349, 327, 259, 231	Unknown
4	13.08	250, 342(s), 522	479		301	Petunidin 3-*O*-glucoside
5	14.19	250, 524	493		463, 331, 315, 301	Malvidin 3-*O*-glucoside
6	14.93	254, 354		479	561 $[CH_3COOH+Na]^+$, 501 $[Na]^+$, 303	Quercetin glucuronide
7	15.33	252, 356		465	487 $[Na]^+$, 303	Quercetin glucoside
8	17.60	252, 354		479	531$[MeOH+Na]^+$, 509 $[479+MeOH]^+$,	Isorhamnetin glucoside
					501$[Na]^+$, 347$[317+MeOH]^+$, 317	
9	18.37	272		695	517, 387, 355, 195, 181, 163	Unknown
10	21.79	254, 534	639		331	Malvidin 3-*O*-*p*-coumaroyl glucoside

Table 2: Spectral characteristics and tentative identification of the major polyphenols detected in the RGP extract analysed.

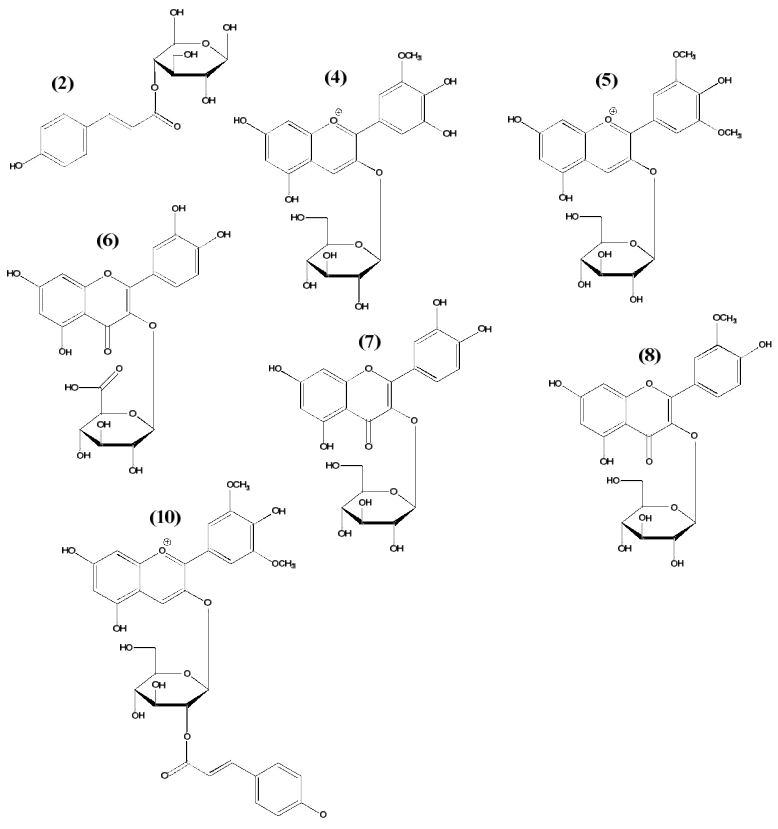

Figure 2: Chemical formulae of the major polyphenolic components tentatively identified in the RGP extract. Assignments are given in Table 2.

diversification, which depends on several factors, such as genetic (varietal) potential, treatment, post-disposal handling etc. Therefore, the examination of such waste materials from various sources might reveal the occurrence of a spectrum of substances. In the study presented herein, red grape pomace originating from the Greek native cultivar V. vinifera var. Agiorgitiko was efficiently extracted with 57% aqueous ethanol, to retrieve polyphenolic compounds. Ten principal polyphenols were detected and seven of them were tentatively identified on the basis of UV-*vis* and mass spectral data. The analyses revealed that the substances detected were a phenylpropanoid, derivative of *p*-coumaric acid, as well as anthocyanin pigments and flavonol glycoconjugates. Further research is needed to better illuminate the complex composition of this particular food industry by-product.

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