An Investigation on Factors Affecting Recovery of Antioxidant Phenolics and Anthocyanins from Red Grape (Vitis vinifera L.) Pomace Employing Water/Ethanol-Based Solutions

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Abstract: Extractions were performed using non-toxic media composed of water/ethanol mixtures and hydrochloric, acetic or tartaric acid. Recovery efficiency was assessed by monitoring the antiradical activity (Aarb) of extracts and several indices related to their polyphenolic composition, including total polyphenol, total flavonoid, total flavanol, total anthocyanin and condensed tannin (proanthocyanidin) content. Extracts with the highest Aarb values were obtained with 57% ethanol, a solvent system that was also favourable in obtaining high total polyphenol and total flavonoid yields, which amounted 7.259 and 7.222 mg/100 g dry weight, respectively. The highest anthocyanin yield was however achieved with 85.5% ethanol (266.2 mg/100 g dry weight). None of the acidification agents used provided extracts with increased polyphenol levels and Aarb. Addition of SO2 (0.01%, w/v) to 57% ethanol, however, resulted in maximisation of Aarb (2.9 mM TRE/g dry weight), although anthocyanin recovery was not maximal (18.6 mg/100 g dry weight). It is suggested that efficient recovery of antioxidant phenolics and anthocyanins from by-products of red vinification can be achieved employing simple extracting media composed of ethanol, but more active, in terms of antioxidant activity, extracts can be obtained with addition of a low amount of SO2. Ethanol is a bio-solvent that can also be obtained from wine-industry wastes and thus the implementation of similar techniques may potentially provide the basis for a sustainable process of integrated exploitation of vinification by-products.

Keywords: Anthocyanins, antioxidants, by-products, polyphenols, red grape pomace, vinification, wastes, wine industry

INTRODUCTION

The food industry produces large volumes of wastes, both in solid and liquid form, as a result of production, processing and consumption of food. These wastes pose increasing disposal and potentially severe pollution problems and represent a loss of valuable biomass and bioactive substances (Laufenberg et al., 2003; Torres et al., 2003). On the other hand, costs of drying, storage and shipment of by-products are, from an economic point of view, significant shortcomings. Thus efficient, inexpensive and environmentally rational utilisation of agri-food industry wastes is of undisputed importance for higher profitability and minimal environmental impact.

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Wine industry wastes, which consist mainly of solid by-products, include mares, pomace and stems, may account on average for almost 30% (w/w) of the grapes used for wine production. All these by-products may bear a considerable burden of phenolic components (González-Parra et al., 2004), depending 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). The last few years, vinification solid wastes have attracted considerable attention as potential sources of bioactive phenolics, which can be used for various purposes in the pharmaceutical, cosmetics and food industry. However, in many instances there is a rather significant lack of appropriate feasibility studies on the exploitation of such wastes and as a result their utilisation is still in its infancy. Studies regarding vinification by-products are mainly focused on the polyphenolic composition of seeds, which are very rich in flavanols (Yilmaz and Toledo, 2006; Guendez et al., 2005), but pomace, which is composed of seeds and skins, has also been evaluated as potential source of antioxidant polyphenols (Alonso et al., 2002; Louli et al., 2004; Kammerer et al., 2005; Pinelo et al., 2005a).

In many instances the methodologies employed for maximal polyphenol recovery may be detrimental to environment, i.e., the use of sulphuric acid, methanol or acetone (Murthy et al., 2002; Cruz et al., 2004; Pinelo et al., 2005b; Pechsl et al., 2006; Yilmaz and Toledo, 2006), be relatively expensive, i.e., the use of enzymes, supercritical fluid extraction and pressurised liquid extraction (Ju and Howard, 2003; Louli et al., 2004; Kammerer et al., 2005) and require multi-step processes, sophisticated equipment and organic solvents, which may further increase processing costs (Borilla et al., 1999; Palenzuela et al., 2004; Pinelo et al., 2005a). On the other hand, the use of simple extracting media composed of ethanol, citric acid and sulphur dioxide have been successfully applied for anthocyanins and other phenolics from fruit wastes (Lee and Wrolstad, 2004).

The scope of the present study was an examination on the possibilities of using non-toxic, cheap and readily available means of recovering phenolics from red vinification solid by-products. On such a basis, the solvent systems tested were composed of ethanol, a vinification co-product that can be obtained after fermentation of the sugar-containing pomace and distillation. Additionally, tartaric acid can be obtained from must or wine dregs and lees, while acetic acid can be produced through acetylation of ethanol solutions or must/wine dregs. For all these products, there is also the possibility for recycling. Hydrochloric acid is a cheap, industrial, non-oxidative product. The implementation of similar techniques may potentially provide the basis for a sustainable process of integrated exploitation of vinification by-products.

**MATERIALS AND METHODS**

**Chemicals**

Folin-Ciocalteu reagent and ascorbic acid were from Fluka (Steinheim, Germany). Trolox® gallic acid, 2,2-diphenyl-picrylhydrazyl (DPPH) stable radical, p-(dimethylamino)-cinnamaldehyde (DMACA) and catechin were from Sigma Chemical Co (St. Louis, MO, U.S.A.). Sodium nitrite and aluminium chloride hexahydrate were from Merk (Darmstad, Germany).

Red grape pomace was from Agiorgitiko cultivar (*Vitis vinifera* sp.), obtained from Gaia Winery (Nemea, prefecture of Corinthia, Peloponnesse) in September 2005. The pomace was left in contact with the fermenting must for 7 days. The material was transferred to the laboratory within a few hours and stored at -40°C until used.

**Extraction Procedure**

A suitable quantity of tissue (approx. 4.5 g) was chopped into small pieces with a sharp, stainless steel cutter to facilitate extraction. The chopped tissue 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 4500 rpm prior to analyses. For control extractions, a solvent system consisted of 0.1% HCl in MeOH/acetone/water (6/3/1, v/v/v) was used. All other procedures were as aforementioned.

**Determinations**

**Moisture content**

Moisture was determined after drying pomace in an air current-heated oven at 95°C for 48 h.

**Total Polyphenols**

Analysis was carried out employing the Folin-Ciocalteu methodology (Armons et al., 2002). Results were expressed as mg Gallic Acid Equivalents (GAE) per 100 g dry weight.

**Total Flavonoids**

A modified protocol of that described by Kim et al., 2003, was used. A 0.1 mL aliquot of extract appropriately diluted was mixed with 0.4 mL distilled water in a 2 mL microcentrifuge tube, added 0.03 mL 5% NaNO₂ and allowed to react for 5 min. Following this, 0.03 mL 10% AlCl₃ was added and the mixture stood for further 5 min. Finally, to the reaction mixture 0.2 mL 1 M Na₂CO₃ and 0.24 mL distilled water were added and the absorbance at 510 nm was obtained against blank prepared similarly, by replacing extract with distilled water. Total flavonoid content was calculated from a calibration curve using catechin as standard and expressed as mg Catechin Equivalents (CTE) per 100 g dry weight.

**Total Flavanols**

Flavanols were determined after derivatisation with p-(dimethylamino)-cinnamaldehyde (DMACA), using the optimised protocol established by Nigel and Glories, 1991. Extract (0.2 mL) suitably diluted with MeOH was introduced into a 2-mL microcentrifuge tube and added 0.5 mL HCl (0.24 N in MeOH) and 0.5 mL DMACA solution (0.2% in MeOH). The mixture was allowed to react for 5 min at room temperature and the absorbance was obtained at 640 nm. Control sample was prepared by replacing sample with MeOH. Results were expressed as mg Catechin Equivalents (CTE) per 100 g dry weight.

**Proanthocyanidins**

The method described by Waterman and Mole, 1994, was used. Butanol reagent was prepared by mixing 70 mg ferrous sulphate (FeSO₄) with 5 mL conc. HCl and made to 100 mL with n-butanol. An aliquot of 0.05 mL sample was mixed thoroughly in a 2 mL, screw-cup vial with 0.7 mL butanol reagent and heated at 95°C in a water bath for 45 min. Following this the sample was cooled, added 0.25 mL n-butanol and the absorbance at 550 nm (A550) was obtained. Results were expressed as mg cyanidic equivalents (CyE) per 100 g dry weight, using as ε = 26900 and MW = 449.2.

**Total Anthocyanins**

The pH-differential methodology was used (Wrolstad et al., 2006). An aliquot of sample was mixed with an appropriate volume of potassium chloride buffer (pH = 1) and the absorbance was read at 520 (A₅20) and 700 nm (A₇0). Extracts were also combined similarly with sodium acetate buffer (pH = 4.5) and the absorbance was obtained at the same wavelengths. Total anthocyanin content was determined as malvin (malvidin-3-O-glucoside) equivalents (MvE) using as ε = 28000 and MW = 529, as follows:

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where:
\[ A = (A_{510} - A_{330})_{pH 1} - (A_{510} - A_{330})_{pH 4.5} \]

\[ F_D = \text{The dilution factor.} \]

**Antiradical Activity (A_{AR})**

A procedure previously reported (Arnous et al., 2002) was employed. Each extract was diluted 1:20 with methanol immediately before the analysis. Sample (0.025 mL) was added to 0.975 mL DPPH solution (73 μM in MeOH) and the absorbance was read at \( t = 0 \) (\( A_{510}^0 \)) and \( t = 30 \) min (\( A_{510}^{30} \)).

Results were expressed as Trolox® equivalents (mM TRE) per g weight using the following equation:

\[ A_{AR} = \frac{0.018 \times \% \Delta A + 0.017}{tw} \times F_D \]  

as determined from linear regression, after plotting \( \% \Delta A_{510} \) of known solutions of Trolox® against concentration;

where:

\[ \% \Delta A_{510} = \frac{A_{510}^{10} - A_{510}^{0}}{A_{510}^{10}} \times 100 \]

\( tw \) = The dry weight (g)

\( F_D \) = The dilution factor (20).

**Statistical Analyses**

All determinations were carried out at least in triplicate and values were averaged and given along the standard deviation (± SD). Correlations were established using regression analysis at a 95, 99 and 99.9% significance level. Differences among polyphenol indices and antiradical activity values were calculated using one-sample t-test at 95, 99 and 99.9% significance level. For all statistics, SPSS™ was used.

**RESULTS**

Previous studies on grape pomace extraction showed that a solvent system composed of acetone/McOH/water and acidified with 0.1% HCl was the most favourable over several other systems, in extracting polyphenols from grape pomace (Ju and Howard, 2003). Such a solution was employed for extraction of the pomace (Table 1) and the extracts obtained served as control samples.

The first step in the optimisation of an efficient solvent system was the examination of the effect of ethanol content (Fig. 1). Ethanol percentage in the solvent systems used varied from 28.5 to 85.5, a region that has been previously shown to provide high yield for grape seed extraction (Shi et al., 2003; Yilmaz and Toledo, 2006). A hydroalcoholic solution of 57% was found to be the most effective for high polyphenol recovery; as this was manifested by estimating TP (p<0.001), TFD (p<0.05), TF (p<0.001) and PC (p<0.001) yields (Table 1), giving also extracts with the highest A_{AR} (Fig. 2). However, the efficiency in extracting TF was not significantly high (p<0.05). Thus solutions composed of 57% ethanol were chosen for further testing the effect of the acidifying agent and S0.
Table 1. Polyphenolic indices of the red grape pomace extracts obtained by employing various extracting media. Values are expressed as mg per 100 g dry weight (dw) and represent means of duplicate determination (x 2).

<table>
<thead>
<tr>
<th>Extracting media</th>
<th>% ETOH</th>
<th>Acidification</th>
<th>% SO2</th>
<th>TPC</th>
<th>TFD</th>
<th>TP</th>
<th>TA</th>
<th>PC</th>
<th>TC/TP</th>
<th>NCTF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0%</td>
<td>-</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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<tr>
<td>1</td>
<td>26.5</td>
<td>-</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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<tr>
<td>2</td>
<td>57.0</td>
<td>-</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>3</td>
<td>85.5</td>
<td>-</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>4</td>
<td>57.0</td>
<td>0.1% HCl (pH 1.10)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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<tr>
<td>5</td>
<td>57.0</td>
<td>1% acetic acid (pH 3.69)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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</tr>
<tr>
<td>6</td>
<td>57.0</td>
<td>1% benzoic acid (pH 3.69)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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<tr>
<td>7</td>
<td>57.0</td>
<td>-</td>
<td>0.01% (pH 6.21)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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</tr>
<tr>
<td>8</td>
<td>57.0</td>
<td>-</td>
<td>0.02% (pH 7.07)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
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</tr>
<tr>
<td>9</td>
<td>57.0</td>
<td>-</td>
<td>0.04% (pH 7.35)</td>
<td>0.0%</td>
<td>0.0%</td>
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* Total polyphenols (mg CTE per 100 g dw), † Total flavonoids (mg CTE per 100 g dw), ‡ Total anthocyanins (mg CTE per 100 g dw), § Monomeric anthocyanins (mg CTE per 100 g dw), 1 extracting medium, 2 control extraction performed employing 0.1% HCl in MacPherson’s water (MSW), 3, 4 Superscripted Greek letters α, β and γ denote statistical differences at α 0.05, 0.01 and 0.005 significance levels, respectively.

Fig. 1: Overview of the procedures carried out for obtaining extracts with optimal antioxidant properties from red grape pomace, employing water/ethanol mixtures. Assignments: AA: Acetic Acid; ETOH: Ethanol; TA: Tartaric acid

Addition of any of the agents tested, including 0.1% HCl, 1% acetic acid and 1% tartaric acid, resulted in decreased polyphenol yield compared with the non-acidified 57% ethanol (Table 1). As can be seen, TP yields were almost 50% reduced compared with that obtained with 57% ethanol. The decrease was even higher on the basis of TFD, but less so with regard to TP and PC. By contrast, TA yields obtained were increased. In addition, all extracts exhibited lower AA compared with both the control extract and that obtained with 57% ethanol (Fig. 2).

Incorporation of 0.01% SO2 in 57% ethanol resulted in less polyphenol recovery in relation with the control and the 57% ethanol extract, respectively, but it was also observed that this combination recovered high TP amounts (p<0.01), 55.7% of which were polymerised. In cases where SO2 was added at levels 0.02 and 0.04%, decreases in TP, TFD and PC were even more pronounced.
Fig. 2: Effect of ethanol content, acidifying agent (0.1% HCl, 1% acetic acid and 1% tartaric acid) and SO₂ concentration on the antiradical activity (Aₐα) of extracts from the red grape pomace. Values are expressed as trolox™ equivalents (TRE) per g of dry weight. Marks *, ** and *** denote statistical difference at a 99.9, 99 and 95% significance level. Assignments: C: Control; AA: Acetic Acid; Aₐα: Antiradical Activity; EtOH: Ethanol; TA: Tartaric Acid

Table 2: Statistical parameters calculated after linear regression between polyphenol groups and antiradical activity

<table>
<thead>
<tr>
<th>Polyphenol groups</th>
<th>R²</th>
<th>p-value</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>0.1388</td>
<td>0.2890</td>
<td>1×10⁻⁴</td>
</tr>
<tr>
<td>TFd</td>
<td>0.2099</td>
<td>0.1013</td>
<td>2×10⁻⁴</td>
</tr>
<tr>
<td>TF</td>
<td>0.1175</td>
<td>0.3322</td>
<td>4×10⁻⁴</td>
</tr>
<tr>
<td>TA</td>
<td>0.0991</td>
<td>0.3757</td>
<td>3.2×10⁻¹</td>
</tr>
<tr>
<td>PC</td>
<td>0.0165</td>
<td>0.7237</td>
<td>1.6×10⁻¹</td>
</tr>
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</table>

Furthermore, the extract obtained was the most active in scavenging radicals (p<0.001). Simple linear regression analysis gave no significant correlation between any of the polyphenolic classes and Aₐα (Table 2), suggesting that the antioxidant potency of extracts does not depend on particular substances. Solutions with ethanol content of 85.5% were proven ideal in extracting anthocyanins, providing the highest yields (Table 1). A 57% ethanolic solution acidified with 0.1% HCl extracted almost 1.2 times more anthocyanins than the non-acidified one, but it was equally less efficient than the 85.5% ethanolic solution in this regard. No improve in anthocyanin yield was seen upon addition of SO₂.

**DISCUSSION**

Grape and wine by-products are exceptionally rich in polyphenolic substances and therefore seeking for environmentally friendly and cost-effective processes of recovery is imminent. The results of this study permitted a deeper insight into this direction, by providing data on the efficiency of water/ethanol mixtures for obtaining extracts from red grape pomace.

The concept underlying the monitoring of the procedure by estimating the antiradical activity was based on the fact that the potential of a complex matrix, such as a red grape pomace extract, in expressing antioxidant effects is not simply attributed to the totality of polyphenols (Qu et al., 2002; Hassimoto et al., 2005; Kallithraka et al., 2005). Indeed, simple linear regression analysis clearly showed that the activity of the extracts obtained did not correlate with any particular polyphenol
group to a significant level (Table 2). These findings are consistent with observations reported for red wines, where in spite of examinations supporting the high antioxidant efficiency of purified fractions rich in specific phenolics (Ghiselli et al., 1998; Saint-Cricq de Gaulejac et al., 1999), other studies showed that the contribution of individual classes may be less pronounced (Burns et al., 2000; Rigo et al. 2006; Landraufet et al., 2001; Amous et al., 2001). This hypothesis may be more clearly illustrated taking into consideration that grape pomace also contains other flavonoid polyphenols, principally flavonols, which have been demonstrated powerful radical quenchers in various systems (Rice-Evans et al., 1995; Rice-Evans and Miller, 1996; Fukumoto and Mazza, 2000).

Large amounts of polyphenols were recovered at an ethanol level of 57%, while the means of acidification was also an important parameter in this regard. Similar ethanol levels were also ideal for the extraction of polyphenols from other tissues, including purple sunflower hulls (Caceca and Mazza 2003), grape seeds (Shi et al. 2003; Yilmaz and Toledo, 2006) and star fruit residues (Shui and Leong, 2006). Contrary to that, anthocyanin extraction was more efficient with higher ethanol levels, but acidification by any means resulted in significantly reduced yields. This is somewhat paradox, because in general acidic solvents are more efficient for anthocyanin extraction compared with neutral ones (Revilla et al., 1998). On the other hand, solvent composition affects profoundly its physical properties, such as density and dynamic viscosity, which affect diffusion and rate of extraction. Also composition influences the dielectric constant. It has been proposed that reduction of dielectric constant of a protic solvent such as water (€ H₂O = 78.5) into the range of intermediate-behavior solvents such as methanol (€ MeOH = 32.6) or ethanol (€ EtOH = 24.3) by modifying pressure or temperature, can improve extraction of anthocyanins (Caceca and Mazza, 2002).

On the other hand, the addition of relatively low amounts (0.01%) of SO₂ in 57% ethanolic solutions had a positive impact, in that it gave extracts with significantly increased A₈₀₀ (p<0.001, Fig. 2). Although sulphured water has been proposed as an efficient means of extracting anthocyanins from black currants (Caceca and Mazza, 2002), in this case the amount of pigments recovered did not differ substantially (p>0.05, Table 1). However, the amounts of SO₂ used were 10 to 12-fold lower than those employed for anthocyanin recovery from black currants. Gao and Mazza (1996) reported that 200 mg L⁻¹ of an aqueous SO₂ solution was the optimum concentration for anthocyanin extraction from purple sunflower hulls. It was stressed that higher concentrations increase pH and consequently the instability of anthocyanins, leading in losses presumably through degradation.

It should be emphasised that the selection of a solvent system that favours obtaining extracts with high scavenging potential was the primary objective, but the efficiency of anthocyanin recovery was considered as an additional, critical criterion for assessing the procedure. Thus deploying the solvents that were shown to be suitable for preparing highly potent extracts may consist a compromise with regard to anthocyanin extraction and vice versa. As a future prospect, it can be supported that the utilization of ethanol, a bio-solvent that is relatively cheap, reusable and non-toxic, could be an environmentally friendly manner for low-cost, low-tech preparation of potentially bioactive extracts from vitification and presumably other agri-food by-products. The last option is contingent on the quality and characteristics of the food processing residual. To the extent similar procedures can be developed on an industrial scale, benefits for industry and environment could be maximized.

CONCLUSIONS

The outcome of this study can be summarised as follows:

- A solvent system consisting of 57% aqueous ethanol was found to be very efficient for red grape pomace extraction, with regard to total polyphenol, total flavonoid and proanthocyanidin recovery.
• Optimal results assessed on the basis of antiradical activity were obtained after addition of 0.01% SO₂ to 57% ethanolic solution. Acidification with either HCl, tartaric or acetic acid was not favourable.
• A simple system consisting of 85.5% ethanol was found ideal for increased anthocyanin yield.
• A solvent system based on water/ethanol mixtures, efficient for preparation of extracts with both high antioxidant potency and high anthocyanin concentration from red grape pomace cannot be proposed. The systems that appear to be suitable for preparing extracts with high antiradical potency may consist a compromise with regard to anthocyanin extraction and vice versa.

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REFERENCES


