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Characterization of phenolics, *in vitro* reducing capacity and anti-glycation activity of red grape skins recovered from winemaking by-products

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HIGHLIGHTS

- Phenolic characterization of red grape skins was performed by UPLC-DAD-MS.
- Glycation was studied by bovine serum albumin-fructose/methylglyoxal model systems.
- Red grape skins had high anti-glycation efficacy compared to nutraceutical products.
- Red grape skins can be cost-effective sources to prevent hyperglycemia's complications.

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ABSTRACT

Red grape skins recovered from ten winemaking processes were analyzed for total phenolic content (Folin Ciocalteu assay), proanthocyanidins (*n*-butanol/HCl assay), individual phenolics (UPLC-DAD-MS), *in vitro* ferric ion reducing capacity and anti-glycation activity by bovine serum albumin/fructose and bovine serum albumin/methylglyoxal model systems. The aim was to assess if these by-products have potential as dietary anti-glycation agents, to prevent the glyco-oxidative stress associated with type-2 diabetes.

Variability was observed in total phenolics (12.1–53.6 g gallic acid Eq/kg), proanthocyanidins (7.2–51.1 g/kg), anthocyanins (2.5–13.8 g malvidin 3-O glucoside Eq/kg), flavonols (0.3–2.6 g quercetin 3-O glucoside Eq/kg) and reducing capacity (103–511 mmol Fe(II) Eq/kg). For all samples, the anti-glycation effectiveness was higher than that of commercial nutraceutical preparations. Hence, in spite of differences in cultivar, location of the vineyard and winemaking procedures, these by-products could be used as a source of cost-effective anti-glycation agent either as a food ingredient or as a nutraceutical preparation.

1. Introduction

The winemaking industry comprises a significant portion of the global food industry. As a result, it generates a substantial volume of by-products, which can be re-utilized as organic fertilizer (Ferrer et al., 2001) substrate for fermentation for biomass production (Fiori et al., 2012) and source of nutritionally-relevant compounds. Their utilization includes the production of both phenolic extracts (Louli et al., 2004; Makris et al., 2007; Spigno et al., 2007; de Campos et al., 2008) and phenolic- and fiber- rich fractions (Saura-Calixto, 2011), which could be used as bioactive food ingredients.

In fact, the "French paradox" demonstrated the inhibition of human low-density lipoprotein oxidation by grape and wine phenolics and initiated numerous studies on these secondary metabolites (Teissedre et al., 1996). The antioxidant properties of grapes have been clearly identified as being responsible for protection towards oxidative stress *in vivo* and inhibition of the progression of atherosclerosis (Vislocky and Fernandez, 2010). Emerging research has also demonstrated that grapes have beneficial effects on other chronic degenerative diseases such as cancer, Alzheimer's disease, age-related cognitive decline and diabetes (Vislocky and Fernandez, 2010).

Abbreviations: AGEs, advanced glycation end-products; AGE-I_{50}, amount of standard or sample that inhibits the glycation reaction by 50%; GAE, gallic acid equivalents; FRAP, ferric ion reducing antioxidant power; BSA, bovine serum albumin; MGO, methylglyoxal; CE, catechin equivalents; BA, Barbera; CR, Croatina; FR, Freisa; DO 1, Dolcetto 1; DO 2, Dolcetto 2; GR, Grignolino; NR, Neretto; NE, Nebbiolo; PI 1, Pinot nero 1; PI 2, Pinot nero 2.

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An overwhelming body of evidence indicates that non-enzymatic glycation of proteins is implicated in a number of biochemical abnormalities associated with both ageing and diabetes and their related pathologies, such as arthritis, atherosclerosis, chronic renal insufficiency, Alzheimer's disease, nephropathy, neuropathy and cataract (Saraswat et al., 2009). The pattern of protein glycation *in vivo* is complex, starting from the reaction of amino groups with glucose or fructose, to give Schiff bases, which then undergo an Amadori rearrangement. Following Amadori rearrangement, reactive α-dicarbonyls are formed, such as methylglyoxal (MGO), which is also formed from other degradation patterns. Despite the complex patterns of protein glycation, the resulting advanced glycation endproducts (AGEs) produce the same chemical outcome, i.e., the formation of covalent cross-links between proteins, which damage their functionality (Singh et al., 2001).

Natural compounds such as polysaccharides (Rout and Banerjee, 2007) and phenolics (Matsuda et al., 2003) have been shown to have anti-glycation activity. Phenolics, especially flavonoids, exert anti-glycation activity, by acting not only as radical scavengers and metal chelators, but also as carbonyl trapping agents (Matsuda et al., 2003). For flavonoids, the structural requirements for maximum anti-glycation activity are only in part the same as for antioxidant activity (Matsuda et al., 2003).

Various phenolic-rich herbs and spices could be regarded as natural sources of anti-glycation agents, either as food ingredients or as nutraceutical preparations (Saraswat et al., 2009). Berries and grapes have also been demonstrated to scavenge reactive carbonyls (Wang et al., 2011; Farrar et al., 2007). Regarding grape-derived products, especially grape pomace, there are wide differences in the phenolic content, depending on grape variety, vineyard location and winemaking procedures. On the other hand, the food industry needs standardized ingredients to be incorporated in specifically-designed foods for target consumers. Therefore, despite grape pomace being an easily available resource, its variability in composition could be a hurdle for its utilization as a natural food ingredient. The current study focuses on UPLC-DAD-MS polyphenol identification and evaluation of *in vitro* reducing capacity and anti-glycation activity of grape skins recovered from different wineries and grape cultivars. The aims were: (a) to characterize these by-products and (b) to assess if efficient anti-glycation extracts can be produced overcoming the differences in raw material quality.

2. Methods

2.1. Chemicals

Malvidin-, cyanidin-, delphinidin-, peonidin- and petunidin- 3-O-glucosides were purchased from Polyphenols (Sandes, Norway). Pycnogenol® (proanthocyanidins from pine bark) was obtained from Horphag (Geneva, Switzerland). Procyanidin dimer A2 and Leucoselect® (proanthocyanidins from grape seeds) were obtained from Indena (Settala, Mi, Italy). All other standard and chemicals were purchased from Sigma – Aldrich (Milan, Italy).

2.2. Grape pomace

Grape pomace samples of Barbera (BA), Croatina (CR), Freisa (FR), Dolcetto (DO 1 and DO 2, corresponding to the same variety processed by two different wineries), Grignolino (GR), Neretto (NR), Nebbiolo (NE), Pinot Nero (PI 1 and PI 2, corresponding to the same variety processed by two different wineries) were kindly provided by different wine-makers located in Northern Italy. At the winery, the pomace samples were sieved (with a 5 mm sieve) to separate the skins from the seeds and frozen to inhibit microbial growth leading to product degradation (Lavelli et al., 2006). Then, the samples were transported frozen to the lab, dried at 50 °C for about 8 h, milled and sieved to obtain fractions having particle sizes in the range: 125–250 μm. These samples were stored under vacuum, in the dark, at 4 °C, until performing characterization studies.

2.3. Moisture content

Moisture content of the red skin powders was determined by drying in a vacuum oven at 70 °C and 50 Torr for 18 h.

2.4. Sample extraction

About 100 mg of grape skin powder was extracted with 8 mL methanol:water:HCl (80:20:0.1, v/v/v), for 2 h at room temperature with continuous stirring. The mixture was centrifuged at 10,000*g* for 10 min, the supernatant recovered and the solid residue was re-extracted using 6 mL of the same solvent twice. The three supernatants were pooled, dried under vacuum at 35 °C and the residue was suspended in 10 mL of methanol:water:HCl (20:80:0.1, v/v/v). The solutions were centrifuged at 2000*g* for 1 min before the UPLC analysis. For a better identification of flavonols, 2 g of grape skin powder was extracted four times according to the above procedure and the final residue suspended in 30 mL.

2.5. UPLC-DAD-MS analysis

The chromatographic system consisted of an UPLC mod. Acquity (Waters, Milford, MA, USA) equipped with a mod. E-lambda photodiode array detector (Waters) and a triple quadrupole mass spectrometer mod. Quattro micro (Waters), operated by Masslynx 4.0 software (Micromass) with Quan-Optimize option for fragmentation study.

2.5.1. Anthocyanins analysis

A 2.6 μm Kinetex C_{18} column (250 × 4.6 mm; Phenomenex) was used for the separation at a flow-rate of 1.8 mL/min. The column was maintained at 40 °C. The separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% trifluoroacetic acid; (B) acetonitrile:trifluoroacetic acid 0.1% (30:70, v/v). The gradient was as follows: 14% B for 15 min, 14–20% B in 10 min, 20–32% B in 10 min, 32–50% B in 10 min; 50–90% B in 3 min; 90% B for 3 min. Data acquisition was performed as described previously (Del Bo et al., 2010). In brief, DAD analysis was carried out in the range of 200–700 nm (integration at 520 nm). Mass spectrometer was operated in positive full-scan mode in the range 200–800 *m/z*. The capillary voltage was set to 3.5 kV, the cone voltage was 20 V, the source temperature was 130 °C, the desolvating temperature was 250 °C and nitrogen was used as carrier gas. The calibration curve was obtained with malvidin 3-O-glucoside and results were expressed as milligrams of malvidin 3-O-glucoside Eq per kilogram of dry product.

2.5.2. Flavonols analysis

A 1.7 μm BEH C_{18} column (150 × 2.1 mm; Waters) was used for the separation at a flow-rate of 0.55 mL/min. The column was maintained at 55 °C and the separation was performed by means of a linear gradient elution. Eluents were: (A) 0.1% formic acid; (B) 0.1% formic acid in acetonitrile. The gradient was as follows: 5–20% B in 9 min, 20–35% B in 3 min, and then 80% B for 3 min. DAD analysis was carried out in the range of 200–450 nm (integration 354 nm). Mass spectrometer operated in negative full-scan mode in the range 100–1000 *m/z*. The capillary voltage was set to 3 kV, the cone voltage was specific for each compound, the source temperature was 130 °C, the desolvating temperature was 300 °C