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White grape pomace extracts, obtained by a sequential enzymatic plus ethanol-based extraction, exert antioxidant, anti-tyrosinase and anti-inflammatory activities

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ABSTRACT

The present work aimed at optimizing a two-step enzymatic plus solvent-based process for the recovery of bioactive compounds from white grape (*Vitis vinifera* L., mix of Trebbiano and Verdicchio cultivars) pomace, the winemaking primary by-product. Phenolic compounds solubilised by water enzyme-assisted and ethanol-based extractions of wet (WP) and dried (DP) pomace were characterised for composition and tested for antioxidant, anti-tyrosinase and anti-inflammatory bioactivities. Ethanol treatment led to higher phenol yields than water extraction, while DP samples showed the highest capacity of releasing polyphenols, most probably as a positive consequence of the pomace drying process. Different compositions and bioactivities were observed between water and ethanol extracts and among different treatments and for the first time the anti-tyrosinase activity of *V. vinifera* pomace extracts, was here reported. Enzymatic treatments did not significantly improve the total amount of solubilised compounds; Celluclast in DP led to the recovery of extracts enriched in specific compounds, when compared to control. The best extracts (enzymatic plus ethanol treatment total levels) were obtained from DP showing significantly higher amounts of polyphenols, flavonoids, flavanols and tannins and exerted higher antioxidant and anti-tyrosinase activities than WP total extracts. Conversely, anti-inflammatory capacity was only detected in water (with and without enzyme) extracts, with WP samples showing on average a higher activity than DP. The present findings demonstrate that white grape pomace constitute a sustainable source for the extraction of phytochemicals that might be exploited as functional ingredients in the food, nutraceutical, pharmaceutical or cosmetic industries.

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1. Introduction

The large volumes of both liquid and solid by-products generated by the food processing industry poses major environmental and economic challenges. The waste load at a food processing plant can be significantly reduced through the application of new or modified industrial processes and reuse methods especially developed to convert by-products into added-value biomolecules and biofuels [1]. In fact, food by-products contain numerous high-value compounds, which could be reused in the food, feed, cosmetic and pharmaceutical industries. Grape (*Vitis* sp.) pomace constitutes the major by-product of the wine making process, representing generally 20–30% of the processed grapes weight [1]. It is mainly made up of pressed skins, seeds and stems of the fruit with a composition that varies considerably depending on grape variety and winemaking technology. Wine-makers sometimes produce spirit from grape by-products, but pomace is also traditionally used as animal feed or fertilizer [1,2]. It has been estimated that more than 9 million tons of grape pomace are generated annually [3], which causes a serious environmental and disposal problem for wineries. However, solid by-products from wine industry are rich in phenolic compounds, in particular

Abbreviations: AA, ascorbic acid; Abs, absorbance; ABTS, 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAT, catechin; DP, dried pomace; DW, dry weight; GA, gallic acid; KA, kojic acid; WP, wet pomace.

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flavonoids (such as anthocyanins and flavanols), phenolic acids and stilbenes [4–6], many of which have been shown to be beneficial to human health due to their antioxidant capacity which is believed to contribute to the prevention of cancer and cardiovascular diseases [7]. Anti-inflammatory, anti-microbial and anti-cholesterol properties have also been attributed to grape polyphenols [4,5,8]. Due to these properties, there is a vast array of potential applications for grape pomace extractable components: as ingredients of functional foods and feeds, cosmetics and nutraceuticals; and as natural colorants and preservatives of foods [4,6]. Moreover, nowadays there is a growing interest in finding phytochemicals than can be used as an alternative to synthetic substances, which are sometimes perceived by consumers as harmful to human health [1,2].

Solvent (mainly water, ethanol and methanol) and supercritical fluid extractions are the most efficient in recovering phenolic compounds from grape pomace [2,9,10]. Available studies regarding phenolic compositions, extractions and applications are mainly focused on pomace from red grape varieties, whereas little attention has been devoted to white grape pomace, which also contains a wide spectrum of potentially bioactive phenols. Moreover, to the authors' knowledge, phenolic extraction by means of cell wall polysaccharide degrading enzyme mixtures has only been reported for red pomace [3,5,11]. In contrast to red wines, white wines are usually made by immediately removing, after grape pressing, the pomace from the must. As a consequence, a much lower phenolic content is present in white wines with respect to red ones. The differences between red and white wine production processes may also affect the amount and availability of polyphenols in the resulting pomace. In fact, as white grape must is not usually fermented in contact with the solid parts of the grape, higher contents of phenolic compounds are present in white compared to red pomace [12,13].

In the present work, enzyme-assisted and ethanol-based extractions were combined in a two-step process aimed at the recovery of phenolic compounds from wet pomace (WP) and dried pomace (DP) of white grapes (*Vitis vinifera* L., mixture of Trebbiano and Verdicchio cultivars). The extracts with the highest amount of compounds were characterised for phenolic composition and tested for antioxidant, anti-tyrosinase and anti-inflammatory bioactivities.

2. Materials and methods

2.1. Materials

White pomace, derived from a mix (60:40) of *V. vinifera* cv. Trebbiano and Verdicchio, was supplied by the Cantine Moncaro wineries (Jesi, Ancona, Italy) immediately after wine production. Pomace was either frozen (wet pomace, WP) or dried (dried pomace, DP) in an industrial vented oven (60 °C for 24 h) and stored at −20 °C until used for analyses. Different WP and DP lots were pulled together before grinding in order to minimise the biological differences.

2.2. Enzyme-assisted and ethanol-based pomace extractions

White pomace was treated as previously described by Ferri et al. [5]. WP was ground in a kitchen blender with the addition of distilled water (1:5 g/mL), while DP was ground directly and rehydrated with distilled water (1:5 g/mL for 1 h) just before enzymatic digestion. Enzyme-assisted extractions of WP and DP pomace suspensions (20 mL aliquots) were carried out by adding different concentrations (0.5, 1 or 2% enzyme volume/pomace DW) of Pectinex 3XL, Pectinex Ultra SPL, Termamyl, Fungamyl, Pentopan 500BG or Celluclast (Sigma-Aldrich, Milan, Italy). The enzymatic treatments were incubated on an orbital shaker (150 rpm) at different incubation times (2, 6, 24 h) and at each enzyme's optimal working temperature (24, 30 or 37 °C) [5]. Controls without the addition of enzymes, were also prepared. After incubation, the supernatant (water extract) and pellet fractions were collected [5]. The pellet was extracted overnight with 30 mL of 95% v/v ethanol at 24 °C, the supernatant was separated from the pellet and stored at −20 °C until further analyses.

2.3. Extract characterisation

Water and ethanol extracts were characterised spectrophotometrically for phenolic [14,15], flavonoid [15,16] and flavanol [17] contents. The results were expressed, respectively, as gallic acid (GA) and catechin (CAT) equivalents by means of calibration curves. The amount of tannins was analysed according to Porter et al. [18]; briefly, two aliquots of 0.3 mL of each sample were mixed with 0.9 mL of reagent (50% *n*-butanol, 50% 12N HCl, 0.015% $FeCl_3$) and incubated for 30 min, one at room temperature and the second at 100 °C. Absorbance (Abs) was measured at 550 nm and tannin content (g/L) was calculated as ($Abs_{100\,°C} - Abs_{room\,temperature}$) x 0.1736.

Both water and ethanol supernatants were analysed for phenolic compounds by HPLC-DAD as previously described [5,19]. The adopted HPLC-DAD separation procedure allowed for the simultaneous analysis of 28 different compounds among stilbenes, phenolic acids and flavonoids [19]: *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid, *trans*- and *cis*-resveratroloside, piceatannol; gallic, protocatechuic, syringic and vanillic acids; caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic and *trans*-cinnamic acids; catechin, epicatechin, epigallocatechin gallate (EGCG), epicatechin gallate, epigallocatechin; vanillin, naringenin, quercetin, rutin, myricetin and kaempferol.

2.4. Determination of biological activities

In vitro antioxidant activities were measured using the 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method with minor modifications [15]. The results were expressed as ascorbic acid (AA) equivalents by means of a dose-response calibration curve.

Anti-tyrosinase activity was assessed by an optimised tyrosinase enzyme inhibition assay [20]. The kinetics of brown colour formation was evaluated by absorbance measurement (490 nm) in reactions containing 10 U of tyrosinase and 2 mM L-DOPA (the substrate) in the presence of the sample. The results were expressed as kojic acid (KA, a well-known tyrosinase inhibitor) equivalents by means of a calibration curve (between 1 and 10 μg of KA).

A bioluminescent cell-based assay for anti-inflammatory activity was performed using human embryonic kidney HEK293 cells ATCC (American Type Culture Collection, Manassas, VA, USA) routinely grown in Dulbecco modified essential medium (DMEM high glucose 4.5 g/L, GE Healthcare) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/μL penicillin, and 50 μg/mL streptomycin. HEK293 cells were plated in 96-well plates the day before transfection at a density of 2×10^4 cells/100 μL of growth medium per well. Co-transfection was performed with plasmid pGL4.32[luc2P/NF-κB-RE/Hygro] containing five copies of the NF-κB response element (NF-κB-RE) driving transcription of the luc2P reporter protein (Promega, Madison, WI) and with plasmid pcDNA.3.1-mcherryPRET9 expressing a thermostable mutant *P. pyralis* luciferase [21], and by using FuGENE®HD (Promega) according to the manufacturer's instructions. During co-transfection, cells were incubated for 24 h at 37 °C with 5% CO_2. After transfection cells were co-incubated for 5 h with 100 μL of

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