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Offline combination of pressurized fluid extraction and electron paramagnetic resonance spectroscopy for antioxidant activity of grape skin extracts assessment☆

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ABSTRACT

A comprehensive characterization of grape skin methanolic and ethanolic extracts prepared by pressurized fluid extraction (PFE) at various temperatures within 40 to 120 °C from two wine grape varieties, St. Laurent and Alibernet was performed. For the first time, an offline combination of PFE and electron paramagnetic resonance (EPR) spectroscopy together with other experimental methods was employed to assess the effect of extraction conditions on numerous extract characteristics including antioxidant or radical-scavenging ability, HPLC profile of anthocyanins, total phenolic compounds content (TPC), tristimulus color values (CIE Lab), and pH values. The properties of extracts depend on the solvent used, the mass of grape skins as well as on the extraction conditions among which the temperature plays a crucial role. In spite of wide interval of extraction temperatures, all extracts still retain their antioxidant and/or radical-scavenging properties, indicating that the extracts prepared by PFE can serve as potential source of functional food supplements or color enhancers.

1. Introduction

Grape skins contain a large number of polyphenolic compounds the concentration of which varies greatly according to the variety of grapevine, cultivar, season and environmental factors. Up to date, more than 8000 different phenolic structures have been recognized. They include both flavonoids (e.g., flavonols, flavan-3-ols as well as polymers of the latter, defined as procyanidins and anthocyanins) and non-flavonoids (e.g., hydroxycinnamates and hydroxybenzoates). Anthocyanins represent the most abundant group of polyphenols and are associated with the color of several aerial and subterranean organs in many plants [1–6].

In grapevines, anthocyanins are accumulated in leaves during ripening and are responsible for the coloration of grape skins in red and rosé cultivars, and in the grape pulp, respectively [1–6]. The most abundant anthocyanins identified in Vitis vinifera grapes and wines are the 3-O-glucosides, 3-O-acetyl glucosides and 3-O-p-coumaroyl glucosides of delphinidin (De), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) as the dominant form. Besides them, tartaric esters of hydroxycinnamic acids, monomeric and dimeric flavanols, flavonols and stilbenes were also identified [4–6].

These pigments are water soluble, revealing also the beneficial effects on human health, including the enhancement of visual acuity, but evinced also anticarcinogenic, antimutagenic or anti-inflammatory action [7–10].

Although grape skins represent the best source for their isolation, only about 30–40% of polyphenols (mainly anthocyanins) are extracted from grapes during the winemaking process [5]. Thus, in order for polyphenols to be used as food supplements, efforts have recently been made to find a suitable extraction system for their isolation from grapes in larger quantities. One also has to respect the limited stability of polyphenols under specific conditions [2,11].

Grape anthocyanins are frequently extracted by conventional extraction techniques using acidified methanol, ethanol, acetone or their aqueous mixtures. However, the use of acidic solvents may lead to the denaturation of cellular membranes, thus facilitating, besides other processes, the solvolysis of anthocyanins. In addition, the extraction process is time consuming and laborious [12,13].

Pressurized fluid extraction (PFE) [14–17] operating at elevated temperatures and pressures was previously used for the extraction of different phenolic compounds from grapes and wines [18–20]. The advantages of PFE over conventional extraction techniques can briefly be summarized as follows: higher temperature increases

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the solubility, diffusion rate and mass transfer of the compounds and decreases the viscosity and surface tension of the extraction solvent. These changes improve the contact of analytes with the solvent and enhance extraction which can be achieved more rapidly with less solvent consumption. Moreover, the elevated pressure improves the contact of solvent with the analytes trapped in the matrix pores [21].

For ecological and economical reasons, ethanol, methanol and water are the most frequently employed solvents in PFE. Ju and Howard [7] investigated the effect of different solvents and temperature on the efficiency of PFE of anthocyanins from the skin of highly pigmented red wine grapes. They concluded that 60% acidified methanol at 60 °C extracted the highest level of anthocyanins [7,22]. The type and polarity of the extracting solvent influence not only the yield and the composition of isolated polyphenols, but also the antioxidant activity of the final extract. As follows from available data, maximum total phenolic extraction yields were obtained with methanol but optimal solvent providing maximum antioxidant activity is required for each substrate [23]. In this context, it should be noted here that different relationships between phenolic content and antioxidant activity have been reported, some authors found a positive correlation while the others were not able to find any relationship [1,2,23–28]. Moreover, as follows from previously published papers, polyphenols can act as either anti- or pro-oxidants depending on the reaction conditions [26,29–31].

Various methods have been used to monitor and to compare the radical-scavenging or antioxidant activity of foods and biosystems, among which electron paramagnetic resonance (EPR) spectroscopy, due to its high sensitivity and selectivity, is considered to be one of the most efficient [32–36]. Most frequently, the ability of the studied system to scavenge different free radicals, e.g., 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl (•DPPH), or hydroxyl radical (•OH) added or generated directly in the experimental system is monitored [8,26,37–39]. As the redox reaction occurring in the experimental system is frequently accompanied by the development or disappearance of color at specific wavelength, UV–vis spectroscopy is frequently effectively involved as well [1,2,11,15–17,24–27,40].

Methods of multivariate statistics, notably principal component analysis (PCA) and canonical discriminant analysis (CDA) [41], represent valuable tools, making possible the categorization of different food samples via the consideration of many variables that can be measured, often in a single analytical level. The analysis of chromatographic, electrophoretic or elemental data was previously effectively used for the discrimination of many kinds of foods, e.g., of cheeses and cheese-derived products or wines, based on the different geographical origin, varieties or quality, but also for the prediction of spices γ-irradiation [42–44].

In this contribution, a comprehensive study of grape skin methanolic and ethanolic extracts prepared by PFE at various temperatures from 40 up to 120 °C from two wine grape varieties, St. Laurent and Alibernet from Velké Pavlovice and Mikulov subregions (South Moravia region, Czech Republic) is presented. To the best of our knowledge, this is the first application of the offline combination of PFE and EPR. The latter was used to assess the effect of the extract preparation procedure on the radical-scavenging ability of the extracts. Other basic characteristics of extracts prepared by PFE, i.e. the HPLC profile of polyphenols as well as total phenolic compounds' content (TPC), tristimulus color values (CIE Lab color space) and pH values were simultaneously monitored as well, in order to obtain as comprehensive information on extracts quality as even feasible.

All the experimental data were processed with PCA and CDA to specify the optimum extraction conditions for extract preparation from the perspective of the potential further application of the extracts as food supplements.

2. Materials and methods

2.1. Sample characterization

Grape skins from two wine grape varieties, St. Laurent and Alibernet from Velké Pavlovice and Mikulov sub-regions (South Moravia region, Czech Republic), collected in 2007 vintage were used in experiments. Harvested grapes were placed into polystyrene boxes filled with dry ice and transported to the laboratory. Grape skins were then manually separated from the pulps at inert atmosphere and lyophilized. The dried skins were ground to a fine powder under liquid nitrogen and stored at −20 °C in dark glass vials.

2.2. Chemicals

The substances used in experiments included 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), freshly distilled before use and stored at −18 °C under argon (Sigma Aldrich Ltd, Milwaukee, WI), stable free radical 1,1-diphenyl-2-picrylhydrazyl (•DPPH) (Fluka, Buchs, Switzerland), 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) salt (ABTS), (Polysciences, Inc., Warrington, PA), $K_2S_2O_8$ (Merck GmbH, Darmstadt, Germany), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma Aldrich Ltd, Milwaukee, WI, USA), H_2O_2, NaOH, $Fe(NH_4)_2(SO_4)_2{\cdot}9H_2O$ (Lachema Brno, Czech Republic), methanol and ethanol of spectroscopic grade purity (AFT, Bratislava, Slovak Republic), and deionized water. The standards for HPLC were Brillant blue FCF (Lachema, Brno, Czech Republic) and 3-O-monoglucosides of malvidin (Mv-3-glc), delphidin (De-3-glc), peonidin (Pn-3-glc), cyanidin (Cy-3-glc), petunidin (Pt-3-glc), and pelargonidin (Pg-3-glc) of analytical grade purity (Polyphenols Laboratories AS, Sandnes, Norway). These 3-O-monoglucosides constitute the principal portion of grape anthocyanins [1–6] and were selected for the present study as a result of trade-off between the need for a representative selection of the antioxidants present in grapes and the resources available. We believe that the selection introduces a tolerable uncertainty into the results described below.

2.3. Pressurized fluid extraction

Static PFE of grape skins was performed using a *one*PSE extractor (Applied Separations, Allentown, PA). A respective portion of grape skin powder (0.5 and 1.0 g, respectively) was placed into 11 ml extraction cell containing inert material (glass beads, 570–700 μm) at the bottom. The extraction was performed using methanol and ethanol as extraction solvents, respectively, under the following conditions: extraction temperature, 40–120 °C (20 °C step); pressure, 15 MPa; extraction time, 3 × 5 min; rinsing time, 20 s; nitrogen purge time, 20 s after each cycle and 120 s after the extraction run. After the PFE run, the extract was cooled to 5 °C and stored in a fridge until further analysis. Two extracts were prepared in parallel under the same conditions for each amount of grape skins. The extracts were diluted with the corresponding solvents, if appropriate. Further details on extracts composition, conditions of preparation as well as their basic characteristics are summarized in Table 1 and Table 2 for methanolic and ethanolic extracts, respectively.

Just for comparison of the extraction methods efficiency from the viewpoint of polyphenols composition and concentrations, conventional Soxhlet extraction was also employed for methanolic and