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Supercritical antisolvent extraction of antioxidants from grape seeds after vinification

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ABSTRACT

The objective of this study is the extraction and concentration of antioxidants (catechin, epicatechin, gallic acid and resveratrol) from grape seeds vinification wastes using supercritical antisolvent extraction (SAE). The grape seeds (*Vitis vinifera*, *Syrah* variety) were defatted, and, then extracted with ethanol. The antioxidants extracted in the resulting solution were concentrated using the SAE technique, in which supercritical CO_2 is used to precipitate selected, non soluble compounds. Several experiments were carried out using pressures ranging from 8 to 15 MPa and temperatures ranging from 35 to 60 °C. The content of the antioxidants in the different extracts was determined using high performance liquid chromatography (HPLC). The determination of the total polyphenols content (TPC) by the method of Folin-Ciocalteau was also accomplished. Operating at 15 MPa and 40 °C, the extracts after SAE processing were enriched in antioxidants of more than 150% with respect to the starting extracts.

1. Introduction

Wine industry produces per year large amounts of residues and by-products in form of skins and seeds that are generally untapped. Indeed, around 1.4 kg of grapes are needed to obtain 1 L of wine, 3 kg of seeds are obtained from 100 kg of grapes and, if the world wine production has reached 264 millions of Hl in 2008 [1], it is easy to conclude that valorizing seed residues could represent an attractive business and an interesting opportunity for Green Chemistry and associated technologies. Traditionally, the seeds are used to feed animals [2] or sold to the oil industries [3], where a multi step processing, including an hexane extraction is used. Grape seed oils contain a large percentage of free fatty acids, mono and diglycerides but also polyphenols. The antioxidants phenolic compounds present in the seeds account for 60–70% of the total polyphenols in the grape [4]; therefore, their recovery should be more than interesting, taking into account that during vinification only a portion of those compounds is extracted [5]. The non negligible quantity of 4 g of catechin can be found per kg of residual grape seeds [6] and similar values should be obtained for other components. Resveratrol, catechin, epicatechin and gallic acid are some examples of polyphenolic compounds with important properties [7–9] that can be found in grape seeds after vinification. Not only does the grape variety affect the compounds content [10,11], but also the recollection time (at veraison the flavan-3-ols content is generally maximum), and the maceration time in the vinification process [5]. In addition, those antioxidants have become more important economically as they are intensively used in cosmetics and nutraceutical industries [12,13].

In these fields, several health and safety requirements have to be fulfilled; therefore supercritical fluids could represent a solution in this area, as organic solvents can be substituted by supercritical carbon dioxide ($scCO_2$) in several extraction processes [14–17]. Grape seeds near critical and supercritical extraction have been thoroughly studied [7]. Sometimes focusing on oil extraction [18] and other times focusing on antioxidant compounds [19–21]. However, since below 500 bars, the solubility of polyphenols in $scCO_2$ is limited [22,23], an organic solvent is added to supercritical extraction (even up to 40–50% (v/v)) to obtain acceptable yields. Casas et al. [24] recovered resveratrol from wine pomace (*Palomino fino* variety) residues by supercritical fluid extraction with CO_2 and ethanol as entrainer obtaining different percentages depending on the starting material (stems, seeds and skins). The optimum conditions for the selective extraction of resveratrol from seeds were 400 bars, 35 °C and 5% (v/v) ethanol obtaining a dry extract containing the 0.60% (w/w) of resveratrol. The total amount of resveratrol per kilogram of dry sample in these conditions was of 83 mg, while the highest recovery of resveratrol occurred at 55 °C (fixing the other

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conditions), for which the recovery of resveratrol was of 111 mg/kg of dry sample. Yilmaz et al. [2] accomplished the supercritical fluid extraction of grape seeds coming from wine pomace (*Carignan* type grapes) with ethanol as modifier in the range of 250–300 bars and 30–50 °C. The influence of the ethanol proportion was analyzed for compositions of 5–20% (w/w). Extraction yields were directly proportional to entrainer proportion and pressure; whereas, temperature played a dual role at those pressures (the volatility of the compounds was balanced by the decrease in the density of the solvent). Highest yields included recoveries of 33, 90 and 43 mg of gallic acid, catechin and epicatechin respectively per kilogram of grape seeds. In spite of the several investigations carried out up to now based on the supercritical fluid extraction of grape materials using $scCO_2$ (with or without entrainer), the problems have not been solved yet, namely, there is still a large amount of organic solvent use and the yields are not successful enough.

To overcome the drawbacks of liquid solvent based processes, the use of supercritical antisolvent extraction (SAE) can be proposed, since as the lipophilic character of CO_2 is advantageous when polar compounds have to be fractionated from an organic solution [25]. This process consists of the continuous contact between $scCO_2$ and a liquid mixture in a pressurized precipitation vessel. A spray of the liquid solution is produced in the supercritical medium to enhance the mixing of the two fluids and, if the process is performed at optimized conditions, the liquid solution can be fractionated by $scCO_2$ and compounds not soluble in the supercritical medium precipitate as solid powder at the bottom of the high pressure vessel. CO_2 and the residual organic mixture are recovered by decompression in a separator downstream the precipitation vessel, operated at a lower pressure. SAE technology has been used for various scopes such as the refining of crude lecithin [26], the purification of phospholipids [27], the fractionation of propolis [28] or the production of ryanodol-rich biopesticidal extracts [29]. Mukhopadhyay and Singh [26] accomplished the highest lecithin enrichment at 298 K and 6.5 MPa from an hexane based feed solution in a range of 283–313 K and 5–6.5 MPa. Aro et al. [27] obtained a SAE product with 72–99% (w/w) in phospholipids starting from a solution of egg yolk previously dissolved in ethanol with a phospholipids content of 60% (w/w). Catchpole et al. [28] obtained fractions containing 20–35% (w/w) in flavonoids, as they are practically insoluble in pure CO_2, but sufficiently soluble in CO_2 plus ethanol to enable their separation from high molecular mass and/or more polar components. Martín et al. [29,30] managed to obtain a 38% ryanodol-rich fraction with excellent insecticide activity in the separator, starting from an ethanol extract containing 7.5% (w/w) and operating at 15.0 MPa and 35 °C.

However, the nearest investigation to our research has been published by Floris et al. [31]. In this study, a SAE process was proposed to selectively extract polyphenols and anthocyanins from lyophilized grape residues; particularly, press grape wastes from *Cannonau* and *Cabernet* cultivars. They accomplished a multiple step process in which first grape wastes were treated with a tartaric buffer to extract the compounds of interest and simultaneously avoid the degradation. After that, the buffer was passed through a C-18 column and the compounds were desorbed with methanol. This methanolic solution went to SAE process. The working conditions were 110 bar and 40 °C as they tried to find a compromise between working in the supercritical phase and obtaining the minimum solubility of the antioxidants in the mixture CO_2 plus methanol. Several polyphenols such as catechin, epicatechin or epicatechin gallate and different anthocyanins such as malvin acetate and malvin cumarate were precipitated. The product recovered by SAE, a powderous solventless precipitate had an anthocyanins content of 15,542 mg/kg and a polyphenols content of 521 mg/kg.

The aim of this work is complementary to the one of Floris et al. [31], to extract antioxidants from grape seeds using the SAE technique. The major difference with the previous work is the starting material that presents different concentrations of the compounds of interest, has completely different consistence (ground seeds of *Syrah*, a different grape variety) and required seed oil elimination. An advantage was the higher stability of the starting material with respect to the whole grape residues. Additionally, ethanol has been selected as a cosolvent greener than methanol and the concentration of the obtained extracts will be optimized by varying the working pressures and temperatures in order to minimize the solubility of the four key compounds in the CO_2 + ethanol mixture [32–35].

2. Materials and methods

2.1. Raw material preparation and characterization

Grape (*Vitis vinifera*) seeds of the *Syrah* variety were obtained as residues from different wine producers in Aragón, Northeast of Spain. They were ground in a refrigerated grinder in batches of 0.2 kg during 5 min (MKM6000, Bosch) and sieved for 60 min with 250 rpm (RETSH AS400) to obtain three different batches of grounded material with an average particle diameter of 375 μm. Ground samples were vacuum stored. The standards of gallic acid, catechin, epicatechin and resveratrol were obtained from Sigma–Aldrich.

2.2. Feed solution preparation

100 g of ground material were stirred 24 h at room temperature (25 °C) in 1 L hexane (>99% purity, Sigma–Aldrich). After defatting, a Soxhlet extraction was performed recirculating 0.3 L of ethanol for 4 h (5 min per cycle). To achieve optimum supercritical antisolvent extraction conditions, the three feed solutions were prepared to contain 3% (w/w) of solids in ethanol, concentrating it to this extent with a rotary evaporator.

2.3. Supercritical antisolvent extraction (SAE) process

SAE tests were performed using the plant previously described [29]. Experiments were repeated twice. The relative expanded uncertainties in recovery (overall mass recovered/mass injected × 100) and in mass composition of selected antioxidants in the SAE extracts (w%) are U_r (rec) × 100 = ±5 and U_r (w%) × 100 = ±5, respectively (coverage factor k = 2). The SAE apparatus is formed of three main parts: a pumping area, a high pressure extraction vessel and a separation area. CO_2, supplied by SON (Società Ossigeno Napoli, IT), is pumped from a reservoir using a Lewa Ecoflow pump (mod. LDC-M-2, max pressure 40 MPa). The feed solution is pumped by a Gilson Pump (Model 305, Gilson FR). The extraction vessel of 500 cm^3 internal volume has a filter on the bottom for the collection of the solid matter. A separator made of stainless steel (AISI 316) working at lower pressures is located downstream from the vessel and allows the recovery of the solvent at time controlled intervals, whereas, the gaseous CO_2 exits through the top of the separator. Separator pressure is measured by a manometer and controlled by a back pressure regulator inserted downstream from the CO_2 exit line. The CO_2 flow rate is measured by a rotameter ASA (mod. N.5-2500, Serval 115022). A typical run consists of the following steps: stabilization of pressure, temperature and CO_2 flow rate in the system; stabilization of liquid flow by injecting liquid solvent (≈10 mL) and then injection of the feed solution (40 mL) to be fractionated. The final step is the injection of pure $scCO_2$ (≈30 min) to wash out the residual solvent. Samples from the separator are collected every 5 min during the experiment. The operation conditions were chosen to work in supercritical homogeneous phase. The experimental conditions