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High-pressure CO_2 assisted extraction as a tool to increase phenolic content of strawberry-tree (*Arbutus unedo*) extracts

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ABSTRACT

Arbutus unedo fruits, commonly known as strawberry-tree fruits, can be used as raw material for the development of bioactive ingredients, besides other traditional applications. In this work, a high-pressure CO_2 Assisted Extraction process (HPCDAE) was investigated in order to find the best process conditions to obtain extracts with a higher phenolic content (TPC) and antioxidant activity. The process parameters studied were pressure (100, 175 and 250 bar), temperature (40 °C, 55 °C and 70 °C) and volume solid:liquid (S:L)/CO_2 ratios (20, 50 and 80%) and the extracts recovered compared with the extract obtained with a S:L conventional extraction. Results show that when the highest pressure (250 bar) temperature (70 °C) and lowest S:L/CO_2 ratio (20%) are used, HPCDAE increases 1.41 times TPC of the extracts, compared to conventional S-L extract. A significant increase in the extraction of galloyl hexoside and 5-O-galloylquinic acid were observed after HPCDAE application. The intensification of both compounds extraction may be related with the increase in the antioxidant activity of the extracts. The results from this study show that HPCDAE is a promising tool to intensify the extraction of bioactive compounds from strawberry tree fruits.

1. Introduction

The strawberry tree (*Arbutus unedo)* is a small (4 m) evergreen plant belonging to the Ericaceae family [1]. It is an endemic specie of the Mediterranean basin, but can also be found in the south of Ireland and in some areas of the southwest of Asia [2]. As it is well adapted to Mediterranean climate conditions, the strawberry tree has the capability to overcome the summer dryness, characteristic of such environments.

The strawberry-tree fruits are spherical berries that vary in color during their maturation, from green to deep red when ripe and yellowish in between maturation stages. The fruits have approximately a 2 cm diameter and have a wrinkled surface in a way that resembles pyramidal shapes [3]. The fruits usually are not consumed fresh due to its perishable character and also because they are tasty only when they are fully ripe. Instead, they are consumed in jams or marmalades, or are used in the preparation of alcoholic beverages such as liquors or a traditional Portuguese spirit called "Aguardente de Medronho" [4–6]. Although it represents an important resource for some populations in the interior and south of the country, strawberry-tree fruits grow mostly wild and numbers of the Portuguese institute for the conservation of nature (ICNF) point out to an area of 15,500 ha of these plants in Portugal. There are no official numbers regarding the production of fruits or spirit, nevertheless in face of the recent wildfires that fustigated Portugal there is a large need for a reforestation based on endemic and profitable vegetable species, and the strawberry-tree fruit can figure as an alternative to common resinous species.

Strawberry-tree fruits have been used in traditional medicine as antiseptic, laxative, diuretic and strawberry tree leaves also as diuretic, depurative, antiseptic urinary and antidiarrheal [2,7]. These properties have been related to phytochemicals with recognized health promoting activity such as phenolic compounds [5,8]. These compounds are known to be secondary metabolites of plants, involved in defense mechanisms and are also responsible for the color of plants and fruits [9]. Phenolic compounds also contribute to the antioxidant activity and can protect membrane lipids from oxidation [10]. The main phenolic compounds present in strawberry tree fruits are gallic acid derivatives [11], namely the forms esterified with quinic and shikimic acids, which are only found in a limited number of plant products. Flavan-3-ols, flavonols, ellagic acid derivatives, and anthocyanins have also been described in literature [5,12,13]. Other compounds as vitamins C and E and carotenoids have also been reported [1,5,6,14,15].

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The method of extraction used for the preparation of extracts influences their chemical composition and consequently their bioactivity [16]. Organic solvents such as methanol, ethanol, water or their combination are the most commonly used for S-L extraction of phytochemicals from natural matrices [17]. Nonetheless, conventional S-L extraction methods are considered solvent and time consuming and may increase the risk of degradation of compounds [18] and on the other hand the need to replace conventional processes for more sustainable ones are a driving force for young researchers. The search for alternative methods of extraction such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) or high-pressure carbon dioxide assisted extraction (HPCDAE) has become important for their effectiveness of extraction allied to the use of a generally recognized as safe GRAS extract. Moreover, the use of high pressure carbon dioxide (HPCD) causes the burst of raw material cell walls, increasing the release of intracellular compounds, besides inactivation of enzymes and microorganisms that can lead to the modification of the extracts composition [19–23]. Xu et al. [23] were the first authors to use high pressure carbon dioxide assisted extraction (HPCDAE) to enhance the extraction of anthocyanins from red cabbage. Also Santos & Meireles [22] studied this process to enhance the recovery of anthocyanins and phenolic compounds from jabuticaba peels. Briefly, the HPCDAE consists in performing an extraction identical to a conventional S/L extraction inside a pressurized vessel with carbon dioxide. Temperature and pressure are used to promote the extraction efficiency by taking advantage of the explosive effect of CO_2 already described by Xu et al. [23]. The rise in pressure causes cell membrane modifications, intracellular pH decrease, disordering of the intracellular electrolyte balance leading to the removal of vital constituents from cells and cell membranes. Both studies, Santos and Meireles [22] and Xu et al. [23] described there were advantages for the use of HPCDAE in comparison to conventional processes. Santos and Meireles [22] demonstrated the higher efficiency of HPCDAE towards jabuticaba recovery of phenolic compounds and anthocyanin's compared to pressurized liquid extraction (PLE) and conventional SL extractions.

The present work investigated the use of high pressure carbon dioxide to intensify the extraction process towards the recovery of phenolics-rich extracts of strawberry-tree fruits. Different process conditions, namely pressure, temperature and volume ratio of solid-liquid/ CO_2 provided by a full factorial experimental design were studied. The effect of these process parameters in phenolic composition and antioxidant activity of the extracts was assessed.

2. Materials and methods

2.1. Plant material

Arbutus unedo fruits were collected in October 2014 from Serra da Gardunha, Portugal (40°07′15.17″N ; 7°27′58.66″W) at approximately 625 m altitude.

The strawberry-tree fruits were dried in an oven with air-circulation at 45 °C during two days. Dried fruits were then minced in an electric knives-miller (Moulinex A320R1, Portugal) and separated by size in an electromagnetic sieve (Retsch A-200, Germany). A fraction with mesh size between 18 and 25 was used for all experiments. The matrix was kept in a vacuum sealed bag inside a desiccator, protected from light.

2.2. Extraction procedures

2.2.1. Solid-liquid extractions

S-L extractions were carried out, prior the HPCDAE, to determine the best conditions (solvent ratios of water:ethanol, solid:liquid ratio and time) to produce a phenolic rich extract from the dried strawberry tree fruits that could serve as a control for the experiments follows. First, S-L extractions were performed in falcon tubes (at atmospheric pressure) using different volume percentages of water:ethanol (100:0; 25:75; 50:50; 75:25; 0:100), a fixed solid:liquid ratio (m/v) of 1:10, and 5 min of extraction in a vortex agitator (witeg Labortechnik GmbH VM-10, Germany) at maximum power (3300 rpm) and ambient temperature. Extracts were tested for phenolic content by the Folin-Ciocalteau method and the best solvent which produce the extract with higher phenolic content was selected for further experiments. The same procedure was done twice to determine the most favorable solid liquid ratio and extraction time. Three different ratios were tested (1:10, 1:20 and 1:30) and at last, three different extraction times were evaluated (5, 15 and 30 min).

After the HPCDAE experiments, further conventional solid-liquid extractions were performed using a temperature of 70 °C and acidified water:ethanol mixture (pH = 3) in order to compare both processes. The selected conditions mimic the highest temperature used in HPCDAE and the effect of pH reduction promoted by the use of carbon dioxide towards aqueous solvents.

2.2.2. High-pressure carbon dioxide assisted extractions (HPCDAE)

The extractions were carried out in a supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SFE-500F-2-C50) comprising a 500 mL cylinder extraction cell (extraction vessel) and two different separators (fraction collector 1 and 2), each of them with 500 mL of capacity, with independent control of temperature and pressure. Liquid carbon dioxide was delivered to the extraction vessel using a TharSFC P-50 high pressure pump (Thar Technology, Pittsburgh, PA, USA). The solvent was preheated on a heat exchanger to the extraction temperature. The pressure on the extraction vessel was maintained by an automated back pressure regulator (TharSFC ABPR, Thar Technology, Pittsburgh, PA, USA), which was located between the extraction vessel and the fraction collector 1.

The experimental design used is a central composite circumscribed design (CCCD) 2^3 and the experiments are presented in Table 2. The Central Composite Design (CCD) is an experimental design used to achieve maximal information about a process from a minimum number of trials. The selected variables were tested simultaneously, using CCD that enable to find interactions between the variables which cannot be identified with classical approaches. MOODE® version 12.0.1.3984 (Sartorius Stedim Data Analytics AB) software was used to calculate the effects of three extraction parameters: pressure (100, 175 and 250 bar), temperature (40, 55 and 70 °C) and volume ratio of solid-liquid / pressurized carbon dioxide ($R_{S\text{-}L/CO2}$(%)) (20, 50 and 80%) by a 2^3 full factorial design, on the recovery of phenolic compounds. These conditions were selected according to previous work already performed at the laboratory [21].

For each experiment, the extraction vessel was filled with the dried strawberry-tree fruit and the mixture of ethanol:water (50:50) at a solid liquid ratio of 1:20. These conditions were selected as described in 2.2.1. The vessel was kept closed and pressurized with CO_2 until the desired pressure. After 15 min the vessel was depressurized and the content removed, filtered and centrifuged at 6000 rpm (Hettich D-78532, Germany), supernatants of extracts were kept at -20 °C until analyses.

2.3. Total phenolic content (TPC)

The total concentration of phenolic compounds in *Arbutus unedo* extracts was determined according to the modified Folin Ciocalteau colorimetric method (Singleton & Rossi, 1965), as previously described by Serra et al. [26]. Briefly, the appropriate diluted solutions of extracts were oxidized with a Folin–Ciocalteu reagent (Panreac, Barcelona Spain), and the reaction was neutralized with sodium carbonate. The absorbance of the samples was measured at 765 nm on a spectrophotometer (GenesysTM 10UV ThermoScientific, Waltham MA, USA) after 30 min, at 40 °C. Gallic acid (Fluka, Seelze, Germany) was used as external standard, and the results were expressed as means of three replicates (mg of gallic acid equiv/g of dry weight of fruit – mg GAE/g