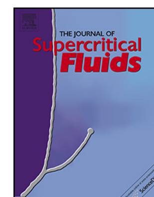
J. of Supercritical Fluids 104 (2015) 122–131

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu



Enzyme-assisted supercritical fluid extraction of phenolic antioxidants from pomegranate peel

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ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form 19 May 2015
Accepted 20 May 2015
Available online 11 June 2015

Keywords:
Enzyme pretreatment
Scanning electron microscopy
Supercritical fluid extraction
Total phenols
Radical scavenging
HPLC-DAD-ESI–MS
Phenolic acids profile

ABSTRACT

This study presents a comprehensive enzyme-assisted supercritical fluid extraction (EASCFE) process developed to extract antioxidant phenolics from pomegranate peel (PP). Pomegranate peel collected from local agro-processing units was treated with various commercial and feed enzyme preparations. The morphological changes in the ultrastructure of PP cell wall after enzyme treatment were visualized by field emission scanning electron microscopy (FESEM). Extraction of antioxidant phenolics from enzyme pretreated PP was carried out by supercritical carbon dioxide (SC-CO_2) with ethanol as a co-solvent. The extracts produced were investigated for their total phenolics (TP), antioxidant activity and phenolic acids profile using HPLC-DAD-ESI–MS. The observed results showed that EASCFE doubled the recovery of crude extracts with increased level of phenolic constituents (TPs), and improved radical scavenging capacity (RSC), trolox equivalent antioxidant capacity (TEAC) and inhibition of linoleic acid peroxidation. HPLC-DAD-ESI–MS characterization of the extracts authenticated the presence of vanillic (108.36 μg/g of extract), ferulic (75.19 μg/g of extract) and syringic (88.24 μg/g of extract) as potent phenolic acids. The results of this study support that EASCFE can be explored as a state-of-the-art green technological process for recovering optimum amounts of antioxidant phenolics from under-utilized pomegranate peel or other such materials. In addition, retention of appreciable level of naturally occurring vanillic, ferulic, and syringic acids in the enzyme-extracted components advocates that EASCFE is a good choice for preparation of PP extracts for food preservation as well as for the development of nutraceuticals and chemo-preventive phytomedicine.

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1. Introduction

The recovery of phenolic compounds from plant materials mainly depends upon the nature and distribution potential of extraction media as well as the technique applied. The conventional organic solvent extraction methods such as Soxhlet and orbital shaking are not only tedious and time consuming but also exert negative impact on environment in terms of emission of organic volatile compounds. Moreover, such conventional extraction techniques are less efficient towards extraction and liberation of bound phenolics from well-organized and complex cellulosic structure comprising sugars (alcohols) and acids (organic and phenolic acids) further fortified by lignin and in certain cases shielded by pectin [1]. Pre-treatments including ultrasound [2], microwave [3] ohmic heating [4] acid [5], and alkaline hydrolysis [6] can be used to attain competitive recovery rates but most of these technological aids have restrictions with regard to consumers health and end-use product safety concerns.

Supercritical fluid extraction (SFE), being a green process, has emerged as a technique of choice for the extraction and isolation of high-value natural products and phytochemicals. It mainly employs supercritical carbon dioxide (SC-CO_2), for the extraction and recovery of valuable natural matrices. The superiority of this technique lies in the recovery of relatively pure and clean extracts especially useful for functional food and nutraceu-

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http://dx.doi.org/10.1016/j.supflu.2015.05.020
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tical/pharmaceutical products [7,8]. Moreover, this technique is relatively safer and applicable for the extraction of thermally labile and oxidation-susceptible plant materials [9]. As far as extraction of plant phenolics is concerned, these compounds being polar in nature are not completely soluble in SC-CO_2. In this regard, although addition of small volume of a co-solvent such as methanol and ethanol can improve the polarity and extractability of SC-CO_2 [10], nevertheless the recovery of covalently or hydrophobically entrapped phenolics (bound forms) is hardly achievable.

Enzymes on the other hand can hydrolyze the cellulosic composite structure of plant cell wall and eventually may enhance the recovery of both bound and free phenolics. The use of enzymes have already been investigated during solvent and aqueous extraction as well as in cold pressing to improve the extraction of polysaccharides [11], edible and non-edible oils [12] proteins [13], and phenolics [14] but as such rarely attempt have been made to utilize enzyme in a highly selective supercritical fluid extraction.

Pomegranate (*Punica granatum* L.); a member of Punicaceae family, is one of popularly consumed fruits all over the world owing to the presence of potent phytochemicals of high medicinal value. Pomegranates have been extensively studied for their phytochemical composition [15], health benefits [16], especially for antioxidant characteristics [17], minerals profile and vitamins content [15]. Peel of pomegranate (PP), constituting more than 40% of whole fruit, is generated in large quantity as result of wide scale fruit consumption and juice production in food industry. However, PP is mostly discarded as an agro-waste rather than used for value-addition purposes [16,18]. Previously published reports reveal that PP contains appreciable amounts of free and bound phenolics [19,20], which prompts the need to optimize the recovery of phenolics from this valuable but under-utilized raw material. In the light of current bio-processing trends, together with the growing demand from the nutraceutical and pharmaceutical sector for the ultra-pure and healthier phyto-extracts, in the present study, therefore, maceration power of different enzymes was utilized to develop an effective, quick, and green extraction protocol for the optimum recovery of phenolic antioxidants from pomegranate peel.

2. Materials and methods

2.1. Procurement of Samples

The pomegranate peel (PP) were collected from local agro-processing units of Faisalabad, Pakistan, washed with distilled water and dried under ambient conditions. The dried material was pulverized into a fine powder using a kitchen grinder and the material that passed through 80-mesh sieve was used for extraction purposes.

2.2. Enzymes, standards/reagents and chemicals

Enzyme complexes including pectinase, protease, cellulase, alcalase, and viscozyme were purchased from Sigma Chemical Co. (St. Louis, MO, USA) whereas, food and feed enzyme formulations comprising acid cellulase, alcalase, pectinex, and kemzyme were procured from Novozyme, Denmark, and Kemin, Germany. The detail of enzyme preparations, constituent units and their related activities is provided in Table 1. The reagent including 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), potassium persulfate, Folin–Ciocalteu reagent, and 2,6-di-*tert*-butyl-4-methylphenol (BHT) and phenolic acid standards such as 3, 4-dihydroxy benzoic, *p*-hydroxy benzoic, gallic, linoleic, vanillic, caffeic, *p*-coumaric, ferulic, syringic, and sinapic acids were supplied by Sigma, St. Louis (USA). All other chemicals, buffers, and solvents used were of analytical and HPLC-grade (Merck, Damstadt, Germany).

2.3. Enzymatic pre-treatment

Accurately weighed 10 g of ground material of PP was taken in petri dishes and separately treated (incubated) with optimum concentration of acid cellulase, kemzyme, pectinex, alcalase, and cocktail enzyme (mixture of pectinase, protease, and cellulase in ratio 25:25:50) in 10 mL of phosphate buffer according to pre-optimized conditions (Table 2). After the required incubation, the enzyme complex was deactivated by heating at 90 °C for 5 min and the recovered enzyme treated PP material was used for extraction purposes.

2.4. Scanning electron microscopy (SEM) of enzyme pretreated PP

The ultrastructure of enzymatically hydrolyzed PP samples under optimum conditions was examined by field emission scanning electron microscopy (FESEM). The samples were dried using liquid nitrogen, fixed on specimen holder by magnetic tape, and coated with palladium in sputter coater. The morphological changes were examined using MIRA3 TESCAN.

2.5. Extraction of phenolics

After enzymatic pre-treatment, the PP samples were extracted using supercritical carbon dioxide (SC-CO_2) extractor (SFT-250 SFE/SFR System; supercritical fluid technologies, Newark, DE, USA) with ethanol as co-solvent (SCFE) under pre-optimized conditions including CO_2 flow rate (2 g/min), ethanol injection (0.2 g/min), temperature (55 °C), pressure (300 bar) and extraction time (30–120 min). For comparison purpose, enzymatic pre-treated PP samples were also extracted using aqueous ethanol (80%) in an orbital shaker (EASE). The general process and schematic diagram followed for SCFE has been illustrated in Fig. 1.

2.6. Ultra micro-essay based determination of total phenolics (TPs)

TPs in the extracts produced by enzyme-assisted supercritical fluid extraction (EASCFE) and enzyme- assisted solvent extraction (EASE) were determined using Folin–Ciocalteu reagent [21] with minor modifications to bring assay at ultra micro level. Briefly, 20 μL of extracts (5%) were mixed with 100 μL of Folin–Ciocalteu reagent (10%) and diluted with 1.5 mL deionized water. The mixture was kept at room temperature for 10 min, and then 500 μL of 20% sodium carbonate (w/v) added. The aliquot was heated in a digital water bath (at 40 °C for 20 min and then cooled in an ice bath. 200 μL of resultant solutions were placed in 96 well plate and the absorbance recorded at λ_{max} 755 using ELISA microplate reader (BioTek, Highland park, USA). Amounts of total phenols were calculated following a calibration curve (R^2 = 0.9978) constructed by analyzing series of standard solutions of gallic acid (10–200 ppm). The results were expressed as milligrams (mg) of gallic acid equivalents (mg GAE)/g of extract.

2.7. HPLC-DAD-ESI–MS profile of phenolic acids

High performance liquid chromatography coupled with diode array detector and electro spray ionization mass spectrometer (HPLC-DAD-ESI–MS) was used to characterize and quantify phenolic compounds in the extracts obtained by EASE and EASCFE, following a previously described method [20] with slight changes.

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