Journal of Chromatography A, 1218 (2011) 7511–7520

Contents lists available at ScienceDirect

Journal of Chromatography A

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

New possibilities for the valorization of olive oil by-products

Miguel Herrero[a,*], Temirkhon N. Temirzoda[a], Antonio Segura-Carretero[b], Rosa Quirantes[b], Merichel Plaza[a], Elena Ibañez[a]

[a] Department of Bioactivity and Food Analysis, Institute of Food Science Research (CIAL-CSIC), Nicolás Cabrera 9, Campus Cantoblanco, 28049 Madrid, Spain
[b] Department of Analytical Chemistry, University of Granada, Campus Universitario de Fuentenueva, 18071 Granada, Spain

ARTICLE INFO

Article history:
Available online 28 April 2011

Keywords:
Environmentally clean extraction techniques
PLE
Olive leaves
UPLC–MS/MS
Phenolic antioxidants

ABSTRACT

In this contribution, the capabilities of pressurized liquid extraction (PLE) using food-grade solvents, such as water and ethanol, to obtain antioxidant extracts rich on polyphenolic compounds from olive leaves are studied. Different extraction conditions were tested, and the PLE obtained extracts were characterized *in vitro* according to their antioxidant capacity (using the DPPH radical scavenging and the TEAC assays) and total phenols amounts. The most active extracts were obtained with hot pressurized water at 200 °C (EC_{50} 18.6 μg/mL) and liquid ethanol at 150 °C (EC_{50} 27.4 μg/mL), attaining at these conditions high extraction yields, around 40 and 30%, respectively. The particular phenolic composition of the obtained extracts was characterized by LC–ESI-MS. Using this method, 25 different phenolic compounds could be tentatively identified, including phenolic acids, secoiridoids, hydroxycinnamic acid derivatives, flavonols and flavones. Among them, hydroxytyrosol, oleuropein and luteolin-glucoside were the main phenolic antioxidants and were quantified on the extracts together with other minor constituents, by means of a UPLC–MS/MS method. Results showed that using water as extracting agent, the amount of phenolic compounds increased with the extraction temperature, being hydroxytyrosol the main phenolic component on the water PLE olive leaves extracts, reaching up to 8.542 mg/g dried extract. On the other hand, oleuropein was the main component on the extracts obtained with ethanol (6.156–2.819 mg/g extract). Results described in this work demonstrate the good possibilities of using PLE as a useful technique for the valorization of by-products from the olive oil industry, such as olive leaves.

1. Introduction

Functional foods are increasingly gaining interest and attention within the food industry. This type of food is able to provide with additional benefits compared to a traditional food. At present, it is possible to find in the market a broad range of these products, including foods claiming antihypertensive, antihypercholesterolemic or antioxidant properties. Nevertheless, a lot of research is nowadays focused on the possible beneficial effects that some natural components might offer if consumed in the diet, such as anti-cancer activities [1,2] or neurodegenerative prevention [3,4], among others. These natural additives are clearly preferred by consumers over their synthetic counterparts. Ideally, in order to develop a new functional food, one or more natural ingredients with demonstrated activity are added to a traditional food in a way in which can exert a substantial beneficial action in the organism [5]. A possibility of obtaining these interesting components is their extraction from natural matrices, such as plants or algae [6,7]. However, another interesting approach is the extraction of such compounds from the food industry by-products, which usually are discarded or employed to produce animal feed. Different food-related by-products have been already studied, and diverse interesting compounds have been identified in some of them, such as lycopene in tomato by-products [8], isoflavones in soybean by-products [9], polyphenols in pomegranate peels [10] or antioxidants in different plants [11], among many others. In this regard, leaves from olive tree (*Olea oleuropaea*) are produced in great amounts as a waste from the olive oil industry which is one of the main food products in the Mediterranean basin. Although the presence of interesting phenolic antioxidants in the olive leaf is well known [12–14], this by-product is still underemployed. The polyphenols present in the olive leaves have been shown to possess important antioxidant [15,16], anti-inflammatory [17,18], anti-atherogenic [19] and antimicrobial activities [20], and even possible anti-cancer effect [14,21,22].

On the other hand, pressurized liquid extraction (PLE) is a widely considered advanced extraction technique which is able to efficiently extract interesting compounds from natural matrices using low volumes of organic solvents, if any, as well as producing high extraction yields in short extraction processes. These good

* Corresponding author. Tel.: +34 910 017 946; fax: +34 910 017 905.
E-mail address: m.herrero@csic.es (M. Herrero).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.04.053

capabilities are a result of the particular extraction conditions used in which the extracting solvents are heated at high temperatures but maintained at high pressures in order to keep their liquid state during the whole extraction procedure. This technique has been already successfully applied to the extraction of phenolic antioxidants from different natural matrices [6]. Of particular interest is the application of PLE using water as solvent. In this case, this completely environmentally friendly technique is also called subcritical water extraction (SWE) or pressurized hot water extraction (PHWE). Here, the main variable is the dielectric constant of water (ε), as a measure of its polarity. When water is heated under pressure and its liquid state is kept, the dielectric constant decreases as temperature is increased. This decrease on the water polarity may effectively modify its solvent properties, decreasing this parameter to values similar to those presented by some organic solvents, such as ethanol or methanol. Thus, the application of this green technique to the extraction of bioactive compounds from olive leaves could be of great interest, not only for the attaining of these natural active compounds but also for the possibility of re-using an important by-product from the industry. Although this technique has been also briefly explored for the extraction of target compounds from olive leaves [23,24], up to now, there is no published report systematically studying the influence of the different extraction conditions on the attainment of phenolic antioxidants from this material by using only food-grade solvents. Thus, the aim of the present study was to test the PLE extraction conditions, using ethanol and water as solvents, to produce phenolic-rich antioxidant extracts from olive leaves and to study the phenolic composition of the PLE extracts, using advanced characterization techniques, and their relationship with the tested antioxidant activity.

2. Experimental

2.1. Samples and chemicals

Olive tree leaves (variety Hojiblanca) generated as by-products from the olive oil industry were dried and provided by Oleoestepa (Sevilla, Spain). The leaves were dried following a traditional procedure as follows: once the leaves were separated from the rest of plant materials, the olive leaves were covered to avoid direct light and left ventilated at ambient temperature to remove humidity for ca. 50 days, depending on the ambient relative humidity. Before extraction, cryogenic grinding of the sample was performed under liquid nitrogen. The samples were stored protected from light at 4 °C until their use. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), potassium persulfate and caffeic acid were obtained from Sigma–Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was provided by Fluka Chemie AG (Buchs, Switzerland). Folin–Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas antioxidant standards, i.e., hydroxytyrosol, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein, quercetin, apigenin and diosmetin were supplied by Extrasynthese (Genay, France). The water used was Milli-Q Water (Millipore, Billerica, MA, USA). For the LC–MS and UPLC–MS/MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed.

2.2. Pressurized liquid extraction (PLE)

Extractions of olive leaves were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Two different solvents (i.e., ethanol and water) were used to obtain extracts with different compositions. In order to avoid any possible oxidation effect and to remove the dissolved oxygen, solvents were sonicated for 15 min prior to use. Extractions were performed at four different extraction temperatures (50, 100, 150, and 200 °C) whereas the static extraction time was maintained for 20 min. An extraction cell heat-up step was carried out for a given time prior to any extraction. The warming up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 50 and 100 °C, 7 min if the extraction temperature was 150 °C, and 9 min if the extraction temperature was 200 °C). All extractions were done using 11 mL extraction cells, containing 2 g of sample. When water was used for the extraction, the extraction cell was filled with sand mixture on the top of the sample (3.0 g of sand) to prevent the clogging of the system.

The extracts obtained were protected from light and stored under refrigeration until dried. For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Virtis Unitop 400 SL, Gardiner, NY, USA) was employed.

2.3. Functional characterization of the PLE extracts

2.3.1. Trolox equivalent antioxidant capacity (TEAC) assay

Two *in vitro* methods were employed to determine the antioxidant capacity of the olive leaves' PLE extracts. Trolox equivalent antioxidant capacity (TEAC) assay was performed as described by Re et al. [25], with some modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with ethanol for the ethanol extracts and with 5 mM phosphate buffer (pH = 7.4) for the water extracts, to an absorbance of 0.70 (±0.02) at 734 nm. Ten microliters of sample (different concentrations) were added to 1 mL of diluted $ABTS^{\bullet+}$ radical solution. After 50 min at 30 °C, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 734 nm in a microplate spectrophotometer reader (BioTek Instruments, Winooski, VT, USA). Trolox was used as reference standard and results were expressed as TEAC values (mmol Trolox/g extract). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were done at least in triplicate.

2.3.2. DPPH radical scavenging assay

The other method employed to measure the antioxidant capacity of the obtained extracts was the DPPH radical scavenging method, based on a procedure described by Brand-Williams et al. [26]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested. Twenty-five microliters of these extracts solutions were added to 975 μL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek). DPPH–methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Therefore, the lower the EC_{50}, the