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Extraction of biologically active compounds from Ñora pepper and their successive concentration by membrane processes

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In this study extraction of polyphenols and flavonoids from Ñora pepper, a variety of sweet pepper that is widely cultivated in Spain, was investigated. Solid-liquid extraction assisted with ultrasound was carried out using ethanol and isopropanol as a solvent. High equilibrium values of the extracted species were obtained - 52.35 mg/(g solid) total phenolics and 46.2 mg/(g solid) total flavonoids with isopropanol. Furthermore, collected extracts containing these biologically active compounds were concentrated using a cross-flow filtration process equipped with a home-made polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was prepared by a phase-inversion precipitation method in a water coagulation bath set up at 60°C. Permeate flux and rejection were monitored during the filtration process.

Keywords: Ultrasound-assisted extraction, Polyphenols, Flavonoids, Ñora pepper, polyvinylidene fluoride (PVDF) membrane

INTRODUCTION

Phytochemicals are chemical compounds generated by plants that are typically involved in plant growth or in the process of protecting them from predators or pathogens [1]. They are secondary metabolites created by plants and they can be divided into the following classes: flavonoids, phenolic acids, phenyl alcohols, secoiridoids, lignans, stilbenes and glycosides, all with somewhat different activities and health benefiting effects (antioxidant, anti-inflammatory, anti-cancer, cardio-protective, antimicrobial, antifungal, chemo-preventive, cardio-protective) [2]. Flavonoids (60 %) and phenolic acids (30 %) constitute the most abundant classes of polyphenols and are characterized by a great diversity of the compounds [3–6]. Nowadays, the use of phytochemicals, especially polyphenols, as alternative anticancer drugs is a promising alternative since they diminish or suppress the adverse effects of the usually more aggressive conventional therapies [2]. The leading sources of polyphenols involve berries, grapes, olive oil, cocoa, nuts, peanuts, propolis and other fruits and vegetables, which contain up to 200–300 mg of polyphenols per 100 g of fresh weight. Furthermore, products manufactured from these fruits such as tea, wine, or beer also include polyphenols in considerable quantities [7].

Ñora pepper is a variety native to the East coast of Spain and is very popular in Mediterranean cuisine. Ñora peppers are small, round and sweet-fleshed red bell peppers. They are dried in strips and used as a spice. Thanks to their sweetness and intense aroma, they are perfect for adding flavour to casseroles and sauces.

Conventional techniques, i. e. extractions using organic solvents, have been thoroughly used to extract antioxidant compounds from plants and vegetables [8]. However, they rely on high temperatures and long incubation times, which usually lead to a low yield in antioxidant activity, while requiring a high energy input [9]. Ultrasound-assisted extraction (UAE) of polyphenols and flavonoids from plant material possesses many advantages over conventional solid–liquid extractions [6]. The use of ultrasound as pretreatment, in general, offers significant advantages in terms of improvement in the yield of biologically active compounds extraction with effects in preserving antioxidant and antimicrobial activities, reduction in the thermal degradation of compounds, reduction in time to extract the products, making the extraction cheaper and environmentally friendly. The beneficial effects of ultrasound on extraction are determined by (i) biomass fragmentation attributed to the collisions between particles and ultrasonic waves, which causes a reduction in the particle size, thereby facilitating mass transfer; (ii) erosion which helps to improve the accessibility of the solvent by imploding the bubbles on the surface of the plant matrix; (iii) sonocapillarity and sonoporation which improve the penetration of liquid through the chan-

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by the bubble implosion and the alteration of the permeability of the cell membranes, respectively; (iv) the sheer stress mechanism produces the collapse of the cavitation bubble into the fluid, due to the oscillation phenomenon [6]. During the UAE process the solutes are in contact with the solvent, so the extraction efficiency is greatly influenced by the interaction time between the two phases resulting in rapid extraction rate so that up to 90% of the polyphenols can be extracted during the first 10-20 min [10]. The traditional approaches used for concentrating of biologically active compounds, extracted from natural products, involve simple steam distillation and vacuum distillation, which commonly require an increased temperature and high energy consumption [5, 11]. The former is inappropriate for heat-sensitive products. These methods may also result in a loss of compounds of low molecular weight, which can be removed together with the solvent during evaporation. Another method described in the literature, particularly for vegetable extract concentration is the lyophilization process [11]. Nevertheless, this method demonstrates some of the disadvantages of the previously mentioned processes, e. g. involves a large amount of energy, comprises incubation at about 70 °C, etc. [11, 12]. A membrane separation process can be utilized as an alternative of the approaches mentioned above. Membrane processes have received a great attention as processes with reduced operation cost, carried out at ordinary temperature. The latter is very crucial because most of the species extracted from natural products are very unstable at elevated temperatures. A possible use of the permeate as an extraction solvent allows to decrease the required rejection of the membranes [3, 5, 13]. For this reason, the aim of the present work is to investigate the amount of polyphenols and flavonoids in Ñora pepper and their possible concentration by employing a PVDF membrane.

Materials

Ñora pepper was purchased from Hacendado, Mercadona brand, Spain. Ethanol (99.2%), isopropanol (99%) and aluminium chloride anhydrous, sodium carbonate, N-methyl-2-pyrrolidone (NMP), gallic acid and quercetin, Folin–Ciocalteu's phenolic reagent were supplied by Merck. Polyvinylidene fluoride membranes (PVDF) used for polyphenols and flavonoids concentration were prepared using a phase inversion precipitation method described in [14], using Solef 6020 polymer supplied by Solvay Specialty Polymers (Bollate, Italy).

Extraction

Before the extraction processes Ñora pepper samples were first dried for 72 h in a Memmert brand oven at 40°C and then they were ground using a Braun MQ 745 Aperitif Multiquick 7 grinder. Successively, solid-liquid extractions of biologically active compounds were conducted in an ultrasonic bath Ultrasounds Selecta 3000683 (50/60 kHz, 100 W) following a protocol described in [6]. Then, the ground material was extracted with liquid–solid ratio 15:1 (ml/g) using ethanol and isopropanol, determined as optimal and kept constant throughout the experiments. The total quantity of extracted material (g extract/g dry solid) was determined gravimetrically after evaporation of the solvent. In order to control the extraction conditions, the temperature changes in the bath caused by the ultrasound were monitored with a thermometer during all UAE experiments.

TP and TF content determination

The total phenolic content (TP) was determined spectrophotometrically according to the procedure reported in [15]. A volume of 0.5 ml of Folin–Ciocalteu's reagent was added to a flask containing 0.5 ml of the sample and 10 ml of H_2O. After 5 min 8 ml of 7.5% aqueous Na_2CO_3 solution was added to the mixture. The prepared samples were kept in dark for two hours at room temperature 22 ± 2 °C and then the absorbance was measured at 765 nm with UV-1800 Shimadzu spectrophotometer (Kyoto, Japan). Three parallel measurements were performed. The results were calculated as gallic acid equivalents, using a standard curve: $Abs = 2.578 \times C + 0.026$, $R^2 = 0.982$, where C [gGAE/ml]. Calibration curve was prepared using standard solution of gallic acid (0.03–0.25 mg/ml).

Total flavonoids content (TF) was determined using a spectrophotometric method based on the formation of aluminium-flavonoid complexes and calculated as quercetin equivalents, following the calibration curve: $Abs = 27.555 \times C + 0.089$, $R^2 = 0.995$, where C is the concentration in µgQE/mL (concentration range 5–26 µg/mL). The following procedure was applied: 0.5 ml of $AlCl_3$ was added to 0.5 ml of diluted sample. The samples were kept in darkness for 1 hour at room temperature 22 ± 2 °C and then the absorbance was measured at 765 nm with UV-1800 Shimadzu spectrophotometer. Three parallel measurements were performed.

Preparation of PVDF membranes

PVDF membrane was prepared by an immersion precipitation method. In summary, PVDF pellets were dissolved in NMP at 80 °C, with vigorous stirring for 48 h, to form a 15 wt % homogeneous casting solution. After air bubbles were removed completely, the resulting solution was cooled to room temperature, 20 ± 2 °C, and spread uniformly onto a glass plate with a non-woven support (15 cm × 20 cm) attached, using a casting knife with a 250-µm gate opening (K Paint Applicator, R K Print Coat Instruments, Ltd., Litlington, UK) and the coating speed set up at 2 m/min. The membrane was immediately immersed (approximately 10 s after coating) into a precipitation bath of deionized water (DW, 3 L) set up at 60 ± 2 °C. Next, the formed solid membrane was thoroughly washed with deionized water to remove residual NMP and dried at about 40 °C for 24 h under vacuum before further application.

Membrane filtration

Concentration of extracted polyphenols and flavonoids was carried out using a self-made stainless steel cross-flow filtration apparatus containing a disk membrane module applying a filtration protocol described in [16]. The effective membrane area in the module was 12.6 cm^2. Figure 1 provides the scheme of the cross-flow filtration equipment. The extract (feed) to the filtration cells was supplied by a piston pump and damped by a pulsation dampener before the membrane cell. During the experiments, the temperature of feed/retentate and permeate, as well as pressure in the membrane cells were controlled and kept constant (22 ± 2 °C, and 5 bar) during all experiments.

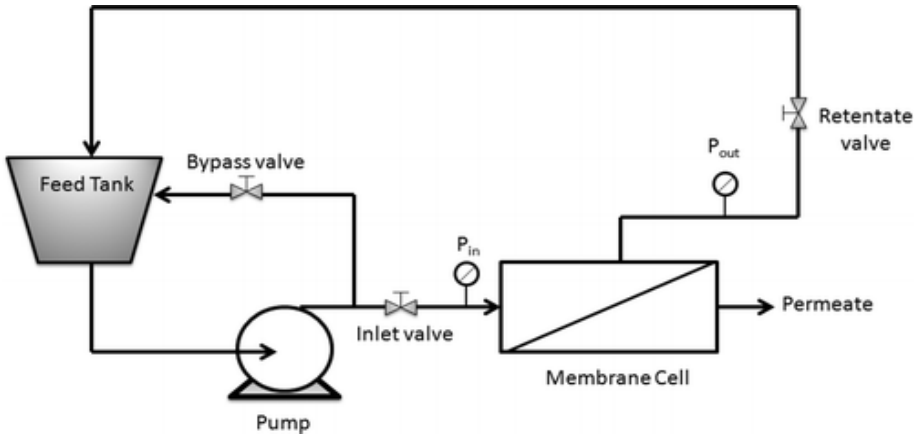

Figure 1. Flow diagram of the cross-flow filtration apparatus.

The flux and rejection experiments were performed using the following procedure: after the initial insertion of the membrane films in the cells, the plant was run first for 15 min without feed pressure in order to condition the system and, hence, for 15 min with a feed pressure of 5 bar, to reach a steady-state permeate flux and to complete the initial membrane compaction. As far as experiments at a pressure of 5 bar are concerned, whenever the solution in the feed tank was changed, the entire apparatus was washed three times with the new solvent before continuing the experiments. In addition, the system was run with a feed pressure of 5 bar for at least 15 min in order to facilitate the removal of the former solvent. Then, compaction at 5 bar feed pressure was done again until steady-state conditions were reached. Membrane performance was assessed on the basis of the solvent flux and rejection experiments. The flux (J) through the membrane can be described by the following equation:

$$J = \left(\frac{V}{A * \Delta t}\right)$$

where V is the permeate volume, A is the membrane area, and Δt is the permeation time. The biologically active compounds rejection rate was calculated using the following equation:

$$R = \frac{Cf - Cp}{Cf} * 100\%$$

where Cp and Cf (mg/mL) are the concentrations of permeate and feed solutions, respectively. The cross-section morphology of the PVDF membrane and the particle size of ground Ñora pepper were characterized by environmental scanning electron microscopy [ESEM (Quanta 600, FEI)] [17, 18]. The cross-sections of the membranes were prepared by fracturing the membranes in liquid nitrogen. The details of this method can be found elsewhere. The real thickness of the membranes (10 measures) and the diameter of the Ñora pepper on the membrane

surfaces (30 measures) were calculated using Image-ProPlus 5® software and the ESEM micrographs.

RESULTS AND DISCUSSION

Figure 2a shows an illustration of the Ñora pepper pieces before extractions, while Figure 2b shows the same sample after 1 h of extraction carried out with ethanol in ultrasound bath.

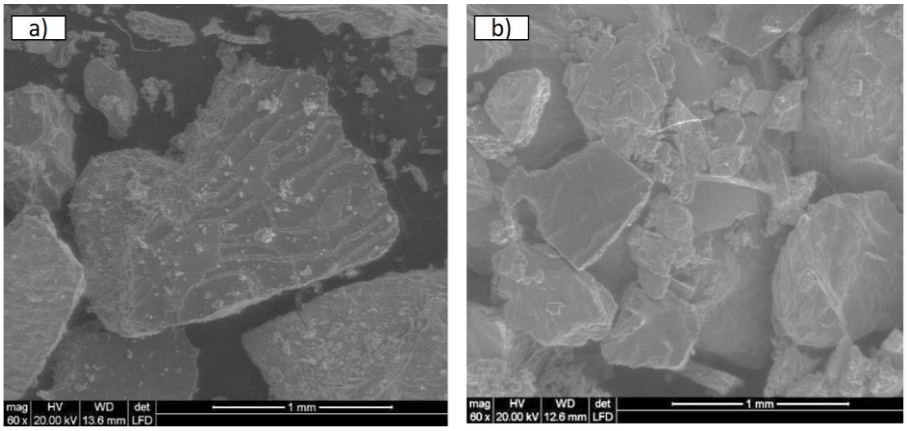

Figure 2. ESEM micrographs of Ñora pepper ground pieces before (a) and after (b) 1 h of ultrasound-assisted extraction with ethanol.

By means of Image-ProPlus 5® software, we analyzed the ESEM micrographs and we found that the pieces used for extraction had diameter fluctuating between 500 μm and 600 μm, while after extraction their size decreased and was in a range of 50 -150 μm. The maximum absolute error of these measurements was 2 μm. It is well known that ultrasound technique helps in the disruption of plant cell walls, improving the solvent permeation and enhancing mass transport across the cell membrane, which leads to higher extract yields. In our opinion, these advantages of using ultrasound energy for extraction not only include more effective mixing and micro-mixing but also could have a significant influence on the mechanical properties of the plant material. The decrease of the particle size and appearance of new small pieces suggest that the ultrasound makes the plant material more fragile and breakable. Indeed, Petigny and co-authors reported that after 2 h of ultrasound-assisted extraction of boldo leaves, the sample was heavily damaged [19]. As we reported in the previous paper, increased temperature of extraction positively affects solubility and enhances the mass transfer both to and into the solid, thus leading to faster kinetics and higher amounts extracted [6]. Ultrasound-assisted extraction experiments were performed in a Selecta 3000683 bath for 1 h. During the experiments the temperature of the ultrasound bath increased from 25 ±2 °C up to 60 ±2 °C. The obtained yield of extraction with ethanol as solvent was 0.0456 g/(g dry solid) while with isopropanol it was by 16% higher. Moreover, 52.36 mg/g of solid for total polyphenols and 46.2 mg/g solid for total flavonoids were achieved during extraction with isopropanol while with ethanol 45.3 mg/g of solid for total polyphenols and 20.7 mg/g solid for total flavonoids were collected. El-Malah *et al.* also reported that extraction performed with isopropanol provides a higher amount of biologically active compounds than that carried out with ethanol as a solvent [20].

In order to concentrate the extracted biologically active compounds we decided to apply the previously investigated PVDF membrane the surface of which is mainly formed by a mixture of TGTG' chains in α phase crystalline domains. This membrane was deeply investigated and reported in [14].

Figure 3 shows the ESEM image of cross-section morphology of the PVDF membrane. From the micrograph can be observed that the selected membrane possesses a compact structure. Moreover, based on the ESEM images, we were able to measure the membrane thickness by means of Image-ProPlus 5® software. The thickness of the employed membrane for TP and TF concentration was 106 ±2 μm. In order to evaluate the rejection of TP and TF extracted from Ñora pepper, their concentrations in the feed and permeate were analyzed. The results are given in Table 1.

Table 1. Content of biologically active compounds in the investigated extracts

	TP content in ethanol solution*(μg/ml)	TP content in isopropanol solution* (μg/ml)	TF content in ethanol solution** (μg/ml)	TF content in ethanol solution** (μg/ml)
Feed	3.02 ± 0.02	1.38 ± 0.01	3.49 ± 0.01	3.08 ± 0.01
Permeate	1.41± 0.02	0.72 ± 0.01	1.16 ± 0.02	1.34 ± 0.02

*TP expressed as the gallic acid equivalent; **TF expressed as the quercetin equivalent.

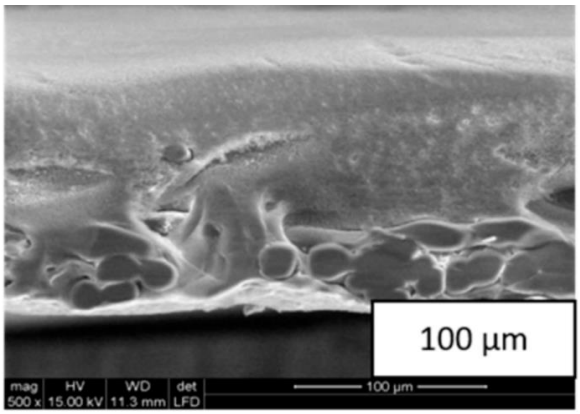

Figure 3. ESEM micrographs of the cross-section of PVDF membrane

Obtained results indicate that only 53% of TP and 48% of TF dissolved in ethanol were rejected by the PVDF membrane. Better results were achieved with isopropanol: 67% of TP and 56% of TF. Permeability flux through the polymeric membrane is strongly influenced by the structures and properties of the solvents. It has been reported that the membrane–solvent interactions can be expected to vary depending on the solvent properties, such as viscosity, dielectric constant, molecular size, dipole moment, solubility parameter, and surface tension [21]. Comparing the flux values for the extracts with isopropanol and ethanol, it can be observed that the flux of ethanol (36 $L/m^2 \times h$) with solvent relative polarity of 0.654 is higher than that of the less polar isopropanol (26 $L/m^2 \times h$) with solvent relative polarity of 0.546. Moreover, it has been reported that the flux values decrease with increasing molecular length, i. e., by lengthening the alcohol structure with additional CH_2 groups, irrespective of the transport mechanism.

CONCLUSIONS

Ultrasound-assisted extraction of biologically active compounds from Ñora pepper was performed. Obtained results indicated that higher amounts of polyphenols and flavonoids were extracted with isopropanol than with ethanol. PVDF membrane with a thickness of 106 μm were obtained by the phase inversion precipitation method at 60° C. By applying this membrane in a cross-flow filtration apparatus we were able to concentrate the BACs.

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