Journal of Food Engineering 263 (2019) 409–416

Contents lists available at ScienceDirect

Journal of Food Engineering

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

A selective extraction of hydroxytyrosol rich olive oil from alperujo

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ARTICLE INFO

Keywords:
Olive oil
Supercritical extraction
Antioxidant capacity
Total phenol content
Food-grade ingredient

ABSTRACT

Alperujo, the solid-liquid waste generated by the current two-phase method of olive oil extraction, was dried, milled and treated with supercritical carbon dioxide (sc-CO_2) to obtain a hydroxytyrosol (HT)-rich oil. At first, extraction rates were analysed as a function of operating variables and the pre-condition of the raw material. Samples with particle size diameter < 0.80 mm and in equilibrium moisture (1%) with the atmosphere, improved oil extraction yield almost 40% compared with samples with the whole range of particle sizes. Extraction yield improved with solvent flow rate, but a minimum residence time was required. The optimum was 0.18 $kg\,h^{-1}$ (7.5 kg CO_2 h^{-1} kg biomass^{-1}). Higher pressures and lower temperatures resulted in higher extraction yields; at 30 MPa and 323 K the extraction curve slope was close to the theoretical oil solubility and the yield was 13%, like that obtained with n-hexane by Soxhlet (14%). However, the HPLC-DAD analysis identified higher HT concentration (1900 ppm) in the supercritical extracts at the highest temperature. Consequently, at 373 K, the total phenol content and the antioxidant capacity of the extracts was uppermost. No HT was found in the n-hexane extracts.

1. Introduction

The Mediterranean diet has been widely studied due to its association with improved human health, as it is very effective against cardiovascular diseases, diabetes, inflammation and ageing (Widmer et al., 2015). Many of the benefits associated with the Mediterranean diet are the result of a high intake of antioxidants and anti-inflammatory elements present in several components of this diet, such as olive oil (Robles-Almazán et al., 2018). The olive fruit contains a wide variety of phenolic compounds. They play an important role in the chemical, organoleptic and nutritional properties of virgin olive oil and table olives (Fernández-Bolaños et al., 2008).

The olive oil production process was based on the so-called three-phase system until the nineties, which produced three streams: olive oil, alpechín and olive pomace. To eliminate the alpechín, a two-phase technology emerged, producing only two effluents: olive oil and alperujo (Altieri et al., 2013; Fernández-Bolaños et al., 2002). A schematic representation of the two and three-phase centrifugation procedures for olive oil extraction from olives can be found in the Supplementary file 1. The alperujo contains all the substances that in the three-phase system were contained in the alpechín and in the olive cake and it still has a significant oil content (Arjona et al., 1999). The yearly production of alperujo from the Spanish (largest producer worldwide) (Bordons and Núñez-Reyes, 2008) olive oil industry may approach four million tons.

The high moisture content of alperujo, together with the sugars and fine solids, give to the alperujo sludge a doughy consistency that makes drying difficult, demanding much energy (Arjona et al., 1999). However, generally the alperujo is used to obtain oil by means of a second centrifugation that, being a mechanical method in cold, generates an oil which requires refining with organic solvents for its consumption.

Most phenols (> 98% in mass fraction) remain in the alperujo, with a concentration of 10 to 100-fold higher than that of olive oil (Fernández-Bolaños et al., 2002, 2008). Therefore, alperujo seems to be an affordable and abundant source of natural antioxidants and interesting compounds, hydroxytyrosol (HT) among the most important ones (Fernández-Bolaños et al., 2008).

HT is an amphipathic phenol with a phenyl ethyl-alcohol structure. It is also called 3,4-dihydroxyphenylethanol, 3,4-dihydroxyphenolethanol or 4-(2-Hydroxyethyl)-1,2-benzenediol by the International Union of Pure and Applied Chemistry (IUPAC) system (Robles-Almazán et al., 2018). The origin of HT is the hydrolysis of oleuropein which happens during the ripening of the olives and during the storage and elaboration of table olives (Brenes and de Castro, 1998; Ramírez et al., 2016). Many studies have demonstrated the antioxidant, antimicrobial, anti-inflammatory and antiatherogenic effects of HT

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https://doi.org/10.1016/j.jfoodeng.2019.07.030
Received 30 October 2018; Received in revised form 13 July 2019; Accepted 29 July 2019

contained in different types of olive oils (De Leonardis et al., 2008; Fernández-Bolaños et al., 2012, 2008; Omar, 2010; Robles-Almazán et al., 2018). Thanks to all these findings, HT has been postulated as a nutraceutical for preventing and treating different diseases (Robles-Almazán et al., 2018). In fact, the European Food Safety Authority (EFSA) approved the following health claim on olive oil polyphenols (Commission Regulation, 2012): "olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil, containing at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil."

So far, several methods and systems aimed at obtaining extracts rich in HT have been developed and patented from the olive and its by-products, as well as from the olive leaf. In many cases, solvent extraction techniques (Allouche et al., 2004) or separation (Russo, 2007) and selective concentration procedures by ultrafiltration (Brenes and Castro, 2004), reverse osmosis (Villanova et al., 2006) and evaporation (Takac and Karakaya, 2010) are used from both the vegetation waters separated from the alperujo and the table olive processing waters. Some of these techniques can be used individually or in an integrated way (Brenes and Castro, 2004). However, all these methods involve many steps, the use of large amounts of organic solvents or have a low yield.

The use of supercritical carbon dioxide (sc-CO_2) as an alternative solvent for extraction has been attracting widespread interest owing to their particular properties (e.g., liquid-like solvent power, negligible surface tension, and gas-like transport properties), and changes in environmental regulations which foster the utilization of green solvents (Brunner, 2005; Sahena et al., 2009). In this field, sc-CO_2 has been specially adopted since it is essentially non-toxic, non-flammable, inexpensive at the industrial level, can be recycled, has easily accessible supercritical conditions, and it is totally dissipated from extracts at atmospheric pressure avoiding the necessity of further expensive and harmful refining treatments (Brunner, 1994). In fact, there are many examples of the use of sc-CO_2 to achieve a selective oil extraction from different plant matrices (Bhattacharjee et al., 2007; Martínez et al., 2008; Özkal et al., 2005; Pederssetti et al., 2011; Rahman et al., 2012).

This work explores the possibility of taking advantage of a by-product of the agroindustrial industry (alperujo) to obtain a valuable product such as an HT-rich oil that could be used in cosmetics and perhaps in food since it would be obtained without the intervention of organic solvents. To that aim, the effect of the main process variables and raw material condition affecting the supercritical fluid extraction (SFE) from alperujo were investigated.

2. Materials and methods

2.1. Chemicals and raw materials

Acetic acid (for HPLC, Sigma-Aldrich, Spain), methanol (CHROMASOLV®, for HPLC, ≥ 99.9%, Sigma-Aldrich, Spain), 1,1-diphenyl-2-picrylhydrazyl (DPPH, Alfa Aesar™, Spain), gallic acid (ACROS Organics™, Spain), sulfuric acid (H_2SO_4, 95%, Sigma-Aldrich, Spain), n-hexane (99%, Sigma-Aldrich, Spain), Folin & Ciocalteu reagent (2 M with respect to acid, Sigma-Aldrich, Spain), heptadecanoic acid (≥98%, Sigma-Aldrich, Spain), boron trifluoride (BF_3, 1.5 M in methanol, ACROS Organics™), sodium hydroxide (NaOH, ≥ 98%, pellets anhydrous, Sigma-Aldrich, Spain), sodium sulphate anhydrous (Na_2SO_4, Sigma-Aldrich, Spain), sea-sand (extra pure, particle size 0.315 mm - 1 mm ≥ 90%, Sigma-Aldrich, Spain), helium (≥99.99%, Air Liquide, Spain) and high purity CO_2 (premier, > 99.995% purity, Air Products Group, Spain) were used for this study. All materials were used as received.

Fresh alperujo was provided by Natac Biotech S.L. (Spain) with an approximate water mass fraction of 70% (0.70 kg water kg^{-1} alperujo) and a fat content of 0.14 kg oil kg^{-1} alperujo on a dry basis (d.b.). The moisture was measured in an oven (Digiheat, J.P. Selecta, S.A., Spain) at 376 K until the weight did not vary more than 0.1%.

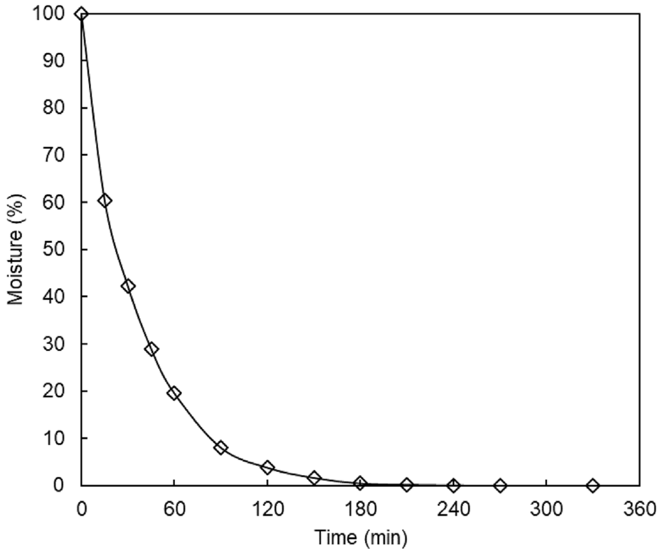


Fig. 1. Drying curve of alperujo at 376 K.

To obtain samples with different moisture content, these were dried by using an oven at 376 K until it reached the desired humidity. For this, a drying curve was performed previously (Fig. 1). The equilibrium moisture of alperujo with the air in Madrid (Spain) was approximately 1.0% in mass fraction. Finally, the dried sample was milled in a commercial coffee blade mill for three cycles of 15 s and then sieved.

2.2. Supercritical fluid extraction

sc-CO_2 extractions from alperujo were carried out at different conditions of temperature (323 K and 373 K), pressure (20 MPa and 30 MPa) and CO_2 mass flow (0.06 kg h^{-1}, 0.18 kg h^{-1} and 0.30 kg h^{-1}). Additionally, the impact of particle size (< 0.80 mm and without sieving) and moisture (equilibrium moisture, 0.1 kg water kg alperujo^{-1} and fresh alperujo) were analysed.

2.2.1. Experimental installation

All SFE were done in a homemade apparatus that consisted of a CO_2 continuous feeding line and an 8 10^{-5} m^3 capacity 316 stainless-steel vessel. A high purity CO_2 was used to avoid large amounts of oxygen in the process, which could degrade interesting compounds during the extraction (Cocero et al., 2000). Moreover, the air from all pipes and valves from the extractor were purged before experiments. A heating jacket was placed around the extractor to control the temperature inside. A membrane pump (Milroyal D; Dosapro Milton Roy, Spain) was used to pump the CO_2 up to the extraction pressure; it had an attached chamber in which a cooling fluid flowed to avoid CO_2 cavitation during pumping. The CO_2 flow rate was measured in the mass-flow meter (M-10 SLPM-D, Alicat Scientific, USA) that was placed at the end of the line, while pressure was controlled by a back-pressure regulator (BPR, 26-1761-24-161, TESCOM Europe, Germany). A schematic representation of the equipment is shown in Fig. 2.

2.2.2. Operation procedure

For each experiment, the extractor was filled with 0.024 kg of alperujo. The sample was introduced with 0.02 kg of sea-sand. The use of sea-sand prevented the sample bed compaction avoiding the formation of preferential channels that reduced the extraction yield. Moreover, no adsorption of the extracts on the sea sand particles was observed. The solid mixture was deposited with a spatula in the extraction vessel

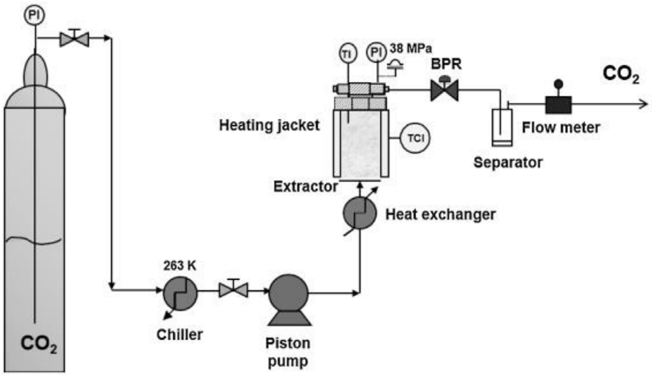


Fig. 2. Schematic representation of the equipment used for supercritical fluid extraction.

forming a fixed bed. A wire mesh was placed on top of the bed to prevent particles entrainment. The extractor was then closed, and the fluid was pumped in to achieve working conditions. Following, the BPR was opened to provide a continuous flow of the fluid through the bed at the selected mass flow. After the BPR, the fluid was depressurized; the solvent power of the CO_2 dropped, and the extract was collected in preweighed vials. The vials were maintained wrapped with aluminium foil and kept in dark at 253 K. The extraction curves were constructed by plotting solvent ratio (kg CO_2 kg alperujo^{-1}) passed through the fixed bed *versus* the oil extraction yield expressed in mg extract g alperujo^{-1}. When a constant mass in the vial was reached, the extraction was stopped.

A similar procedure was used to obtain the theoretical oil solubility data at 323 K and 373 K, both at 30 MPa. For this, the vessel was filled with cotton, which was impregnated with 0.003 kg of the oil previously extracted from the alperujo. The CO_2 flowed through the bed at 0.18 kg h^{-1} and the solubility values were obtained as the maximum slope of the extraction curves constructed.

Separate assays were performed to get samples for the phenol content and the antioxidant activity. In this case, the extraction was carried out all at once to obtain a single sample that was immediately analysed. This was done to avoid possible degradation of the antioxidant compounds. Samples were stored at 253 K before HPLC analysis.

A summary of the pre-treatment of alperujo and the subsequent sc-CO_2 extraction to obtain an HT-rich oil from alperujo is shown in the block diagram of Fig. 3. The alperujo retained most of the HT derivatives that were distributed in the other two residues in the old procedure. Thus, the alperujo contained approximately 98% of the total hydroxytyrosol derivatives initially contained in the pressed olives (Fernández-Bolaños et al., 2002, 2008). After removing most of the water contained in the alperujo, the HT fraction is concentrated in the dried sample, making it readily available for subsequent supercritical extraction.

2.3. *Soxhlet extraction*

Soxhlet extractions were performed using 0.01 kg of dried alperujo. The sample was transferred into a cellulose thimble and inserted into a Soxhlet assembly fitted with a 2.5 10^{-4} m^3 flask. A 1.5 10^{-4} m^3 volume of n-hexane was added and the whole assembly was heated for 8 h using a heating jacket (FIBROMAN-C, SELECTA, Spain). Finally, the extracts were concentrated using a rotary evaporator R-210 (BUCHI, Switzerland) and then immediately analysed.

2.4. *Total phenol content*

The total phenol content of samples coming from the Soxhlet and SCF extractions was analysed according to the Folin–Ciocalteu method (Folin and Ciocalteu, 1927), with minor modifications. In brief, 2.5 10^{-7} m^3 of oil extract diluted in a mixture methanol - H_2O (L L^{-1}) was well mixed in a vortex (Heidolph REAX top, Heidolph Instruments, Germany) with 2.5 10^{-6} m^3 of distilled water and 5 10^{-7} m^3 of the Folin–Ciocalteu stock reagent. After, 1 10^{-6} m^3 of the Na_2CO_3 reagent (75 kg m^{-3}) was added to the mixture and then incubated at room temperature for 30 min. Then, the absorbance was measured spectrophotometrically (MRC, model UV-1800, Tel-Aviv, Israel) at 765 nm. The total phenol content was expressed in milligrams of gallic acid equivalents per gram of oil extract (mg GAE g oil extract^{-1}). The calibration curve was established from 0 kg gallic acid m^{-3} to 0.1 kg gallic acid m^{-3}.

2.5. *Free radical scavenging capacity*

The method principle relies on the fact that the scavenging of free radicals by samples or standards causes a reduction in DPPH absorbance. Briefly, 2 10^{-7} m^3 of a methanolic solution of the sample was added to 3.8 10^{-6} m^3 of a freshly prepared methanolic solution containing 60 μM DPPH. This solution exhibited a deep purple colour with maximum absorption at 517 nm. The reaction mixture was then kept at room temperature in a dark chamber for 30 min. The change in colour from deep violet to light yellow was measured at 517 nm in the spectrophotometer. A standard curve was prepared using the reference synthetic antioxidant Trolox diluted in methanol, and the antioxidant capacity of the extracts equivalent to that of millimoles of Trolox equivalent per gram of extract (TEAC) was calculated. All measurements were repeated at least twice.

2.6. *HPLC analysis of hydroxytyrosol content*

An acid hydrolysis of the extract was performed before the analytical quantification to convert the HT derivatives into free HT and tyrosol. For that, 1 g oil was mixed with 2 mL of a 1 M H_2SO_4 solution. The mixture was maintained in a water bath at 353 K for 2 h, and then, diluted to 10 mL with methanol. Then, it was vortexed for 2 min and centrifuged for 10 min at 3500 rpm. Afterwards, the supernatant was filtered through a 0.45 μm membrane into an HPLC vial. To measure the HT content, samples were analysed through high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD), equipped with a Luna C18 (2), 150 mm × 4.6 mm x 5 μm column, purchased from Phenomenex (Torrance, USA). Methanol was used as mobile phase A and 0.2% in a volume fraction of acetic acid as mobile phase B. The elution program was A (%)/B (%): 10/90, 0 min; 37/63, 10 min; 10/90, 11 min–15 min; eluent flow 1 10^{-6} m^3 min^{-1}. The injection quantity was 2 10^{-8} m^3. The wavelength was fixed at 280 nm.

2.7. *Fatty acids analysis*

First, the samples were derivatized following the procedure described by the AOAC Official Method 969.33 (AOAC, 1995). For this, approximately 1 10^{-4} kg of oil was placed into a screw cap glass tube. 1 10^{-5} kg of heptadecanoic acid as internal standard and 4 10^{-6} m^3 of NaOH in methanol (0.5 N) were added into the tube and then nitrogen was blown for 15 s. The tube was covered tightly, vortexed, heated in a water bath W350 Memmert (Memmert GmbH and Co, Schwabach, Germany) for 5 min at 358 K and then cooled. After 5 10^{-6} m^3 of BF_3 in methanol (12% in mass fraction) was added to the tube, vortexed and then nitrogen was blown. The tube was covered tightly, heated for 15 min at 358 K and then cooled. In this stage, fatty acids were converted to fatty acid methyl esters (FAMEs). Then, 3 10^{-6} m^3 of hexane was added as extracting solvent. Furthermore, 3 10^{-6} m^3 of saturated NaCl solution was added and then the solution was vortexed. Finally, the upper layer was transferred to a small amount of anhydrous Na_2SO_4 (placed on the top of a filter liner) into a test tube with a Pasteur

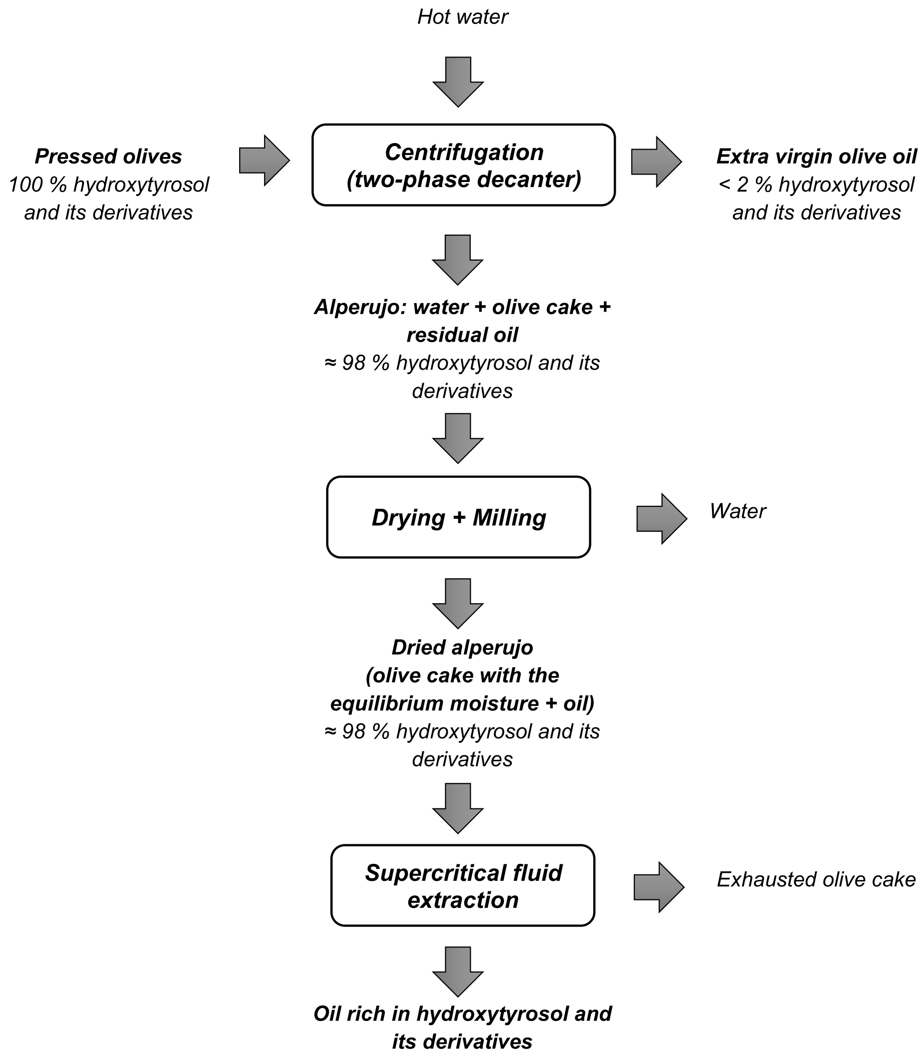


Fig. 3. Scheme for obtaining oil rich in hydroxytyrosol from alperujo with sc-CO_2.

pipette.

Subsequently, the samples were analysed by gas chromatography (GC) with a Shimadzu 2010 Plus gas chromatograph (Shimadzu Corporation, Japan) equipped with a Flame Ionization Detector (FID) and a Zebron ZB-1HT capillary column (20 m × 0.18 mm i.d. × 0.18 μm film thickness). The separation was carried out with helium ($1.8\ 10^{-6}\ m^3\ min^{-1}$) as a carrier gas. The column temperature was programmed starting at a constant value of 393 K during 3 min, heated to 458 K at 3 K min^{-1}, held at 458 K during 3 min, heated again to 523 K at 15 K min^{-1} and finally held at 523 K for 5 min. The equilibration time was 5 min. A split injector (50:1) at 523 K was used. The FID was also heated at 553 K. The injection volume was $1\ 10^{-9}\ m^3$. The compounds were quantified related to the area of the internal standard.

2.8. Statistical analysis

The experiments were repeated three times. Analyses were performed in duplicate for each replicate ($n = 3 \times 2$). Means and standard deviations were calculated for all data. The maximum standard deviation on the supercritical extraction yield was 1.0%.

3. Results and discussion

The extraction yield and rate of the oil from alperujo as a function of the solid pre-treatment: particle size and moisture, and extraction conditions: pressure (P), temperature (T), and CO_2 mass flow rate (Q) are first described. The oil composition is compared to virgin olive oil. Following, the extraction yield and extract purity are compared to those obtained with conventional extraction using a Soxhlet apparatus with n-hexane as the organic solvent. Finally, the oil is analysed in terms of its phenolic content and its antioxidant capacity. The content of HT is specifically followed since it is the most interesting antioxidant compound.

3.1. Effect of particle size

The particle size distribution of the milled and dried Alperujo is shown in Fig. 4. A wide particle size distribution with three maximums was obtained. Alperujo is made up of different types of waste from the olive oil industry such as pulp, pit, seed, peels, leaves and others (Alburquerque, 2004) that produces particles with dissimilar sizes.

SFE from the alperujo without sieving and with a particle size less than 0.80 mm were performed to check out the effect of particle size on the oil recovery. The two extraction curves are compared in Fig. 5a, where the extraction yield (expressed as mg extract g of alperujo^{-1}) is plotted against solvent ratio (kg CO_2 kg alperujo^{-1}). The oil yield increased with the lowest particle size.

Mass transfer in sc-CO_2 extraction from solid substrates in most cases depends heavily on the transport rate within the particle. Particles with a diameter greater than 0.80 mm may hinder the penetration of

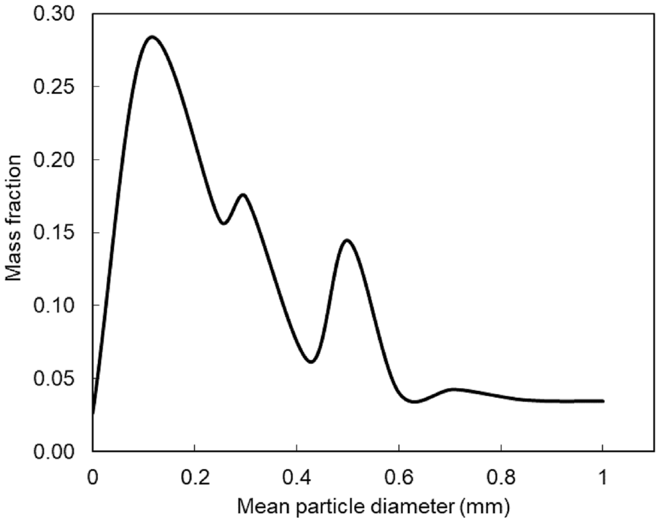


Fig. 4. Particle size distribution of the dried and milled alperujo.

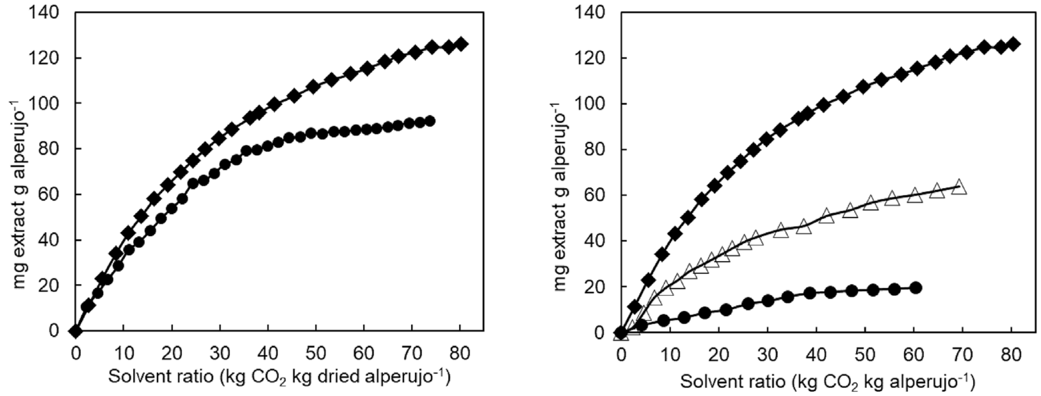
the supercritical solvent and the solubilisation of the solute (Brunner, 1994). On the contrary, particles smaller than 0.40 mm could represent a risk of formation of preferential channels and blockages during extraction (Lack et al., 2001); this fraction of particles represented slightly more than 60% of the particles size distribution (See Fig. 4). Shorter milling times and mortar grinders were used to prevent this size distribution; however, the results were similar, taken into account that the alperujo was composed of several types of waste from the olive oil industry, as it was previously mentioned. Alperujo with a particle size (PS) less than 0.80 mm was used for the rest of the assays.

3.2. Effect of the initial content of water

The excess of water can create a barrier to mass transfer when the raw material is subjected to SFE. However, drying is a very expensive and energy consuming operation. Therefore, the initial water content of alperujo must be optimised. Fig. 5b shows the extraction curves, working at 30 MPa, 323 K, 0.18 kg h^{-1} CO_2 flow rate and different initial moisture content: 1%, 10% and 70%, expressed as the mass fraction of water. The last one corresponded to the fresh alperujo. The fastest extraction was achieved when the sample had a water mass fraction of 1%, which was the equilibrium moisture with the environment humidity in Madrid. Therefore, to improve the extraction rate it was necessary to dry. This should be taken into account for the economic viability of the whole process.

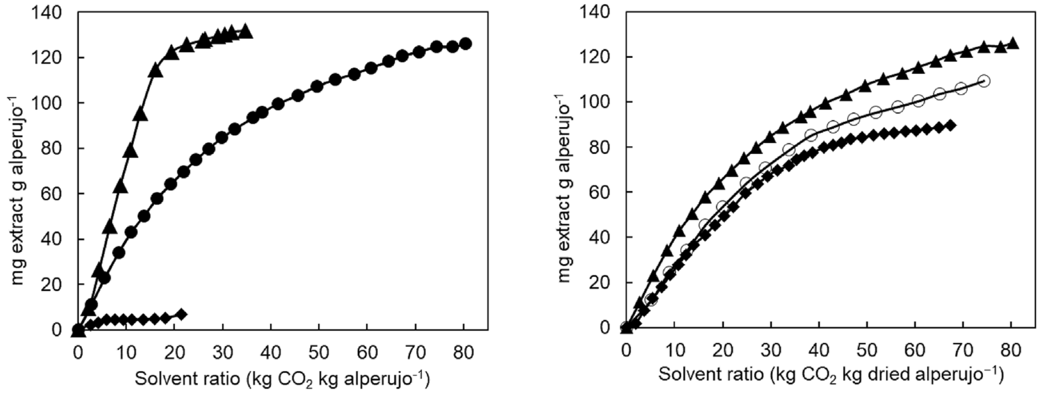
3.3. Effect of operating conditions

The effect of pressure on the oil extraction yield from alperujo was investigated with sc-CO_2 at a constant temperature of 373 K and



a) < 0.80 mm (♦) and whole range of particle sizes (●). Conditions: P = 30 MPa; T = 373 K; Q = 0.18 kg h^{-1}; alperujo moisture = 1 %.

b) 1 % (♦), 10 % (Δ), and 70 % (●). Conditions: P= 30 MPa, T = 323 K; Q = 0.18 kg h^{-1} and PS < 0.80 mm.



c) 20 MPa and 373 K (♦); 30 MPa and 323 K (▲) and 30 MPa and 373 K (●). Conditions: Q = 0.18 kg h^{-1}; PS < 0.80 mm; alperujo moisture = 1 %.

d) 0.06 kg h^{-1} (♦), 0.18 kg h^{-1} (▲) and 0.30 kg h^{-1} (O). Conditions: P = 30 MPa; T = 373 K; PS < 0.80 mm; alperujo moisture = 1 %.

Fig. 5. Supercritical carbon dioxide extraction of hydroxytyrosol-rich oil from alperujo as a function of **(a)** particle size, **(b)** initial water content, **(c)** pressure and temperature and **(d)** CO_2 flow rates.

Table 1
Influence of temperature on oil solubility and maximum extraction load of the supercritical carbon dioxide at 30 MPa.

T [K]	Density [kg m^{-3}]	Pure oil solubility [g kg^{-1}]	Maximum slope on extraction curves [g kg^{-1}]
323	870.43	8.0 ± 0.1	7.9 ± 0.2
373	661.87	2.9 ± 0.0	2.7 ± 0.1

pressures of 20 MPa and 30 MPa. The results are presented in Fig. 5c. As expected, the extraction yield significantly increased with pressure, according to the basic principles of SFE (Brunner, 1994) and similar to the other studies on extraction from olive residues (de Lucas et al., 2002; Le Floch et al., 1998; Şahin et al., 2011). In general, at a constant temperature, the solubility of a substance in an SCF increases with pressure due to the subsequent increase of the solvent density.

The influence of temperature from 323 K to 373 K (at a constant pressure of 30 MPa) on the cumulative content of oil extracted is also shown in Fig. 5c. During sc-CO_2 extraction, the increase in temperature decreases the density of the CO_2, but increases the vapour pressure of the solutes, and favours the desorption of the solutes from the vegetable matrices. At the operating pressures of this work, the highest extraction yield was observed at the lowest temperature. This was mainly due to the high solvent power of sc-CO_2 caused by its highest density at 323 K (see Table 1).

On the other hand, pure oil solubility was determined by carrying out extraction experiments at 323 K and 373 K, both at 30 MPa by using the extracted oil from alperujo of the previous assays. The oil was soaked on cotton and placed in the extractor, by way of the methodology described in Section 2.2.2. As Table 1 shows, oil solubility was highest at the lowest temperature.

A comparison of the theoretical solubility with the maximum slope of the extraction curves at the two temperatures revealed that the slope was close to the theoretical solubility (see Table 1), which means that probably the equilibrium controlled the rate of the extraction at the beginning. This also indicates that the pre-treatment used was enough to make a good cell opening.

3.4. Effect of the supercritical solvent flow rate

For commercial purposes, extraction time and solvent consumption must be optimised since both significantly affect production costs. Besides, the size of pumps and heat exchangers needed to condition the solvent dependency on the solvent flow rate, directly influencing the investment costs. As such, the price of the SCF installation increases as the square root of the solvent flow rate as it has been demonstrated by Perrut (2000). Therefore, it is necessary to optimize this parameter in order to design and run a profitable facility.

Fig. 5d shows the extraction curves that resulted from the use of three CO_2 flow rates that ranged from 0.06 kg h^{-1} to 0.30 kg h^{-1}, operating at 30 MPa and 373 K. The alperujo bed in the extractor had a length-to-diameter ratio of approximately 4:1. An increase in flow rate led to a higher extraction yield and to shorter extraction times. However, the highest yield and rate were maxima when working at 0.18 kg h^{-1}. These results are confirmed in Fig. 6; at a solvent ratio of 7.5 kg CO_2 h^{-1} kg dried alperujo^{-1}, which corresponded to 0.18 kg h^{-1}, the extraction rate was the highest at every moment.

On the one side, the flow rate rise enhanced superficial solvent velocity, which benefited turbulence and reduced the film layer of stagnant fluid around the solid, thus increasing the external mass transfer coefficients. On the other hand, an excessively large flow rate (0.30 kg h^{-1}) lessened the residence time in the extractor, which reduced the time for oil solubilisation and transport into the bulk of the solvent, as it was also demonstrated by Viguera et al. (2016) in the extraction of oil from microalgae.

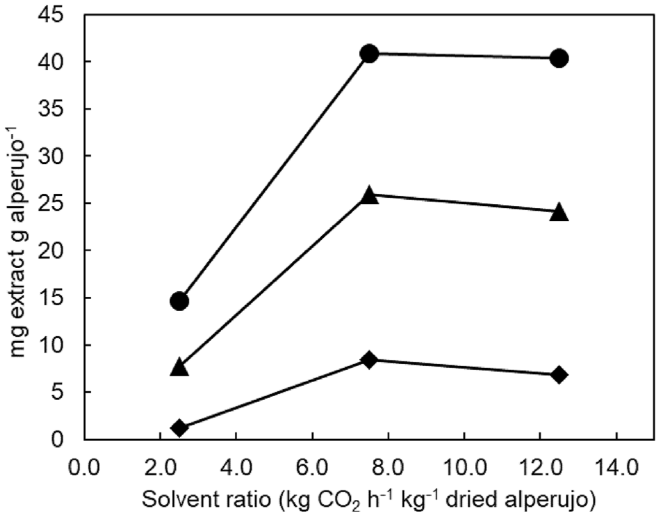


Fig. 6. Impact of increasing solvent ratios on the extraction rate at three extraction moments: 10 min (◆), 30 min (ж), 50 min (O). Conditions: P = 30 MPa; T = 373 K; PS < 0.80 mm; water mass fraction of alperujo = 1%.

3.5. Comparison of the supercritical and soxhlet extraction yield

The extraction yield reached at the most adequate conditions with supercritical extraction was 13% ± 1% d.b., which was close to that achieved by conventional Soxhlet extraction with n-hexane (14% ± 0% d.b.). The slightly higher oil extraction yield with the organic solvent was probably due to the lower selectivity of this latter procedure, as discussed and demonstrated by other authors (Nisha et al., 2012; Soh and Zimmerman, 2011; Viguera et al., 2016). Their findings showed that the extract obtained with n-hexane contained apart from neutral lipids, other polar lipids as well as pigments like chlorophyll.

3.6. Composition of the oil extracted with supercritical CO_2

The comparison of the fatty acid profile between the supercritical extracts and a commercial olive oil is shown in Table 2. The standard deviation for fatty acid composition was on average 6% in mass fraction. The analysis of the oil extract obtained by SFE from alperujo showed that the fatty acid composition was close to the extra virgin olive oil.

3.7. Total phenol content and antioxidant capacity of the extracts

The amount of phenols (expressed as mg gallic acid equivalent g $extract^{-1}$) and the antioxidant capacity (expressed as μM Trolox equivalent g $extract^{-1}$) were examined in the oily extracts resulting from the supercritical extraction. These analyses were also done in the Soxhlet extracts with n-hexane. The results in terms of the total phenol yield and the DPPH radical scavenging activity are presented in Table 3.

During the SFE, the highest values of total phenol content and antioxidant capacity were found in the oil obtained at the uppermost

Table 2
Fatty acid profile of the supercritical extracts and of a commercial virgin olive oil.

Fatty acid	Supercritical extract (%)	Extra virgin olive oil (%)
Palmitic acid	14	14
Stearic acid	3	2
Oleic acid	69	71
Linoleic acid	11	9
Linolenic acid	1	< 1

Table 3
Hydroxytyrosol, total phenol content and antioxidant capacity of the extracts from alperujo.

Type of extraction	mg hydroxytyrosol g extract^{-1}	mg gallic acid equivalent g extract^{-1}	μM Trolox equivalent g extract^{-1}
SFE at 20 MPa and 323 K	Not detected	1.3 ± 0.0	720 ± 18
SFE at 20 MPa and 373 K	0.7 ± 0.0	2.7 ± 0.0	1454 ± 209
SFE at 30 MPa and 323 K	1.2 ± 0.1	3.5 ± 0.0	1944 ± 85
SFE at 30 MPa and 373 K	1.9 ± 0.1	9.6 ± 0.1	6726 ± 156
Soxhlet	Not detected	1.9 ± 0.6	222 ± 37

pressure (30 MPa) and temperature (373 K), being 2.7 times higher than those at 323 K. Le Floch et al. (1998) and Şahin et al. (2011) on the SFE of phenol compounds from olive leaves obtained similar results and related them to the fact that the temperature improved the mass transfer of this type of compounds from the cell. These results contrasted with the total extraction yield, which was better at low temperatures. Thus, the extracts obtained at higher temperature were more concentrated in the phenolic compounds. Consequently, the antioxidant capacity of the supercritical extracts was also higher at 373 K and 30 MPa.

On the other hand, it can be seen in Table 3 that the SFE at 30 MPa and 373 K improved the extraction of phenolic compounds more than five times than the Soxhlet extraction with n-hexane, demonstrating the greater selectivity of the sc-CO_2 for these compounds.

3.8. Content of hydroxytyrosol in the oil

The HT content in the supercritical and in the n-hexane extracts are also reported in Table 3. An example of the chromatogram of the sc-CO_2 extracts is shown in Supplementary file 2. Only the two representative peaks of HT and tyrosol were detected as the samples were hydrolysed before the HPLC analysis. The UV–Vis spectrum of the sample and that of the HT analytical standard are also provided to demonstrate that only free HT was quantified. The samples prior to hydrolysis showed an essentially zero amount of free HT in most of them, indicating that the source of the detected HT in the extracts came from the derivatives contained in the starting alperujo. The HT yields in the supercritical extracts drastically increased with pressure and temperature. In the sample obtained with the lowest pressure (20 MPa) and lowest temperature (323 K), the HT could not be detected; while at 30 MPa and 373 K (the highest pressure and the highest temperature explored), it was 1.9 mg g extract $^{-1}$. Furthermore, the HT content in all the extracts was directly related to the total phenol content and antioxidant capacity.

The high HT concentration in the sc-CO_2 oil extracts could be explained by the amphipathic nature of the HT and the lipophilic characteristic of its derivatives, which is reported in several papers (Fernández-Bolaños et al., 2002, 2008; Procopio et al., 2011; Robles-Almazán et al., 2018). In fact, the high antioxidant character of HT and derivatives created by the conjugation of HT with fatty acids of different molecular weights is partly due to this lipophilic characteristic (Procopio et al., 2011). As some of the HT derivatives are highly lipophilic (Grasso et al., 2007), e.g. some flavonoids and the aglycon derivatives of oleuropein and dimethyl oleuropein, they are also more extractable by sc-CO_2. Moreover, the oil itself contained in the alperujo (about 14% d.b.) could have acted as matrix cosolvent easing the HT extraction. This is already shown for other polar bioactive compounds. Vasapollo et al. (2004) claimed the extraction of lycopene from tomato using sc-CO_2 in the presence of hazelnut oil. Sun and Temelli (2006) used canola oil as cosolvent during the sc-CO_2 extraction of carotenoids from carrot. Krichnavaruk et al. (2008) reported the sc-CO_2 extraction of astaxanthin from *Haematococcus pluvialis* by using vegetable oils (soybean oil and olive oil) as co-solvents. We have recently described the extraction of oil rich in carotenoids from *Dunaliella salina* with solely sc-CO_2 (Tirado and Calvo, 2019).

On the other hand, HT could not be detected in the samples extracted in the Soxhlet extraction with n-hexane. In general, n-hexane is not a good solvent for phenolic compounds (Şahin et al., 2011; Babbar et al., 2014). Another possible reason to not find HT in the extract from this solvent was the harsh conditions and the prolonged duration of the Soxhlet method. The temperature in the distillation flask in contact with the heating element was probably too high; the degradation temperature of HT starts at approximately 536 K (Tu and Yuan, 2018). Moreover, the assays were conducted in the natural light of the laboratory as the Soxhlet apparatus was made of glass. HT is also sensitive to light and oxidation (Fernández-Bolaños et al., 2008; Robles-Almazán et al., 2018). In the sc-CO_2 extraction, high purity CO_2 was used to avoid the presence of oxygen that could promote the oxidation of the HT. The operating temperature was moderate (< 373 K) and the extract was protected from light in all parts of the installation.

Finally, the HT content found in the supercritical extracts of this work was higher compared to other values reported in the literature. The maximum HT concentration of those analysed in this work (1900 ppm), was greater than the value reported by De Marco et al. (2007) in olive mill wastewater (1224 ppm) and significantly superior than that reported by Owen et al. (2000) in extra virgin oil (232 ppm). The difference in HT content would be mainly related to the different raw material, harvesting conditions or other environmental factors; although it could be connected to the way the extraction was carried out.

4. Conclusions

This work shows the possibility of a green extraction of an oil rich in antioxidants from alperujo using supercritical CO_2. In particular, it is possible to obtain an oil rich in polyphenols and in hydroxytyrosol, which is the most potent natural antioxidant. The operation requires the pre-treatment of the alperujo, specifically drying and milling. These expensive operations together with the supercritical extraction itself can lead to high production costs. But the oil quality in terms of fatty acid composition similar to olive oil and with a high antioxidant potential could be used to formulate nutraceuticals, cosmetics or even pharmaceuticals with a high added value. It should not be forgotten that the raw material is a large volume of waste in the Mediterranean countries. Moreover, the cost of the conventional methods (extraction and downstream) to obtain olive oil of much worse quality and the posterior treatment of the wastewater are surely also high.

Acknowledgements

Diego Felipe Tirado Armesto thanks the “Bolívar Gana con Ciencia” program from the Department of Bolívar (Colombia) for an international PhD grant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfoodeng.2019.07.030.

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