Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization.

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Abstract

In the present work, the possibility of using oregano leaves as natural source to obtain nutraceuticals with antioxidant activity has been investigated using an environmentally friendly extraction technique such as the Subcritical Water Extraction (SWE). The extraction of antioxidants from oregano leaves has been studied considering different temperatures (25, 50, 100, 150 and 200°C) to test the selectivity of the process. Different parameters have been tested to evaluate the antioxidant activity of the extracts such as *in vitro* assays (using the radical scavenging DPPH method) and the total phenolic content (measured with the Folin test); results showed a strong antioxidant activity of almost all the extracts obtained at the different temperatures being the highest the one obtained at the highest temperature, 200°C (EC₅₀ equal to 10 µg/ml). It is important to consider that, at these extraction conditions, the extraction yield was also the highest (54% dry weight). The total phenolic content showed no differences among the different extraction conditions tested, therefore, it can be concluded that the amount of phenolic compounds extracted was similar but the type and structure of the phenolics was different, providing in this way different antioxidant activity. The characterization of the chemical composition of the extracts was performed using HPLC coupled to DAD and by comparing the results with those previously published in our research group dealing with the extraction of antioxidant compounds from oregano using supercritical fluid extraction with CO₂. Some compounds could be tentatively identified, proposing some probable chemical structures for some of them, such as flavanones, dihydroflavonols, favonols and flavones.

Keywords: subcritical water extraction, oregano, antioxidant activity, flavonoids

Introduction

A nutraceutical is usually defined as any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. In the last few years, there has been an increasing interest towards the search and characterization of new nutraceuticals from natural sources able to provide some additional benefit for human health such as antioxidants, anti-inflammatories antihypertensives, etc. [1]. Antioxidant compounds are usually employed in the food industry to prevent undesirable changes due to oxidation reactions, but the main importance of such compounds is related to their health benefits [2]. In recent years, there has been a wide interest in finding natural compounds that could replace synthetic antioxidants such as BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole), which are commonly used in foods, because of its possible toxicity and due to a suspected action as promoters of carcinogenesis [3,4]. In this sense, some studies have reported the interest of herbs and spices, commonly employed as food ingredient to flavour different types of food preparations, since they contain a wide variety of compounds that have shown to have beneficial health effects [4]. It has recently become clear that one of the values of many spices is that they contain natural antioxidants which provide protection against harmful free radicals. Despite the scientific documentation indicating possible antioxidant effects of many spices such as rosemary, sage, thyme or oregano, only some of them have been widely studied as nutraceuticals sources [5-8].

Oregano is an herbaceous plant native to the Mediterranean regions used as a medicinal plant that belongs to the Labiatae family. Some healthy properties have been attributed to this plant, such as its powerful anti-bacteria and anti-fungal effects [9, 10] that seemed related to some of the primary components of oregano essential oil, namely, carvacrol and thymol [11]. Also, some antioxidant activity has been detected in these compounds [12]. However, little is known about the antioxidant activity of oregano compounds. Some authors have found free radical scavenging activity in aqueous

and organic extracts of oregano leaves [13, 14]. In a recent work done in our research group, we studied the antioxidant activity of CO₂ supercritical fluid extracts of oregano and some of the compounds responsible of the antioxidant activity were tentatively identified as dihydroquercetin, eriodictiol, dihydrokaempferol, naringenin, quercetin and other flavones and phenolics [15].

Extraction of antioxidants from plant tissues has usually been accomplished by conventional extraction processes such as solid-liquid extraction employing methanol, ethanol, and acetone and also through steam distillation. Recently, there has been an increasing interest in the use of environmentally clean technologies able to provide high quality and high activity extracts while precluding any toxicity associated to the solvents. In this sense, both, supercritical fluid extraction (SFE) with carbon dioxide (CO₂) as a solvent and subcritical water extraction (SWE) meet the requirements to be considered clean and safe processes [16-18].

SWE, i.e., extraction using hot water under pressure sufficient to maintain water in the liquid state, has demonstrated its ability to selectively extract different classes of compounds depending on the temperature used, with the more polar extracted at lower temperatures while the less polar compounds were extracted at higher temperatures. The selectivity of SWE allows for manipulation of the composition of the extracts by changing the operating parameters and has been used for essential oil isolation [17, 19-23] as well as for antioxidant extraction [24, 25].

The goal of the present investigation was to study the selectivity of SWE at several temperatures to extract antioxidant compounds from oregano leaves. SWE is proposed in this work as a feasible process to concentrate and isolate antioxidant compounds to be used as nutraceuticals. The extraction process has been evaluated in terms of activity of the extracts and total phenolic composition. LC-DAD was used to characterize the extracts in terms of their chemical composition.

Experimental

Samples and chemicals

The oregano sample (*Origanum vulgare* L.) consisted of dried oregano leaves obtained from Murciana de Herboristeria (Murcia, Spain). Oregano leaves were collected during September and then dried by using a traditional method previously described [26]. Cryogenic grinding of the sample was performed under carbon dioxide. The size of the particle (between 250 and 500 μm) was determined by passing the ground plant material through sieves of appropriate size. The whole sample was stored in amber flasks at -20°C until use (a maximum of 2 months).

1,1-diphenyl-2-picrylhydrazyl (DPPH, 95% purity) was from Sigma-Aldrich (Madrid, Spain). Methanol was from Fluka. Milli-Q water was obtained using a purification system (Millipore Corporation, Billerica, MA, USA).

Subcritical water extraction

To perform the extractions an Accelerated Solvent Extraction system ASE 200 equipped with a solvent controller unit from Dionex Corporation (Sunnyvale, CA, USA) was used. Extractions were carried out in triplicate using water. Two different extraction procedures have been used depending if individual extractions (at a chosen temperature) or sequential experiments were performed.

Individual temperatures considered were 25, 50, 100, 150 and 200°C with 30 minutes extraction time. Sequential extractions were performed using the same sample considering the following temperatures: 25, 50, 100, 150 and 200°C and 15 minutes extraction time at each temperature. Previous to each experiment an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature (the heat-up time is automatically fixed by the equipment). Namely, 5 minutes heat-up was used when extraction temperature was set at 50 and 100°C , 7 minutes

at 150 °C and 9 minutes at 200°C. Likewise, all extractions were performed in 11 mL extraction cells, containing 750 mg of sample.

The extraction procedure was as follows: i) sample is loaded into cell, ii) cell is filled with solvent up to a pressure of 1500 psi, iii) initial heat-up time is applied, iv) static extraction with all system valves closed is performed (for 30 or 15 min, depending if individual or sequential extraction was considered), v) cell is rinsed (with 60 % cell volume using extraction solvent), vi) solvent is purged from cell with N₂ gas and vii) depressurization takes place. Between extractions, a rinse of the complete system was made in order to overcome any extract carry-over. For solvent evaporation a freeze dryer (Freeze Dry System, Model 79480 de Labconco Corp., Kansas City, MO 64132, USA) was employed. The collected extracts were kept protected from light, at 4°C until use.

Determination of antioxidant activity

The antioxidant activity was determined using the procedure described by Brand-Williams et al. [27] that consists in the neutralization of free radicals of DPPH by the antioxidant extracts. The procedure was the following: 23,5 mg of DPPH were dissolved in 100 ml of methanol. This solution was diluted 1:10 with methanol; different concentrations of oregano extracts were tested and 0,1 ml of these solutions along with 3,9 ml of DPPH solution were placed in test tubes to complete the final reaction media (4 ml). Reaction was completed after 4 h at room temperature and absorbance was measured at 516 nm in a UV/VIS Lambda 2 Perkin Elmer Inc. spectrophotometer (Wellesley, MA, USA). Methanol was used to adjust zero and DPPH-methanol solution as a reference sample.

The DPPH concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression (n= 7; r= 0,9999):

$$[\text{DPPH}] = (\text{Abs} + 0,0029) / 0,0247.$$

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 EC₅₀. The lower the EC₅₀, the higher the antioxidant power.

HPLC-DAD analysis of the extracts

Analysis were performed with an Agilent 1100 series with manual injection equipped with a Zorbax C18 column 4.6x150 mm, 5 µm particle size. The mobile phase was a mixture of solvent A (50% acetonitrile in water) and solvent B (10 mM acetic acid in water) according to a step gradient lasting for 35 min, starting from 50% B at 5 min to 30% B at 15 min and 0% B at 30 min. Flow rate was kept to 0,6 ml/min. Detection was accomplished by using a diode array system at a wavelength of 230 nm.

Folin method for total phenol determination

Total phenols were determined as described by Yang et al (2002) [28]. Freeze-dried powder, from SWE extractions, was dissolved in a solution of 5 ml of 1.3% HCl in methanol/deionised water (60:40, v/v) and the resulting mixture (100 µl) was added to 2 ml of 2% aqueous sodium carbonate solution. After 3 min, 100 µl of 50% Folin-Ciocalteau's reagent (Sigma) was added to the mixture. After 30 min standing, absorbance as measured at 750 nm. Content of total phenols was calculated on the basis of the calibration curve of gallic acid (Sigma).

Results and Discussion

Two different sets of experiments were studied: sequential extractions and individual extractions at a single temperature [24]. The first approach consists of the sequential extraction of oregano leaves at different temperatures ranging from 25 to 200°C for 15 minutes. In this approach, the same material is extracted sequentially at higher temperatures (25, 50, 100, 150 and 200°C), providing information about selectivity. The second approach allows direct comparison of the total antioxidant activity obtained with a single extraction temperature, as might be used in an industrial process, thus allowing the determination of the different compounds that have been extracted at each temperature.

Tables 1 and 2 show, respectively, the results obtained for individual and sequential extractions in terms of antioxidant activity (EC_{50}), extraction yield (dry weight) and total phenols (expressed as mg of gallic acid/mg extract). As can be seen comparing the two different sets of experiments, the extraction yields achieved at individual temperatures extractions are much higher than when sequential extractions are applied, increasing with the temperature and reaching values higher than 50% at 200°C (see Table 1). This behavior is not observed when sequential extraction is used since compounds that can be extracted at 50°C have most probably been extracted at 25°C. In this sense, the different selectivity can be seen when comparing yields achieved at 100 and 200°C since at this high temperature, yield increases because other compounds different from those that have been extracted at lower temperatures can be recovered.

By analyzing the results corresponding to the antioxidant activity of the different extracts (EC_{50} values) as a function of temperature, it can be seen that the highest antioxidant activities (lower EC_{50} values) were achieved when working at the highest temperatures (150 and 200°C) for individual extractions. For sequential extractions, an increase in EC_{50} values was observed when increasing the temperature up to a maximum value of 25 $\mu\text{g/ml}$ at 150°C, similar to the value obtained when working at 200°C. One important aspect that should be considered is that working at high temperatures with

pressurized fluids does not necessarily imply degradation or oxidation of the bioactive compounds in the oregano leaves. This fact was previously confirmed working with rosemary [24] where carnosic acid, which is the more potent antioxidant in this plant, was preferentially extracted at 200°C. In this regard, it is important to point out that the antioxidant activity of the subcritical water extracts of oregano was higher than the one obtained with supercritical CO₂ extraction of the same plant (EC₅₀ values higher than 53 µg/ml) [15].

The Folin analysis of oregano extracts obtained by individual subcritical water extractions reveals the presence, in all of them, of phenolic compounds (Table 1). Regarding to the total amount of phenols, statistical differences (using one-way ANOVA) were not found among the different extracting conditions. However, the highest content in phenols corresponded to the extraction carried out at 25 °C, where phenols represented more than 18 % of dry weight (corresponding to 0,182 mg of gallic acid per mg of extract, see Table 1). The lowest percentage of phenols was found in the extraction performed at 200 °C in which phenols represented approximately 14 % of dry weight (corresponding to 0,149 mg of gallic acid per mg of extract, see Table 1). By analyzing the data of Table 1 and 2 it can be concluded that the antioxidant activity of the oregano extracts (referred as EC₅₀) does not correlate with the total phenols parameter. This can be explained by the extraction of different type of phenolic compounds, at the different temperatures tested. As a result, polyphenols with a different relationship structure-antioxidant activity can come out; in this sense, the relationship between the chemical skeleton of flavonoids and the ability to scavenge radicals has been suggested elsewhere [29, 30].

Nevertheless, when extractions are carried out sequentially, the highest amount of phenols (18% of dry weight, corresponding to 0,182 mg gallic acid /mg extract) is obtained at 50 °C, which is interpreted as an increase on extraction efficiency due to temperature; it is important to point out that, at these conditions, also the best antioxidant activity was obtained. Higher temperatures do not increase the yield of total phenols as the matrix progressively exhausts and only residual phenols are extracted. On the other hand, the lowest yield in phenolics is obtained at 150°C (8% of dry weight, corresponding

to 0,084 mg gallic acid/mg extract) that also corresponded to the fraction with lower antioxidant activity. However, at temperatures of 200 °C there is an increase in the amount of extracted phenols that reach 12% of DW, being interpreted as an increase in the extraction of low polarity compounds due to the change of the solvent characteristics of water near the critical point. The behaviour is similar to that observed when other plant materials were used (data not shown).

Since as demonstrated above, individual extractions of Table 1 were able to provide both, better antioxidant activities and higher yields, these extracts were selected in order to characterize their chemical composition by HPLC with diode array detection. Therefore, oregano extracts obtained by SWE at 25, 50, 100, 150 and 200 °C were analyzed and the results are shown in Table 3. As can be seen, different compounds (characterized for their retention time (t_R)) were detected in the different extracts and they show the UV-absorbance maxima and shoulders given in Table 3. A putative flavonoid skeleton, for the pertinent compounds, is proposed and shown in Table 3. The aglycon structures are suggested on the basis of their corresponding UV spectra and using also data obtained in a previous work done by our research group by exhaustively characterizing oregano supercritical extracts by LC-MS [15].

Thus, peaks at 3,16, 3,43, 3,58, 3,75, 4,56 and 6,07 min showed UV maximum at wavelengths between 285-290 nm and a shoulder between 300-340 nm, which is consistent with spectra corresponding to flavanone and dihydroflavanol skeleton [31]. These results are consistent with other previously reported in which the presence of flavanone/dihydroflavanol structures, such as dihydroquercetin, eriodictyol and dihydrokaempferol were detected in oregano extracts obtained with supercritical CO₂ [15]. However, further structural elucidation is required to fully identify these structures.

Other compounds with retention times at 3,91 and 3,96 min respectively showed maximum at wavelengths between 280-360 nm and a shoulder between 250-305 nm. These spectral characteristics are consistent with those corresponding to flavonols [31], which have been widely reported in oregano

under the form of quercetin [15, 32, 33]. A less represented class of flavonoides in the extracts of oregano are flavones. However, a peak with retention time at 6,28 min showed one maximum at wavelengths between 310-350 nm and a second one between 250-285 nm, which is consistent with a flavone skeleton [31] and our previously reported results [15].

As mentioned, Table 3 shows the relative percentage (referred to the total area of the selected components based on DAD peak area at 230 nm) of each compound in the extract depending on the temperature of extraction and provides information about the semi-quantitative composition of the extracts. Studying the data in Table 3, the profiles of the different extracts can be observed. For example, extracts obtained at high temperatures (150, 200 °C) showed a higher relative abundance of compounds eluted at longer t_R , (and, therefore, of low polarity when referred to the used HPLC conditions) compared with those ones obtained at lower temperatures (25, 50 °C). This variation on the polarity of the extracted compounds depending on the extraction temperature is explained by the previously suggested compound class selectivity achieved by using subcritical water. This selectivity exists because extraction depends on solvation of the target compounds in the liquid state of the water. As a polar fluid, water normally solvates more polar compounds more readily than non-polar compounds. Higher temperatures reduce the polarity of water, thus increasing its ability to solvate non-polar compounds. At the lowest temperature, the more polar compounds, such as some flavanones/dihydroflavonol structures (such as dihydroquercetin, eriodictyol and dihydrokaempferol) were preferentially extracted ((FLA-1 and FLA-3 main compounds extracted at 25, 50 and 100°C). When water was heated up to 200°C, the dielectric constant of water was reduced to values similar to those of methanol or acetonitrile which increased the solubilities of less polar compounds such as those eluting at longer retention times in a reversed-phase HPLC system (from NI-6 to NI-9) by several orders of magnitude, resulting in such compounds being the major constituents of these fractions.

Results obtained during the chemical characterization of the subcritical water extracts of Table 3, strongly support the theory of a different type of compounds being extracted at the different

temperatures tested. Thus, it is reasonable to think that even if the total phenols is similar in those extracts performed at individual temperatures and these data do not correlate with the antioxidant activity, the structure of the different flavanoids extracted at the different conditions will finally determine the antioxidant activity of the whole extract. Also, an important aspect to consider is the synergies between different compounds that could also influence the final activity of the extracts.

Conclusions

In the present study, it is demonstrated that the combination of SWE together with biological and chemical analysis is a suitable protocol to obtain and characterize nutraceuticals from natural sources. Thus, the possibility of tuning the extraction selectivity of pressurized water by changing its temperature has been demonstrated for the extraction of antioxidant compounds from oregano using SWE. Also, the process described here allows obtaining and characterizing extracts enriched with different types of nutraceuticals with important antioxidant activities. Moreover, the SWE process proposed in this work has clear possibilities of being used at industrial scale for nutraceuticals production.

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Table 1. Values of EC₅₀ (µg/ml), extraction yield (% dry weight) and total phenols (as mg gallic acid/mg extract) obtained for extractions at a fixed temperature (individual extractions) for 30 min and 1500 psi. Values are the average of 3 replicates.

Extraction Temperature (°C)	EC ₅₀ /(µg/ml)	Extraction yield (%)	Total phenols (mg gallic acid/mg extract)
25	17 ± 3	29,2 ± 0,4	0,182
50	13 ± 3	33,3 ± 1,6	0,175
100	11 ± 2	38,1 ± 1,5	0,168
150	10 ± 1	48,8 ± 0,4	0,179
200	10,2 ± 0,3	54 ± 1,3	0,149

Table 2. Values of EC₅₀ (µg/ml), extraction yield (% dry weight) and total phenols (as mg gallic acid/mg extract) obtained for sequential extractions for 15 min and 1500 psi.

Values are the average of 3 replicates.

<i>Extraction</i> <i>Temperature (°C)</i>	EC ₅₀ /(µg/ml)	Extraction yield (%)	Total phenols (mg gallic acid/mg extract)
25	14 ±2	29,1 ±1,7	0,120
50	9,0 ±0,2	3,8 ±0,4	0,182
100	12 ±2	3,4 ±0,04	0,136
150	25 ±4	12,5 ±0,1	0,084
200	22 ±6	11,2 ±0,1	0,120

Table 3. Characteristic parameters of the compounds detected in the subcritical water oregano extract analyzed by LC-DAD. Relative percentage (normalized areas (%)) of the compounds corresponding to individual extractions at different temperatures.

tr	Spectra*	Compound **	Normalized Areas (%) 25 C	Normalized Areas (%) 50 C	Normalized Areas (%) 100 C	Normalized Areas (%) 150 C	No Ar
2,95	287M 326M	NI-1	25,0	30,2	-	-	
3,16	286M 325Sh	FLA-1	-	-	-	1,4	
3,28	268M 288M 330M	NI-2	-	-	-	5,1	
3,30	262M 292M 330Sh	NI-3	20,6	3,1	-	-	
3,43	290M 330Sh	FLA-2/FLA-3	19,6	33,1	29,0	14,3	
3,56	280M 330Sh	NI-4	-	-	-	-	
3,58	288M 325Sh	FLA-4	-	-	-	1,9	
3,75	286M 325Sh	FLA-5	-	-	-	2,8	
3,91	270Sh 288M	FOL-1	-	-	-	-	
3,96	305Sh 340M	FOL-2	12,8	5,9	6,0		
4,21	268M 288Sh 330M	NI-5	-	-	-	2,8	
4,56	290M 330Sh	FLA-6	0,8	0,9	6,6	8,0	
6,07	288M 340Sh	FAL-7	-	-	-	1,3	
6,28	285M 340M	FLN	3,9	3,0	10,7	3,8	
13,62	288M	NI-6	-	-	-	8,7	
17,43	290M 345M	NI-7	5,9	4,3	12,9	8,9	
26,38	290M 332Sh	NI-8	-	-	-	6,5	
31,38	276M	NI-9	11,4	19,14	34,7	34,5	

* M: maximum, Sh: Shoulder. ** FLA:Flavanone, FLN: Flavone, FOL: Flavonol, NI: non identified