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

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Abstract

In the present work, oregano leaves (*Origanum vulgare* L.) are explored as natural source of nutraceuticals with antioxidant activity. To do this, subcritical water extraction (SWE), a new environmentally friendly technique, is employed as extraction procedure and HPLC coupled to DAD is used for the chemical characterization of the extracts. Moreover, the radical scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and the determination of the total phenolic content (measured with the Folin test) are applied to evaluate the antioxidant activity of the extracts. The extraction of antioxidants from oregano leaves by SWE is studied considering different temperatures (25, 50, 100, 150 and 200 °C) to investigate the selectivity of the process. The highest antioxidant activity is observed for the extract obtained at the highest temperature, 200 °C (EC_{50} equal to 10 μg/ml). Moreover, the extraction yield was also the highest (54% dry weight) at these extraction conditions. The total phenolic content showed no differences among the different extracts, concluding 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. 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; Phenolic compounds

1. 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 diseases. 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 and 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 commonly used in foods such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), 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 ingredients to flavour different types of food preparations, since they contain a wide variety of compounds that can have beneficial health effects [4]. Recently, it has been shown that one of the main values of spices is that they can contain natural antioxidants which provide protection against harmful free radicals [5–8]. 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].

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Oregano is an herbaceous plant native to the Mediterranean regions that belongs to the Labiatae family. Some healthy properties have been attributed to this plant, such as its powerful antibacteria and antifungical effects [9,10] related to carvacrol and thymol compounds that are primary components of oregano essential oil [11]. Also, some antioxidant activity has been detected in these compounds [12]. However, little is known about the antioxidant activity of other oregano compounds, although some authors have found free radical scavenging activity in aqueous and organic extracts of oregano leaves [13,14]. In a recent work, our research group studied the antioxidant activity of CO_2 supercritical fluid extracts of oregano and some of the compounds responsible of the antioxidant activity were tentatively identified as dihydroquercetin, eriodyctiol, 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 (CO_2) 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 it 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 (*Origanum vulgare* L.). 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 antioxidant activity of the extracts and their total phenolic composition. HPLC–DAD was used to characterize the extracts in terms of their chemical composition.

2. Experimental

2.1. Samples and chemicals

The oregano sample (*O. 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).

2.2. 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 min extraction time. Sequential extractions were performed using the same sample considering the following temperatures: 25, 50, 100, 150 and 200 °C and 15 min 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 min heat-up was used when extraction temperature was set at 50 and 100 °C, 7 min at 150 °C and 9 min 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 N_2 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, USA) was employed. The collected extracts were kept protected from light, at 4 °C until use.

2.3. 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 ($n = 7$; $r = 0.9999$):

$$[\text{DPPH}] = \frac{\text{Abs} + 0.0029}{0.0247}.$$

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 EC_{50}. The lower the EC_{50}, the higher the antioxidant power.

2.4. HPLC–DAD analysis of the extracts

Analyses were performed with an Agilent 1100 series with manual injection equipped with a Zorbax C18 column 4.6 mm × 150 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 280 nm.

2.5. Folin method for total phenol determination

Total phenols were determined as described by Yang et al. [28]. Freeze-dried powder, from SWE extractions, was dissolved in a solution of 5 ml of 1.3% HCl in methanol/deionized 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 was measured at 750 nm. Content of total phenols was calculated on the basis of the calibration curve of gallic acid (Sigma).

3. Results and discussion

Two different sets of experiments were studied: sequential extractions and individual extractions. The first approach consists of the sequential extraction of oregano leaves at different temperatures ranging from 25 to 200 °C for 15 min. 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 at a given 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 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% dry weight 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 already extracted at 25 °C what reduces the extraction yield. In this sense, differences in selectivity can be deduced comparing yields achieved at 100 and 200 °C since at the highest temperature yield increases because other compounds different from those extracted at 100 °C can be recovered. As for the difference in precision observed for the same experiment (at 25 °C) when individual or sequential extractions are compared (Tables 1 and 2, respectively), it can be due to the different extraction times used (30 and 15 min, respectively); for instance, a higher precision can be associated to a longer extraction time depending on the extraction rate and on the amount of extractable material.

Table 1
Values of EC_{50} (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

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

Values are the average of three replicates.

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) for individual extractions (see Table 1) were achieved when working at the highest temperatures (150 and 200 °C). For sequential extractions (see Table 2), an increase in EC_{50} values up to a maximum value of 25 μg/ml was observed when increasing the temperature 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 CO_2 extraction of the same plant (EC_{50} values higher than 53 μg/ml) [15].

Table 2
Values of EC_{50} (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

Extraction temperature (°C)	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

Values are the average of three replicates.

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 gallic acid/mg 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 gallic acid/mg extract; see Table 1). By analyzing the data of Tables 1 and 2 it can be concluded that the antioxidant activity of the oregano extracts (referred as EC_{50}) does not correlate with the total phenols parameter. Regarding the relationship between phenolic content and antioxidant activity, different results have been reported depending on the raw material and on the method used to measure the antioxidant activity; some studies did not found correlation working, for example, with citrus residues [29] or plant extracts [30]. In our study, this can be explained by the extraction of different type of phenolic compounds, at the different temperatures tested, giving, as a result, polyphenols with a different relationship structure–antioxidant activity; in this sense, the relationship between the chemical skeleton of flavonoids and the ability to scavenge radicals has already been suggested [31,32].

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 phenols 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 in Table 2) 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 dry weight, 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.

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 of a previous work from our group working with oregano supercritical extracts and HPLC–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 and 290 nm and a shoulder between 300 and 340 nm, which is consistent with spectra corresponding to flavanone and dihydroflavanol skeleton [33]. These results are consistent with other previously reported in which the presence of flavanone/dihydroflavonol structures, such as dihydroquercetin, eriodictyol and dihydrokaempferol were detected in oregano extracts obtained with supercritical

Table 3
Characteristic parameters of the compounds detected in the subcritical water oregano extract analyzed by LC-DAD

Peak no.	t_R	Spectra[a]	Compound[b]	Normalized areas (%)				
				25 °C	50 °C	100 °C	150 °C	200 °C
1	2.95	287M 326M	NI-1	25.0	30.2	–	–	–
2	3.16	286M 325Sh	FLA-1	–	–	–	1.4	
3	3.28	268M 288M 330M	NI-2	–	–	–	5.1	7.3
4	3.30	262M 292M 330Sh	NI-3	20.6	3.1	–	–	–
5	3.43	290M 330Sh	FLA-2/FLA-3	19.6	33.1	29.0	14.3	
6	3.56	280M 330Sh	NI-4	–	–	–	–	2.4
7	3.58	288M 325Sh	FLA-4	–	–	–	1.9	–
8	3.75	286M 325Sh	FLA-5	–	–	–	2.8	–
9	3.91	270Sh 288M	FOL-1	–	–	–	–	12.6
10	3.96	305Sh 340M	FOL-2	12.8	5.9	6.0		
11	4.21	268M 288Sh 330M	NI-5	–	–	–	2.8	3.5
12	4.56	290M 330Sh	FLA-6	0.8	0.9	6.6	8.0	6.5
13	6.07	288M 340Sh	FLA-7	–	–	–	1.3	1.2
14	6.28	285M 340M	FLN	3.9	3.0	10.7	3.8	4.7
15	13.62	288M	NI-6	–	–	–	8.7	8.9
16	17.43	290M 345M	NI-7	5.9	4.3	12.9	8.9	10.1
17	26.38	290M 332Sh	NI-8	–	–	–	6.5	6.9
18	31.38	276M	NI-9	11.4	19.14	34.7	34.5	35.6

Relative percentage (normalized areas (%)) of the compounds corresponding to individual extractions at different temperatures.
[a] M: maximum; Sh: shoulder.
[b] FLA: flavanone; FLN: flavone; FOL: flavonol; NI: non identified.

CO_2 [15]. However, further structural elucidation is required to undoubtedly identify these structures.

Other compounds with retention times at 3.91 and 3.96 min showed, respectively, maximum at wavelengths 288 or 340 nm and a shoulder at 270 or 305 nm. These spectral characteristics are consistent with those corresponding to flavonols [33], which have been widely reported in oregano under the form of quercetin [15,34,35]. A less represented class of flavonoids in the extracts of oregano are flavones. However, a peak with retention time at 6.28 min showed one maximum at wavelengths between 310 and 350 nm and a second one between 250 and 285 nm, which is consistent with a flavone skeleton [33] 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 and 200 °C) showed a higher relative abundance of compounds eluting at longer retention times (and, therefore, of low polarity when referred to the used HPLC conditions) compared with those ones obtained at lower temperatures (25 and 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 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-3 is the main compound extracted at 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 (from NI-6 to NI-9) by several orders of magnitude, resulting in such compounds being the major constituents of these fractions. As an example, Fig. 1 shows a typical chromatographic profile of a subcritical water extract of oregano obtained at 150 °C.

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 flavonoids extracted at the different conditions will finally determine the antioxidant activity of the whole extract. Several authors have demonstrated that, for example, polymeric polyphenols are more potent antioxidants than simple phenols [36,37]. As a general trend, improved stabilization of the phenoxyl radical is desirable; the antioxidant effect of the phenolic compounds has been described to depend on the number and position of hydroxyl and methoxyl groups in the benzene ring and the possibility of electron delocalization in the double bonds [38]. Another important aspect that has to be considered in such complex extracts is the synergy between different compounds that could also influence the final activity of

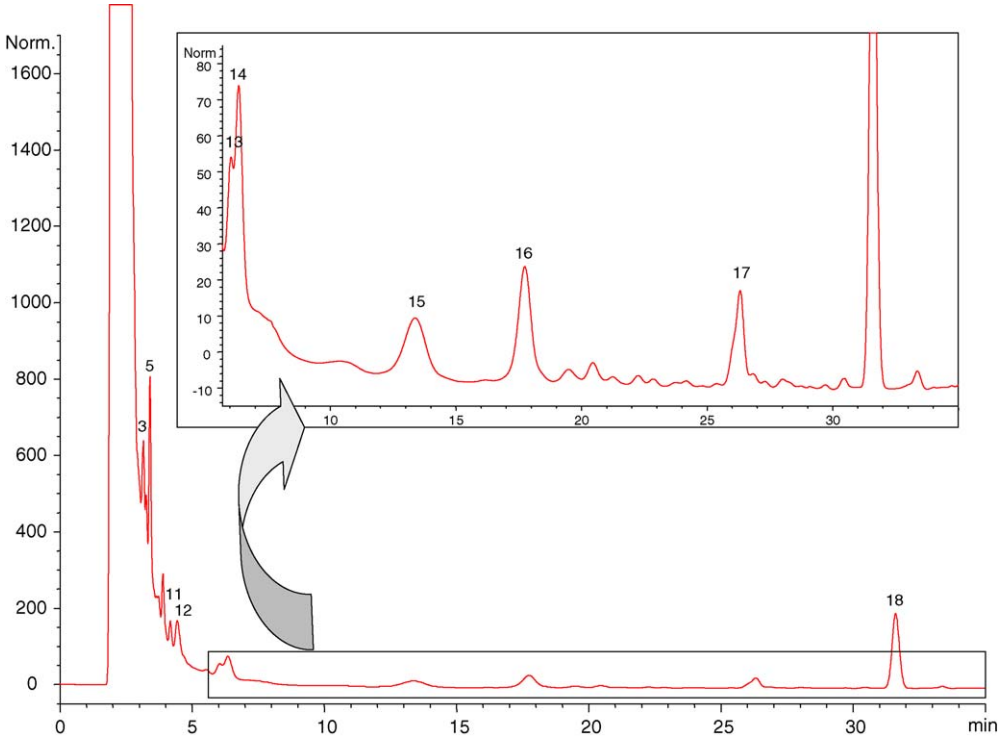

Fig. 1. HPLC chromatogram, at a wavelength equal to 280 nm, of a subcritical water extract obtained at 150 °C. Peak data as in Table 3.

the extracts. This fact has been proved by other authors working with both, crude and simulated extracts [39].

4. Conclusions

In the present study, it is demonstrated that the combined use of SWE together with biological and chemical analytical procedures 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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