Assisted extraction of rosemary antioxidants with green solvents

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Abstract

The use of natural antioxidants in the food industry has increased in the last years and there is a growing interest in improving the extraction processes using GRAS (general recognize as safe) solvents. In this work the extraction of antioxidants from rosemary with ethanol and water as solvents has been studied using different extraction processes (conventional, microwave assisted – MAE – and ultrasound assisted – USAE –) and plant pretreatments (deoiled and milled, deoiled and fresh plant). Total phenolic compounds in the extracts were determined by the Folin–Ciocalteu assay and HPLC with UV detection was employed for the quantification of the main antioxidant compounds: rosmarinic acid and carnosic acid. The antioxidant activity of the extract was determined by the DPPH• scavenging assay. The double pretreatment, deoiling by solvent free microwave extraction (SFME) and milling, has shown to be essential to overcome inner mass transfer limitations. Extraction efficiency can be additionally enhanced by microwave and ultrasound assisted extraction process, being this latter more significant in aqueous extracts.

Keywords
Ultrasound assisted extraction; microwave assisted extraction; phenolic antioxidants; Rosemary;

1. Introduction

Oxidation is one of the most important processes involved in food degradation. Antioxidants are compounds capable of scavenging free radicals delaying, retarding or preventing auto-oxidation. The growing interest of consumers in more natural foods and the concern of some human health professionals about potential toxicological long-term effects for the synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated Hydroxytoluene (BHT), have fostered more efficient and cleaner extraction processes to isolate natural antioxidants.

Natural antioxidants are mainly polyphenolic compounds, aromatic secondary plant metabolites. In rosemary, the most important ones are rosmarinic acid and carnosic acid. They are found mainly in rosemary leaves. Other parts such as stem, roots and flowers have little content of polyphenols. Only carnosic acid has a higher concentration during spring and summer in flowers (Del Baño et al., 2003). Carnosic acid is found in chloroplasts, subcellular organelles with their own double membrane (Munné-Bosch and Alegre, 2001). Valued traditionally as a spice, rosemary is now being studied because of its antioxidant properties in the conservation of fresh, cooked, frozen or pre-cooked frozen fish and meat (Vareltzis et al., 1997; Sebranek et al., 2005).

The most common lab scale technique of obtaining natural antioxidants from plant materials is soxhlet extraction, carried out at the solvent boiling point. The usual solvents are methanol and acetone (Chang et al., 1977; Erkan et al., 2008) as they provide a high antioxidant yield due to their hydrogen-bonding ability (Tena et al., 1997) which is crucial for the extraction of phenolic diterpenes responsible for antioxidant properties in many plant materials, such as rosemary leaves. This method has some drawbacks including high temperature during long processing time, low selectivity and elimination of solvent residues that are often prohibited by food regulations. Recent investigations are focused on the use of solvents accepted in the food industry, such as water at boiling temperature (Chen et al., 2007; Dorman et al., 2003) and ethanol, by leaching at low temperature (Navarrete et al., 2011; Visentín et al., 2011). However, due to the low extraction yields, the performance of the so called assisted extraction techniques has been studied: presurized liquid extraction (PLE) or accelerated solvent extraction (ASE) (Herrero et al., 2010), microwave assisted extraction (MAE) with water and its mixtures (40: 60 v/v) with organic solvents: methanol, acetone and ethyl acetate (Proestos and Komaitis, 2008), and ultrasonic assisted extraction (Tena et al., 1997; Albu et al., 2004). Supercritical carbon dioxide (SC-CO_2) has been also used as green solvent for direct extraction of polyphenols from rosemary alone (Carvalho et al., 2005; Herrero et al., 2010) or with ethanol as co-solvent (Braida et al., 2008; Herrero et al., 2010) because of the low solubility of the main antioxidants in pure supercritical CO2 (Cháfer et al., 2005; Rižnar et al., 2008). A more recent approach in order to obtain highly concentrated extracts is the fractionation of ethanolic extracts by SC-CO_2 (Visentín et al., 2011).

Water is a usual solvent in food industrial extraction plants; some of them are multipurpose plants that work with seasonal crops. These plants have versatile equipment for pretreatment, extraction and drying steps to get the final product. The extension to new applications is limited by the extraction solvent as the use of organic solvents is not possible with conventional extractors and dryers. The possibility of using their equipment for the extraction of antioxidants is an interesting alternative to increase productivity. Consequently research focused in improving the extraction with water over more conventional alcohol extraction is interesting, as well. Although the extraction efficiency could by limited by the lower solubility, the process efficiency can be increased by the use of pretreatment steps.

It should be bear in mind that extraction from natural solid material is a mass transfer process involving transport of the solvent into the matrix (inner transport), dissolution of the solutes (solubility) and release of solutes from a solid matrix to the global solvent phase (external transport). The above mentioned assisted solvent extraction techniques aim to reduce mass transfer limitation and increase the yield of extraction. As it is explained in detail below, microwaves assisted extraction reduce inner mass transfer limitations and ultrasounds assisted extraction mainly reduces external transport limitations, and also can break cell membranes reducing control of inner mass transport. In this sense, the pre-treatment of the plant material is also essential to further reduce inner mass transfer limitations, reducing particle size by milling and breaking cell membranes to facilitate the access of the solvent to the antioxidants. As an example, the use of de-oiled rosemary in conventional extraction of antioxidants with ethanol has shown to improve the extraction yield significantly (Navarrete et al., 2011).

The aim of this work is to compare the use of water and ethanol for the extraction of polar compounds from Rosmarinus officinalis leaves subjected to different pre-treatment: deoiled and milled, deoiled and fresh plant. Solvent extraction at low temperature has been compared to microwave assisted extraction (MAE) and ultrasound assisted extraction (USAE) to evaluate whether assisted extraction techniques can dispense with the pretreatment of the plant material. To the best of authors' knowledge, pure water has not been previously used in MAE and USAE from Rosmarinus officinalis leaves. The analysis of the extraction process takes into

consideration the location of antioxidants in the plant material and the increase in mass transfer for each pretreatment and extraction techniques. Finally, the extracts were compared in terms of global yield, total phenolic content, antioxidant composition and antioxidant activity.

1.1. Microwave extraction

Microwave-assisted extraction (MAE) can result in a yield increase in shorter time at the same temperature using less solvent. Owing to their electromagnetic nature, microwaves possess electric and magnetic fields which are perpendicular to each other. The electric field causes heating via two simultaneous mechanisms, namely, dipolar rotation and ionic conduction. Dipolar rotation is due to the alignment on the electric field of the molecules possessing a dipole moment (either permanent or induced by the electric field) in both the solvent and the solid sample. This oscillation produces collisions with surrounding molecules and thus the liberation of thermal energy into the medium, the resulting heating is very fast. Indeed, the larger the dielectric constant of the solvent, the higher the heating effect. Consequently, unlike classical conductive heating methods, microwaves heat the whole sample simultaneously and homogeneously. In the case of extraction, the advantage of microwave heating is the disruption of weak hydrogen bounds promoted by the dipole rotation of the molecules. A higher viscosity of the medium lowers this mechanism by affecting molecular rotation. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the yield of extraction (Kaufmann and Christen, 2002; Spigno and De Faveri, 2009). However, there exists an opposite opinion, according to which microwave-transparent solvents, i.e. low dielectric constant solvents, are better than microwave absorbing ones. Thanks to the moisture content of the sample, the heat will be distributed fast through the extraction matrix , and then it will be transferred to the solvent, which remains cold during extraction reducing the temperature in the matrix (Proestos and Komaitis, 2008; Wang and Weller, 2006).

1.2. Ultrasounds assisted extraction

The benefit of using ultrasound in plant extraction has already been applied to a number of compounds of interest in both the pharmacology and food industries (Vinatoru et al., 1999). The observed enhancement of extraction of organic compounds by ultrasound is attributed to an intensification of mass transfer due to the phenomenon of cavitation produced in the solvent by the passage of an ultrasonic wave.

During the rarefaction cycle of the sound wave cavitation bubbles are produced which fill with solvent vapour. During the compression cycle the bubbles and the gas within them are also compressed resulting in a significant increase in temperature and pressure. This finally results in the collapse of the bubble with a resultant ‘shock wave’ passing through the solvent and enhanced mixing occurring. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the plant body. This, coupled with enhanced mass transfer and significant disruption of cells, via cavitation bubble collapse, has the effect of releasing cell contents into the bulk medium (Albu et al., 2004).

Ultrasound may also produce some chemical effects due to the production of free radicals within the cavitation bubbles. Sonication of water results in the formation of highly reactive hydroxyl radicals which can combine to form hydrogen peroxide which may or may not be beneficial to the extraction process itself (Paniwnyk et al., 2001). Nevertheless, in this work sonication with water has been carried out for comparison purposes and because the most active antioxidants from rosmary herb, carnosic acid and rosmarinic acid, are degraded into products like rosmanol, galdosol and carnosol, which also exhibit antioxidant activity (Albu et al., 2004). Other solvents - as ethanol, ethyl acetate or butanone - produce fewer free radicals

than water under similar sonication conditions and it has already been observed that the extraction of carnosic acid is significantly improved by sonication (Albu et al., 2004).

2. Materials and Methods

2.1. Materials

Rosemary was collected in October 2010, in Peñafiel (Valladolid, Spain). Plants were stored at 4°C until needed for the extractions. For every experiment only the leaves were used, which were removed from the stems.

The solvent, ethanol of 96% purity, Folin-Ciocalteu reagent, gallic acid and sodium carbonate were purchased from Panreac Química (Spain). All products were used as received. Cromatographic standards, rosmarinic acid and carnosic acid, were purchased from Sigma-Aldrich. Acetonitrile, acetic acid and methanol (all HPLC gradient grade) were purchased from Panreac Quimica (Spain). Water was Milli-Q quality. These solvents were degassed and filtered through a 0.20 µm filter before their use.

2.2. Extraction procedures

2.2.1. Pretreatment: Essential oil extraction

Two different ways of pretreatment have been tested next to the fresh plant material, deoiled and deoiled + milled.

The essential oil was removed from the plant by solvent free microwave extraction (SFME) as this procedure improves the antioxidants extraction yield. The extraction was carried out as described by Navarrete et al., 2011 in a modified domestic microwave oven (Panasonic NN-GD 566 M): 100 g of fresh plant were subjected to microwave heating at 1000W for 5 min.

The milling was carried out in a two blade coffee grinder (Braun) at ambient conditions. The powder was sieved and the fraction between 0.850 – 0.212 mm was selected.

2.2.2. Conventional solvent extraction (CSE)

Extraction was performed according to Navarrete et al., 2011. Rosemary leaves, subjected to the corresponding pretreatment, were preheated in a water bath at 40 °C for 15 min. Then, preheated solvent (either water or ethanol 96%) was added (ratio 1:6 w/w) and the mixture was rotated at 50 rpm to assure the mixture. After a period of 4 hours, the extract was filtered (pore size 0.45 µm) by vacuum at 20 mbar. The liquid phase was recovered and stored at 4°C.

2.2.3. Microwave-assisted extraction (MAE)

Plant samples (25g) were mixed with the solvent in a ratio of 1:6 w/w and irradiated with microwaves (250W) in 30s ON/OFF cycles to a global time of 7 min, using the same microwave apparatus as in the pre-treament. The extract was vacuum filtered (pore size 0.45 µm) and the liquid was recovered and stored at 4°C.

The temperature increase was monitored by a fiber-optical thermo-sensor (FoTemp 4, OPTOcon GmgH, accuracy 0.1K).

2.2.4. Ultrasounds assisted extraction (USAE)

It was carried out keeping the same plant to solvent ratio (1:6 w/w) and same energy input as in the MAE process (ca. 300 J/g). A Hielscher ultrasonic processor UP400S (400 watts, 24kHz) with a horn of 22 mm in diameter was used.

Two operational procedures were tested: a discontinuous process, with 30s ON/OFF cycles to a total time of 7 min, as in the MAE process, and a continuous process at 40°C using a jacketed vessel for 7 min. As in previous experiments, extracts were filtered at vacuum with a 0.45µm membrane and afterwards, they were stored at 4ºC until they were analyzed.

As in the MAE process, temperature was measured during the process by the fiber-optical thermo-sensor (FoTemp 4, OPTOcon GmgH, accuracy 0.1K).

2.3. Analysis

2.3.1. Extraction yield

An aliquot of 1mL of each ethanolic extract was weighed and oven dried at 50 ºC during 24 hours and then new weight was registered. Aqueous extracts were dried for 48 hours. The extraction yield was expressed as grams of dried extract in 100 mL of sample. Values are presented as the mean of duplicate analyses.

2.3.2. Total phenolics content

Total phenolics were determined as gallic acid equivalents (GAE) (Singleton et al., 1999). The 20 µL of solvent extract were diluted in water (1.5 mL) to which 100 µL undiluted Folin-Ciocalteu reagent were added. After 1 min, 300 µL of a saturated solution of Na_2CO_3 were added. After 0.5 h incubation at 40ºC, the absorbance was measured at 765 nm and compared to a prepared gallic acid calibration curve in the same solvent used for the extractions, either ethanol 96% or water. Values presented are means of duplicate analyses.

2.3.3. HPLC analyses

Major components of rosemary extract, rosmarinic acid and carnosic acid, were determined by HPLC analyses, according to the method of (Wellwood and Cole, 2004) adapted from Cuvelier et al., 1996. It was performed on a reversed phase C18 Hypersil- ODS column (25 cm x 4.6 mm, 5 µm pore size; Supelco). 20 µL of liquid extract were injected. The mobile phase was programmed with a linear gradient from 90% A (840 mL of deionized water with 8.5 mL of acetic acid and 150 mL of acetonitrile), 10% B (methanol), to 100% B in 30 min, with a flow rate of 1.5 mL/min. The system was left to stabilize for 3 min between consecutive injections. The column oven temperature was 25 °C. The samples were detected by UV at 284 nm. The compounds were identified by comparison with the relative retention time of standards in both solvents and with reference to a published chromatogram (Cuvelier et al., 1996). Both standards were calibrated between 0.2 and 20 mg/mL in ethanol and 0.2 to 1.5 mg/mL in water. Before HPLC analysis, the samples were filtered through a 0.2 µm nylon membrane filter (Millex GN). The presented value is a mean of three independent analyses.

2.3.4. DPPH• scavenging assay

The ability of the extracts to scavenge DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical was assessed spectrophotometrically as described by (Almeida et al., 2010).
Briefly, the liquid ethanolic rosemary extracts were diluted in ethanol and mixed with 1 mL 0.3 mM 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) ethanol solution, to give final concentrations of 5, 10, 25, 50, 125 and 250 µg of dry extract per milliliter in a total volume of 3.5 mL. After 30 min of reaction at room temperature, the absorbance values were measured at 517 nm in spectrometry (Genesys, 10 VIS, Rochester, NY, USA) and converted into percentage of antioxidant activity (% AA) according to equation 1.

$$\%\ AA = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\} \quad (1)$$

Where Abs_{blank} is the absorbance of the solvent, $Abs_{control}$ is the absorbance of DPHH• solution diluted to 3.5 mL without extract and Abs_{sample} is the absorbance of the sample at a given concentration.
In aqueous extracts as DPHH• is insoluble in water, the extracts have been diluted in adequate water- ethanol mixtures in order to obtain a final concentration of 50% water in volume. At

higher water ratios (70–90% (v/v)) unreal low antioxidant activities are measured, since part of the DPPH• can form aggregates and it will not react with the antioxidants (Staško et al, 2007). The results are expressed as IC_{50} value that represents the extract concentration that shows 50% AA, i.e., the antioxidant potential is inversely proportional to IC_{50} value. The IC_{50} value was calculated from the linear regression of the % AA curves obtained for all extract concentrations.
The presented value is the mean of three independent analyses.

3. Results

The results of the different extraction procedures in terms of extraction yield, extract composition (total phenols, rosmarinic acid and carnosic acid) and antioxidant activity are shown in Tables 1 to 3 for the different pretreatments.

3.1. Extraction yield and composition

Without any pretreatment, ethanol is the better choice as solvent and the extraction is quite improved using any of the assisted extraction techniques, being the MAE the one that performs better taking into account all the analyzed parameters. However, when only the de-oiled pre-treatment is carried out, the extracts produced by the conventional and MAE processes are quite similar. Nevertheless, according to a kinetic study of the extraction process (Figure 1), the outcome of the assisted process can be improved increasing the energy input, either by a longer extraction time or higher power input, as the concentration of polyphenols (carnosic or rosmarinic acid) has not reached a plateau as in the conventional process. However, longer processing times with the actual MW setup are not advisable as the ethanol starts boiling after 5 minutes processing. A refrigeration column with reflux should be implemented to avoid evaporation (open systems) or overpressure (close systems). The increase in temperature when using US is slower; a temperature of 69°C is reached after 7 minutes processing. Operating temperatures using water as solvent are ca. 10°C lower to those of ethanol processing for MAE and USAE.

The global yield of extraction is not improved by the pre-treatment when using ethanol as solvent in conventional extraction (CSE), although there is a clear increase in the extraction of the target compounds, rosmarinic and carnosic acid, when the leaves are de-oiled by Solvent Free Microwave Extraction, in agreement with Navarrete et al., 2011. Also, the milling process increases the yield of these compounds, although to a lower degree.

If both pretreatments are carried out, the water extraction shows better performance than the extraction with ethanol in terms of yield and total polyphenol content. Also the content of rosmarinic acid is highly increased with respect to ethanol extractions; however, the concentration of carnosic acid is usually below the detection limit (0.0035 mg/mL). This can be explained on the basis of hydrophobicity of each compound, carnosic acid with two –OH groups and a –COOH group is much more hydrofobic than rosmarinic acid with four –OH groups and a –COOH group. Thus the solubility of carnosic acid in water is much lower than that of rosmaniric acid.

Moreover, the total amount of rosmarinic acid extracted by any of the solvents by the MAE and the USAE presented procedures (45 – 145 mg/g dried extract) is higher than obtained by other assisted techniques as presurized liquid extraction (PLE) with a maximum of 16 mg/ g dried extract (Herrero et al., 2010). On the other hand, the amount of carnosic acid extracted with ethanol is of the same order (70 -80 mg/g dried extract) of that extracted by PLE, and higher than that extracted by longer ultrasonic procedures, 14 mg/g in 15 min, using ethanol as solvent a 50ºC and a slightly higher solvent to leaves mass ratio (8:1) (Albu et al., 2004).

Proestos and Komaitis, 2008 also used MAE to extract antioxidants from rosemary and other aromatic plants, finding that water was a better solvent and its mixtures (60: 40 v/v) with organic solvents (acetone, methanol, ethyl acetate). They used dried and grinded rosemary obtaining an extract with a total phenol content of 20 mg GAE/g rosemary. This value is approximately 30 fold the value obtained in this work for fresh plant; however, the energy input is about 30 fold higher, as well. On the other hand, from extract from de-oiled and grounded material, the energy input used in this work is 2.5 fold smaller, whereas the phenolic content is around 2.5 fold higher (50 mg GAE/ g rosemary) showing a higher efficiency in the use of the energy.

Further it has to be noted that, in general, results from cyclic and continuous ultrasound processes are quite similar so results for this technique were referred globally in the previous discussion. The continuous process has the advantage of a better control of the temperature, avoiding high temperatures that may degrade the antioxidants.

These results can be explained taking into account the steps of the extraction process.
The milling process reduces inner mass transfer limitations. Total phenol content of ethanolic extracts from CSE is increased by a factor of 2, by a factor of 3 within extracts from MAE and by a factor near to 4 within the extracts from USAE process (Data from Table 1 and Table 2). The factors of MAE and USAE are higher because these techniques improve the inner and outer solvent transport, respectively. USAE further improves the inner transport by disruption of cells via cavitation, although to a lower extend.
De-oiling by SFME also improves the inner mass transfer because the membranes of the cell and chloroplasts are broken by internal superheating, which facilitates liberation of solutes from the matrix. Total phenol content of ethanolic extracts from CSE is increased by a factor of 3, by a factor of only 1.5 within extracts from MAE and by a factor of 2 within the extracts from USAE process (Data from Table 2 and Table 3). The factors of MAE and USAE are lower because these techniques already reduce solvent transport limitation, as previously mentioned.
It can be also noticed that without any pretreatment, total phenol content of ethanolic extracts from MAE is about the double of the content of CSE extract, because of the decrease in inner transport resistance. This effect is less pronounced in aqueous extracts, maybe because water-soluble phenols are readily available after milling and de-oiling process and the effect of external transport is more significant (USAE).
This shows that the controlling step of the extraction process is the inner mass transport.

3.2. Antioxidant activity

In general, the aqueous extracts show better antioxidant activity against the DPHH• radical than ethanolic extracts. It is also higher than the activity reported in previous works (Dorman et al., 2003; Chen et al., 2007) for aqueous extracts (236 ± 8 µg/ mL; 366 ± 2 µg/ mL) obtained after conventional processes at boiling temperature for long times (2h).
Regarding the effect of the pre-treatment step, the general trend is that the pretreatment increases the antioxidant activity in agreement with the higher concentration of antioxidants, although no clear relationship can be established between the total content of polyphenols and the antioxidant activity (Figure 2). This is in agreement with investigations on antioxidant activity of plant extracts from other authors (Erkan et al.,2008; Spigno and De Faveri, 2009; Herrero et al., 2010), due probably to synergistic effects between the different compounds extracted. In this sense, even extracts from non-pretreated materials with low content of carnosic and rosmarinic acid have quite good antioxidant activity values.
From Figure 2, it is also clear that the antioxidant activity is related mainly to the pretreatment carried out than to the extraction technique used.

It is also observed that aqueous extracts, with no content of carnosic acid, have the highest antioxidant activity, although carnosol and carnosic acid have been suggested to account for over 90% of the antioxidant properties of rosemary extract (Richheimer et al., 1999). This is because in aqueous systems, as in the DPPH procedure used, rosmarinic acid exhibits the highest antioxidant activity, whereas in lipid systems, extracts with higher phenolic diterpene content, i.e. carnosic acid, are more effective (Del Baño et al., 2003).

4. Conclusions

Raw material (rosemary leaves) pretreatment, de-oiling by solvent free microwave extraction (SFME; 3000 J/g) and milling, is essential to maximize the extraction efficiency using water and ethanol as solvents, because the controlling step of the extraction process is the inner mass transport. The selection of the solvent is mainly related with the future use of the extract: aqueous extracts, rich in rosmaric acid, will be effective as antioxidant in hydrophilic systems, while, in lipophilic systems, ethanolic extracts will be favorable due to its higher content in carnosic acid.

Ethanol extraction can be further improved by the use of low energy input (300 J/g) and short time (7 min) assisted process like microwave assisted (MAE) and ultrasound assisted extraction (USAE). Internal mass transport is additionally increased by MAE whereas USAE enhances external mass transport, which is more significant in aqueous extracts.

The proposed extraction procedure, solvent free oil extraction and grinding followed by an assisted solvent extraction with a benign solvent (water or ethanol), provides an extract of rosemary with equal or higher antioxidant content as those produced by other assisted extraction techniques or different procedures of the same processes (MAE and USAE) with an amount of rosmarinic acid between 50 – 140 mg/g dried extract, a carnosic acid content in ethanolic extracts about 80 mg/g dried extract and a total phenolic content between 110 – 180 mg GAE/ g dried extract. Moreover, the proposed process takes short times, below 15 minutes, and shows a higher efficiency in the use of the energy in comparison with similar processes. Additionally, the duration of the process can be optimized to maximize the amount of antioxidants extracted.

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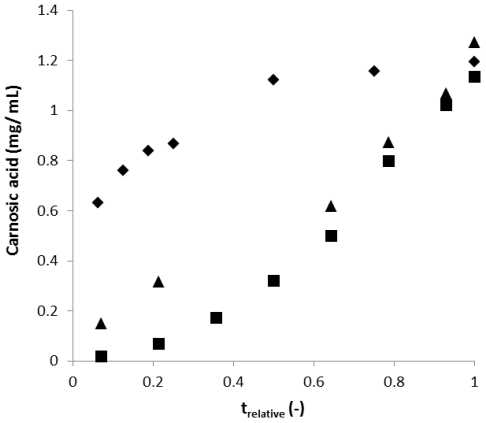
Figure captions list

Figure 1. Evolution of carnosic acid concentration with dimensionless extraction time (extraction time to total extraction time) for deoiled rosemary leaves for the different process: CSE (◆), MAE (■) and USAE- cycles (▲), using ethanol 96 %wt. as solvent.

Figure 2. Antioxidant activity plotted versus total polyphenol content organized according to: a) Procedure: CSE (◆), MAE (■) and USAE- cycles (▲). b) Pretreatment: De-oiled and milled (◆), De-oiled (■) and fresh rosemary (▲). Full symbols represent ethanolic extracts and empty symbols denote water extracts. Note: extreme values are not presented.

Figures



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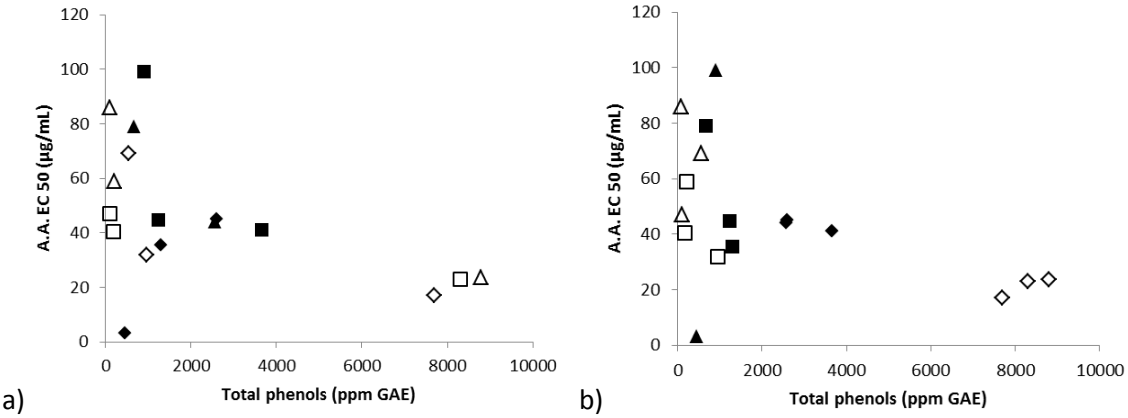

Figure 2. Antioxidant activity plotted versus total polyphenol content organized according to: a) Procedure: CSE (◆), MAE (■) and USAE- cycles (▲). b) Pretreatment: De-oiled and milled (◆), De-oiled (■) and fresh rosemary (▲). Full symbols represent ethanolic extracts and empty symbols denote water extracts. Note: extreme values are not presented.

Tables

Table 1. Results of extraction from de-oiled and milled rosemary leaves.

Extraction Technique	Solvent	Extraction yield (% w/v)	Total phenols (ppm GAE)	Rosmarinic acid (mg/mL)	Carnosic acid (mg/mL)	A.A. EC50 (µg/mL)
Solvent extraction	Etanol	2.4 ± 0.2	2600 ± 700	0.70 ± 0.03	2.11 ± 0.06	45 ± 2
	Water	3.89 ± 0.07	7700 ± 900	6.50 ± 1.3	N.D.	17 ± 9
Microwave	Etanol	3.3 ± 0.2	3662 ± 8	1.55 ± 0.08	2.46 ± 0.06	41 ± 4
(ON/OFF cycles)	Water	4.6 ± 0.8	8300 ± 800	6.20 ± 1.3	N.D.	22.8 ± 0.5
Ultrasounds	Etanol	2.70 ± 0.02	2570 ± 80	1.77 ± 0.10	2.21 ± 0.06	44 ± 2
(ON/OFF cycles)	Water	6.61 ± 0.09	8790 ± 300	6.36 ± 1.3	0.09 ± 0.02	23.6 ± 0.9
Ultrasounds	Etanol	2.35 ± 0.02	2040 ± 40	1.10 ± 0.06	2.21 ± 0.06	49 ± 2
(continuous)	Water	3.500 ± 0.007	8440 ± 70	5.10 ± 1.4	N.D.	24.3 ± 0.5

Table 2. Results of extraction from de-oiled rosemary leaves.

Extraction Technique	Solvent	Extraction yield (% w/v)	Total phenols (ppm GAE)	Rosmarinic acid (mg/mL)	Carnosic acid (mg/mL)	A.A. EC50 (µg/mL)
Solvent extraction	Etanol	2.135 ± 0.007	1290 ± 80	1.07 ± 0.05	1.2 ± 0.03	35.4 ± 1.9
	Water	0.600 ± 0.014	960 ± 90	0.031 ± 0.018	0.0035 ± 0.0003	32.0 ± 1.1
Microwave (ON/OFF cycles)	Etanol	2.050 ± 0.016	1240 ± 170	0.87 ± 0.05	1.13 ± 0.03	44.6 ± 1.8
	Water	0.13 ± 0.03	179 ± 3	0.0120 ± 0.0009	0.0035 ± 0.0003	40.6 ± 0.7
Ultrasounds (ON/OFF cycles)	Etanol	1.70 ± 0.08	670 ± 17	0.079 ± 0.004	1.27 ± 0.03	79 ± 1.8
	Water	0.14 ± 0.09	211.0 ± 1.3	0.28 ± 0.01	N.D.	59 ± 2
Ultrasounds (continuous)	Etanol	1.54 ± 0.03	664 ± 11	0.084 ± 0.004	1.48 ± 0.04	69 ± 2
	Water	0.31 ± 0.03	218 ± 2	0.11 ± 0.01	N.D.	108 ± 2

Table 3. Results of extraction from fresh rosemary leaves.

Extraction Technique	Solvent	Extraction yield (% w/v)	Total phenols (ppm GAE)	Rosmarinic acid (mg/mL)	Carnosic acid (mg/mL)	A.A. EC50 (µg/mL)
Solvent extraction	Etanol	2.5 ± 0.9	450 ± 60	0.050 ± 0.003	0.36 ± 0.02	3.2 ± 0.2
	Water	0.605 ± 0.007	550 ± 110	0.014 ± 0.002	0.0035 ± 0.0003	69 ± 5
Microwave	Etanol	3.1 ± 1.2	902 ± 32	0.62 ± 0.03	0.32 ± 0.07	99 ± 2
(ON/OFF cycles)	Water	0.095 ± 0.007	110 ± 6	0.004 ± 0.0002	0.0035 ± 0.0003	47 ± 3
Ultrasounds	Etanol	2.70 ± 1.5	330 ± 70	0.101 ± 0.004	0.105 ± 0.002	500 ± 10
(ON/OFF cycles)	Water	0.077 ± 0.004	92 ± 36	0.0040 ± 0.0002	0.0035 ± 0.0003	86 ± 5
Ultrasounds	Etanol	1.10 ± 0.02	195 ± 9	0.015 ± 0.019	0.220 ± 0.006	350 ± 40
(continuous)	Water	0.075 ± 0.007	92 ± 48	0.004 ± 0.0004	N.D	75.3 ± 0.7