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Green processes for the extraction of bioactives from Rosemary: Chemical and functional characterization via ultra-performance liquid chromatography-tandem mass spectrometry and in-vitro assays

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ABSTRACT

In this contribution, the performance of three different extraction procedures towards the extraction of antioxidants from rosemary (*Rosmarinus officinalis*) is presented. Namely, pressurized liquid extraction (PLE), using water and ethanol as solvents, supercritical fluid extraction (SFE), using neat CO_2 and supercritical CO_2 modified with ethanol, as well as a novel extraction process called Water Extraction and Particle formation On-line (WEPO) are directly compared. Different extraction conditions including temperatures, times and pressures have been studied. The produced extracts have been characterized in terms of extraction yield, antioxidant activity (using the DPPH radical scavenging method) and total phenols (using the Folin method). Besides, all the extracts have been chemically characterized using a new quantitative UPLC-MS/MS method. This method allowed the determination of the main antioxidants present in rosemary, including, among others, rosmarinic acid, carnosic acid and carnosol, attaining detection limits as low as 2 ng/mL. The results obtained in this study show that PLE using ethanol at high temperatures (200 °C) was able to produce extracts with high antioxidant activity (EC_{50} 8.8 μg/mL) and high yield (ca. 40%) while efficiently extracting antioxidants of diverse polarity, among them, carnosic and rosmarinic acids, regarded as the most important antioxidants present in rosemary. Nevertheless, in this work, the ability of the three studied environmentally friendly extraction techniques to obtain bioactives from natural sources is demonstrated.

1. Introduction

Nowadays, the demand for natural bioactive compounds is increasing due to their use in the functional food industry. Natural components from plants and other organisms, such as algae and microalgae, are employed, including different functional activities, for instance, antioxidant activity [1], antimicrobial activity [2], antihypertensive [3], anti-cancer [4], or neurodegenerative diseases prevention [5], among others [6–8]. Rosemary (*Rosmarinus officinalis*) is one of the most appreciated natural sources for this kind of compounds. This plant has been widely studied due to the potent antioxidant activities associated to some of its components; among them, phenolic diterpenes have attracted more attention [9–11]. Both carnosic and rosmarinic acids have demonstrated to possess potent antioxidant activities although other compounds also present in rosemary (i.e., carnosol, rosmanol, epi-rosmanol, among others) positively influence the total antioxidant activity. Due to these activities, some rosemary extracts have been recently commercialized, and the influence of these extracts on different foods has been already tested [12,13]. In this sense, rosemary extracts have been proposed to enhance the oxidative stability of sunflower oils in order to eliminate the use of synthetic antioxidants [12]; they have been also used to maintain a low lipid oxidation level in irradiated beef burgers [13]. Nevertheless, besides its antioxidant activity, other functional activities have been observed in different rosemary extracts, mainly anti-inflammatory and anti-tumor [14] and antimicrobial activities [15,16]. For all these reasons, it is easy to understand why the interest on attaining bioactive extracts from rosemary is increasing.

Different extraction techniques have been applied to obtain antioxidant extracts from rosemary. Conventional solvent extraction [17,18] and ultrasounds assisted extraction have been employed [19,20]; this technique was also compared with steam distillation [21] and Soxhlet extraction [22]. Other extraction methods such as hydrodistillation or microwave hydrodiffusion have been also applied to obtain the rosemary essential oil [23–25] although, generally, the aim of these works was not the extraction of phenolic antioxidants.

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Even if these techniques are able to provide with bioactive extracts, more environmentally friendly and selective extraction techniques are nowadays preferred. Among them, supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been widely applied to natural bioactives extraction [7]. SFE has important advantages over traditional extraction techniques, mainly considering that low volumes of organic solvents, if any, are employed and the fact that a solvent free extract can be obtained. On the other hand, the use of PLE employing water as solvent allows the attainment of generally higher extraction yields also limiting the use of toxic organic solvents. Both techniques have been applied to antioxidants extraction from rosemary [26,27]. The effect of different extraction parameters on the supercritical fluid extraction (SFE) of rosemary was assessed [28,29], so it was its economical viability from a commercial point of view [30]. PLE using water at different temperatures was tested in order to study the selectivity that could be attained to extract the carnosic acid present in rosemary [31,32].

In the present work, the extraction performances of PLE and SFE as well as a new procedure developed at our lab, called Water Extraction and Particle formation On-line (WEPO) to obtain antioxidant extracts from rosemary are directly compared for the first time. The shortcomings and advantages of each of these environmentally friendly extraction processes are critically commented in terms of extraction time, solvent consumption, extraction yield, antioxidant activity and total polyphenols of the obtained extracts. Moreover, the phenolic antioxidants present on the extracts are quantified using a new UPLC-MS/MS method.

2. Experimental

2.1. Samples and chemicals

The rosemary samples (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves obtained from Herboristeria Murciana (Murcia, Spain). Rosemary leaves were dried using a traditional method, as follows: once collected, the plants are ventilated to remove humidity, covered with a blanket to avoid sunlight, and allowed to dry in a ventilated place for 20–30 days, depending on the season. Cryogenic grinding of the sample was performed under liquid nitrogen and particle size was determined by sieving the ground plant material to the appropriate size (between 999 and 500 μm). The whole sample was stored at −20 °C until use.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma–Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Alemania) whereas antioxidant standards, i.e., gallic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, rosmarinic acid, carnosol and carnosic acid were supplied by Sigma–Aldrich (Madrid, Spain). CO_2 (N-48) was provided by Praxair (Madrid, Spain). The water used was Milli-Q Water (Millipore, Billerica, MA, USA). For the UPLC-MS/MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed.

2.2. Supercritical fluid extraction (SFE)

Extractions were carried out in a pilot scale supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 2 L cylinder extraction cell and two different separators (each of 0.5 L capacity) with independent control of temperature and pressure. The extraction device also included a CO_2 recirculation system, where CO_2 is condensed and pumped up to the desired extraction pressure. For each experiment, the extraction cell was filled with 0.5 kg of rosemary.

Three different experiments were carried out. For all experimental assays, temperature was set to 40 °C in the extraction vessel as well as in both separators, CO_2 flow rate was set to 60 g/min. According to previous kinetic studies (data not shown) extraction time was set to 5 h to ensure high extraction yield. In the first experiment, extraction pressure was 400 bar; pressure in the first separator was fixed at 100 bar, while in second separator decompression up to recirculation pressure was accomplished. In the second experiment, extraction pressure was 300 bar, decreasing the pressure in the first separator to 100 bar, while in second separator decompression up to recirculation pressure was again accomplished. In the last experiment, extraction pressure was 150 bar, but in this case 7% ethanol was used as modifier. Only a separator was employed in this case, decompressing down to recirculation pressure. When the decompression was carried out in two steps, two different extracts were collected and analyzed separately. For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using ethanol as modifier. All extracts were diluted with ethanol and kept under N_2, at −20 °C and protected from light until analysis.

2.3. Pressurized liquid extraction (PLE)

Extractions of rosemary were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Two different solvents (i.e., ethanol and water) were used to obtain extracts with different compositions. In order to avoid any possible oxidation effect and to remove the dissolved oxygen, solvents were sonicated for 15 min prior use. Extractions were performed at four different extraction temperatures (50, 100, 150, and 200 °C) whereas the static extraction time was maintained for 20 min. An extraction cell heat-up step was carried out for a given time prior to any extraction. The warming-up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 50 and 100 °C, 7 min if the extraction temperature was 150 °C, and 9 min if the extraction temperature was 200 °C). All extractions were done using 11 mL extraction cells, containing 1 g of sample. When water was used for the extraction, the extraction cell was filled with sand mixture on the top of the sample (2.0 g of sand) to prevent the clogging of the system.

Extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with solvent up to a pressure of 1500 psi (1 psi = 6894.76 Pa), (iii) heat-up time was applied, (iv) static extraction takes place (i.e., at 20 min) in which all system valves are closed, (v) cell is rinsed (with 60% cell volume using extraction solvent), (vi) solvent is purged from cell with N_2 gas and (vii) depressurization took place. Between extractions, a rinse of the complete system was made in order to overcome any carry-over. The extracts obtained were protected from light and stored under refrigeration until dried.

For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Labconco Corporation, Missouri, USA) was employed.

2.4. WEPO extraction

The Water Extraction and Particle formation On-line process (WEPO) [33] combines two different processes: firstly a dynamic water extraction and secondly the drying of the extract produced

under hot N_2 (70 °C) using a supercritical CO_2 assisted nebulization. The extraction cell (1 g of sample mixed with 2 g of sand) was placed inside an oven operated at 200 °C. Subcritical water was passed through at 0.2 mL/min of flow rate using a modified Suprex Modifier Pump (Suprex, Pittsburg, PA). Supercritical CO_2 at 80 bar was delivered using a modified PrepMaster (Suprex) extractor. The total extraction time was 20 min. A more detailed description of the process and the devices can be found elsewhere [33].

2.5. UPLC-MS/MS analysis of the extracts

The UPLC-MS/MS analyses were carried out using an Accela (Thermo Scientific, San Jose, CA) liquid chromatograph equipped with a DAD and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer via an electrospray interface. The analytical conditions employed consisted of the use of a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 μm) (Thermo Scientific) using as mobile phases ACN (0.1 formic acid, A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 40% B; 6.2 min, 5% B; 6.5 min; 5% B; 7 min, 95% B; 9 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 5 μL. The diode array detector recorded the spectra from 200 to 450 nm.

To quantify the antioxidants, the mass spectrometer was operated in the negative ESI multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM using scan width 0.010 Da and scan time of 0.040 s. The values corresponding to the tube lens voltage and collision energy were optimized for each quantified compound as indicated below.

2.6. Total phenols and antioxidant activity determination

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g extract [34]. The total volume of reaction mixture was miniaturized to 2 mL. 1.2 mL water and 20 μL of sample were mixed, to which 100 μL undiluted Folin-Ciocalteau reagent was subsequently added. After 1 min, 0.3 mL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 2.0 mL with water. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve elaborated in the same manner. The data were presented as the average of triplicate analyses.

For the antioxidant activity determination of all the extracts produced, the DPPH radical scavenging method was employed that consists in the neutralization of free radicals of DPPH by the antioxidant extracts. The particular method was based on a procedure described by Brand-Williams et al. [35] and modified by Herrero et al. [36]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This solution was further diluted 1:10 with methanol; different concentrations of rosemary 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 Beckman DU-70 spectrophotometer (Beckman, Fullerton, CA). Methanol was used to adjust zero and DPPH–methanol solution as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. 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}. Therefore, the lower the EC_{50}, the higher the antioxidant activity. Measurements were done, at least, in duplicate.

3. Results and discussion

As mentioned, three different environmentally friendly extraction techniques have been studied in the present contribution to obtain phenolic antioxidants from rosemary plants. Although SFE and PLE using water as solvent had been used before, this is the first comparison directly established concerning these two advanced extraction procedures together with a new process called WEPO. This fact is of importance, considering that the same raw material has been used during the same narrow period of time. Therefore, the performance of each technique in terms of antioxidants extraction and yield produced can be compared minimizing the possible strong effect of the sample origin. It has been already shown that the antioxidant activity produced as well as the particular chemical composition of the extracts obtained from rosemary can vary in great extent depending on the origin and year of production of the sample [37]. This fact makes difficult to effectively compare the results presented in different works that can be already found in the literature. Here, the extraction yield produced, the extracts antioxidant activity, their total phenol content as well as the main polyphenolic antioxidant compounds, quantified using a new UPLC-MS/MS method, are calculated for each extract obtained using the different extraction protocols.

3.1. Rosemary extraction and functional activity

The conditions employed to carry out the rosemary extraction as well as the results obtained in terms of extraction yield produced, antioxidant activity and total phenol content for all the extracts obtained are shown in Table 1. Preliminary experiments showed that the extraction yields produced by SFE using an analytical instrument were extremely low, above all compared to those obtained by PLE. For this reason, the SFE extractions were finally carried out using a pilot scale instrument, in order to get enough amount of the final extract to perform both the chemical and the functional characterizations. A wide range of conditions were tested when using PLE and SFE in order to have a more precise idea of the influence of the diverse extraction conditions on the outcome of the extraction. First of all, in SFE three different extraction procedures were carried out; two of them with neat CO_2 at 300 and 400 bar, respectively, whereas the depressurization was carried out in two separators, being the pressure in the first one 100 bar. This means that the extract collected in the first separator corresponded to the fraction extracted at the final pressure, and the one obtained in the second separator corresponded to the components still soluble in the neat CO_2 below 100 bar. The last extraction procedure was carried out using 7% ethanol as cosolvent at 150 bar, so that the influence of a modifier could be appreciated. As it can be observed in Table 1, the addition of ethanol as modifier significantly increased the extraction yield produced, being this value by far higher than the rest obtained with neat CO_2. Besides, the antioxidant activity of this extract was the highest of those attained by SFE. It is worth to mention the extremely low extraction yield produced at high pressures using neat CO_2. In fact, as it has been already commented, most compounds extracted from rosemary by SFE at high pressures were still soluble in CO_2 below 100 bar, and therefore, were not collected in the first separator. The functional characterization of these extracts showed that at 400 bar higher antioxidant activity was obtained when compared to the rest of the supercritical CO_2 extractions. However, the total phenol values did not correlate well with the antioxidant activity obtained. The highest phenol amount was achieved below 100 bar. Therefore, it can be guessed that some compounds other than phenolic antioxidants, included in the extracts obtained at 400 bar, had a positive influence on the total antioxidant activity of the extract obtained.

Table 1
Values of extraction yield (% dry weight), EC_{50} (μg/ml) and total phenols (as mg gallic acid/mg extract) obtained for the different extractions performed at the indicated conditions.

Extraction technique	Solvent	Temp. (°C)	Time (min)	Pressure (bar)	Extraction yield (%)	Antioxidant activity, EC_{50} (μg/mL)	Total phenols (mg gallic acid/mg extract)
PLE	Ethanol	50	20	100	17.8	11.4 ± 0.3	0.078 ± 0.009
		100	20	100	22.9	10.3 ± 0.5	0.094 ± 0.009
		150	20	100	29.1	9.6 ± 0.6	0.120 ± 0.006
		200	20	100	38.6	8.8 ± 0.9	0.111 ± 0.011
	Water	50	20	100	21.1	6.4 ± 0.4	0.157 ± 0.014
		100	20	100	24.0	5.3 ± 0.2	0.168 ± 0.006
		150	20	100	37.3	5.9 ± 0.3	0.174 ± 0.020
		200	20	100	37.9	7.0 ± 0.6	0.183 ± 0.002
SFE	CO_2	40	300	<100	3.1	12.1 ± 0.0	0.123 ± 0.003
		40	300	300	0.5	18.2 ± 0.1	0.070 ± 0.006
		40	300	400	0.9	10.5 ± 0.2	0.078 ± 0.004
	CO_2 + 7% ethanol	40	300	150	6.5	8.1 ± 0.1	0.121 ± 0.004
WEPO	Water	200	20	0.2[a]	4.0	7.4 ± 0.6	0.153 ± 0.004

[a] Water flow rate (ml/min).

Nevertheless, the extremely low yields produced (0.9%) limited in great extent the use of these conditions in order to get a significant amount of bioactives.

On the other hand, two different solvents, i.e., ethanol and water, were tested for the PLE extractions. Although water was previously employed to extract antioxidants from rosemary by PLE [31], to our knowledge, this is the first time that other food-grade solvent, ethanol, is studied. Four temperatures (namely, 50, 100, 150 and 200 °C) were investigated at a constant extraction time (20 min). The extraction time was not considered a variable in these experiments given the results published in the literature where it is possible to see how the extraction time had little or no influence on the outcome of the PLE extractions of similar materials [38]. As it can be appreciated in Table 1, the highest the extraction temperature, the highest the extraction yield produced independently of the solvent employed. It was observed that higher yield was produced when using water as solvent, although the maximum yields obtained for both of them were very similar. Concerning the functional characterization of the PLE extracts, some interesting observations can be made. When using ethanol as solvent, the antioxidant activity obtained using the DPPH radical scavenging method increased with the extraction temperature, as it did the amounts of total phenols. However, no difference was observed between the total phenols extracted at 150 and 200 °C. It looks like the maximum amount of phenols that can be extracted with ethanol is reached already at 150 °C, although increasing the temperature up to 200 °C resulted in the extraction of some components, which enhanced slightly the antioxidant activity produced. On the other hand, when water was selected as extraction solvent, the amount of total phenols kept increasing with the extraction temperature. Besides, the amount of phenols that could be obtained with water was significantly greater than with ethanol even at the lowest temperatures tested. The antioxidant activity of the water extract was also higher than that of the ethanol extracts, although the highest values were obtained at mild temperatures. This effect has been previously observed [39]. Probably, some kind of degradations related to the temperature employed could decrease somewhat the total antioxidant activity. Nevertheless, the obtained values were still better that those provided by ethanol extracts.

Concerning the WEPO process developed at our lab [33], although its development is still ongoing, in this work a novel application is shown. Briefly, the process is based on the dynamic subcritical water extraction (SWE) of the natural material and its continuous drying. At the exit of the extraction chamber the flow coming from the extractor is mixed with supercritical CO_2 in an expansion vessel in which a flow of hot N_2 is also introduced. These conditions allow the on-line precipitation of the extracts forming particles whose diameter could be tuned by varying the drying conditions. The basic advantage of this procedure is that it is a single-step process, which eliminates the need of any lyophilization procedure typical of SWE processes (PLE processes using water as solvent) saving a lot of time. The experiments were carried out at 0.2 mL/min water flow rate, setting the oven temperature at 200 °C. The extraction yield produced was lower than that of the static SWE procedure (see Table 1) although both, the amount of total phenols extracted and the antioxidant activity produced were comparable. These results could be explained given the different procedure employed in each case. While in PLE an equilibration step was followed by the static extraction step, in the WEPO process the raw material was not pre-equilibrated. This observation may suggest that, probably, the combination of a relatively short static extraction step followed by a dynamic extraction phase would provide with better results. The optimization of the lab-made WEPO instrument is still under development.

Comparing the three techniques, PLE using water seemed to be the most appropriate technique in order to maximize both the extraction yield obtained and the antioxidant activity produced. Nevertheless, the use of pressurized ethanol at high temperatures provided also with good results. Regarding the selectivity of each technique, the influence of the solvent and temperature employed (PLE) or the pressure (SFE) could be better appreciated from the results of the chemical characterization of the extracts by UPLC-MS that will be commented later. The WEPO process provided better results than SFE but significantly lower yields than PLE. However, the results are promising, above all, considering the time-saving produced due to the absence of a later drying process. If the ongoing development of the instrument can provide with higher extraction yields, this technique will represent a useful and effective alternative to the commercial PLE instruments. Moreover, it could be concluded that the addition of a relatively small proportion of modifier (7%) to the supercritical CO_2 had a significant influence both on the extraction yield and on the antioxidant activity produced.

3.2. UPLC-MS/MS method development and optimization

First of all, a conventional LC-MS method was set up, with the aim to separate and identify the possible antioxidants present on the extracts. Two PLE extracts were chosen as representative obtained with ethanol and water at 150 °C for being one of the most active and with a higher content on total phenols (see Table 1). The method was based on the use of ACN (with 0.1% formic acid) and water (with 0.1% formic acid) as mobile phases in

Table 2
Identification of antioxidant compounds in rosemary PLE extracts. UV–vis and MS characteristics.

ID	Retention time (min)	Identification	UV–Vis maxima (nm)	$[M-H]^-$	Main fragments detected
1	18.8	Caffeic acid[a]	323, 295	179.31	135.3
2	24.0	Homoplantaginin	333, 270	461.47	359.4
3	24.4	Rosmarinic acid[a]	328, 290, 225	359.24	
4	27.9	Scutellarein	344, 267	285.21	
5	30.0	Tryhydroxycinnamic acid derivative	325, 299	207.21	
6	34.5	Cirsimaritin	334, 274	313.19	
7	34.9	Rosmanol	282	345.27	300.9, 283.1
8	36.0	Epi isorosmanol	271	345.3	
9	37.7	Genkwanin	335, 268	283.55	
10	44.4	Carnosol[a]	284	329.88	
11	46.7	Rosmarinic acid methyl ester	329, 268	373.46	329.5, 293.3
12	49.2	Carnosic acid[a]	285	331.48	287.4
13	41.9	Methyl carnosate	282	345.45	301.4
14	18.0	Gallocatechin	286, 313	305.00	

[a] Identification confirmed using commercial standards.

a step-wise gradient as indicated in Section 2. A conventional C_{18} column (150 mm × 4.6 mm, 3 μm d.p.) was employed. The chromatograms obtained are shown in Fig. 1. As it can be seen in this figure, several phenolic compounds could be separated in analysis times of ca. 60 min. The careful analysis of the separated compounds, using the information provided by the DAD detector as well as the MS detector installed in series, together with the information that could be found in the literature and from commercial standards (when available) allowed the identification of 14 antioxidant compounds in both extracts. The identified compounds as well as their chemical characteristics that allowed their correct identification are shown in Table 2. All these compounds were detected using an electrospray interface operated in negative ionization mode. Other compounds could be detected but no conclusive identification was reached. Besides these MS experiments, a $m/z = 162$ neutral loss scan experiment was performed in order to reveal possible glycosilated phenolics. The results of this experiment showed that there were not any of these compounds present on the extracts.

From the identified compounds, it is interesting to note that rosmarinic acid and carnosic acids were amongst the main peaks in the ethanol extract. Interestingly, some compounds could be only

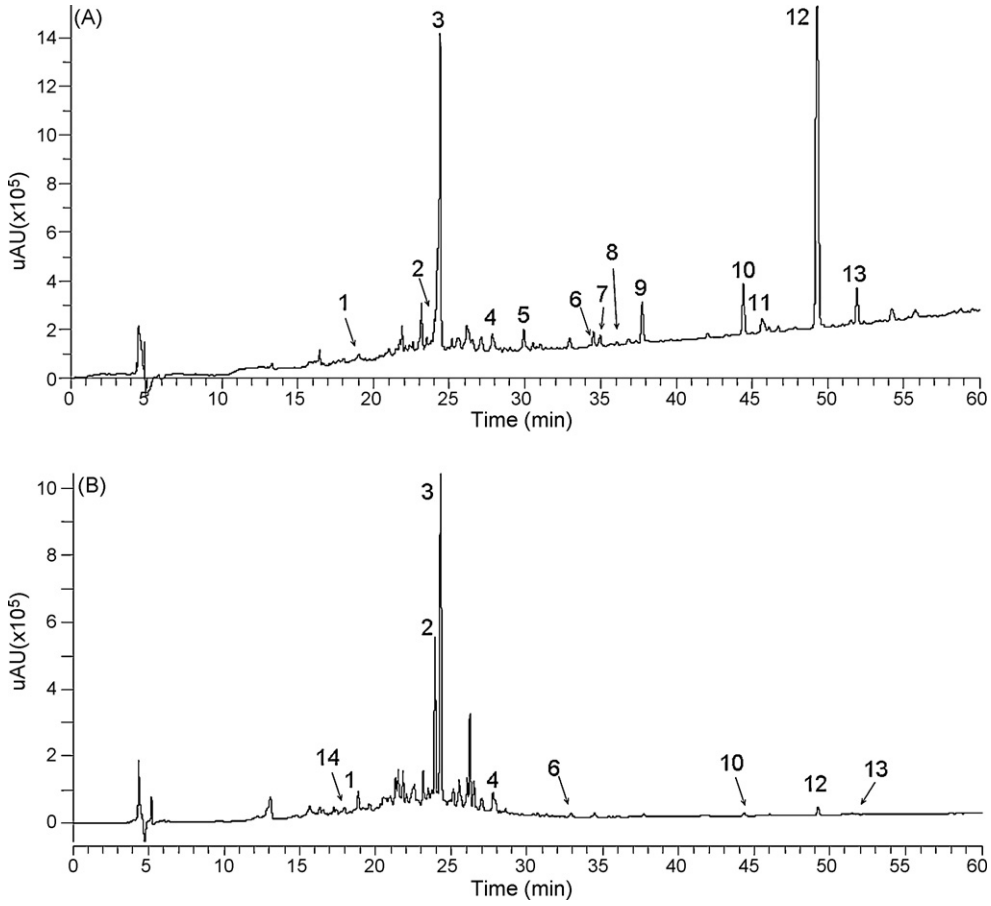

Fig. 1. Chromatograms (230 nm) corresponding to the LC-DAD-MS analysis of the PLE extracts obtained at 150 °C using ethanol (A) and water (B) as extracting solvents. Mobile phases: ACN (0.1% formic acid) and water (0.1% formic acid); column: C_{18} (150 mm × 4.6 mm, 3 μm d.p.). Rest of the analytical conditions in Section 2. For peaks identification see Table 2.

Table 3
Main optimized parameters for the MS detection of the antioxidants quantified and the optimum values employed for each one.

Compound	Parent ion [M−H]⁻	Product ion	Collision energy (V)	Tube lens offset (V)
p-Coumaric acid	163.1	119.341	11	64
Gallic acid	168.9	125.084	23	23
Caffeic acid	179.1	135.102	22	66
Carnosol	329.4	285.497	19	44
Carnosic acid	331.4	286.847	21	44
Chlorogenic acid	353.0	191.087	15	66
Rosmarinic acid	359.3	160.972	18	82

detected in one of the extracts (for instance, see caffeic acid or rosmanol), suggesting a difference on the selectivity of the technique when using different solvents. This particular point will be further discussed below.

Once the identification of the main peaks was concluded, the re-optimization of the analytical method was carried out with the aim to transfer the conventional HPLC method into UPLC. By doing this, a significant time and solvents saving was expected. A C_{18} short column (50 mm × 2.1 mm, 1.9 μm d.p.) was employed. The gradient was adapted and subsequently modified in order to not to lose resolution and speeding up the separation by using a 400 μL/min flow rate. Typical chromatograms of the different samples analyzed can be observed in Fig. 2. As it can be appreciated, the analysis time could be reduced nearly 8 times, whereas the use of the MS detector avoided the loss of information due to possible co-elutions. The optimized method was then applied to the quantification of the main antioxidants in all the extracts produced using the three environmentally friendly extraction techniques.

3.3. Antioxidants quantification by UPLC-MS/MS

The use of a triple quadrupole to quantify the main antioxidants present on the extracts allowed attaining low limits of detection thanks to its extremely high selectivity. Unfortunately, commercial standards for all the identified compounds were not available. Thus, the quantification of the main rosemary antioxidants, rosmarinic and carnosic acids together with carnosol and caffeic acid also identified in the extracts, was carried out in order to compare the performance of the extraction techniques studied in terms of antioxidant compounds extracted. Besides, with the aim to demonstrate the full potential of the powerful analytical tool employed in terms of sensitivity, UPLC-MS/MS using a triple quadrupole analyzer, three additional phenolic antioxidants whose presence could not be formerly assessed were included. These compounds were chlorogenic acid, *p*-coumaric acid and gallic acid. These phenolic antioxidants were selected because they were readily available and because they were previously identified in Lamiaceae herbs, like rosemary [39]. The seven standard compounds were analyzed to optimize their MS detection conditions. This step would allow the decrease of the limits of detection and quantification by improving both the electrospray conditions as well as the detection conditions for each compound by selected reaction monitoring (SRM). The parameters optimized included parent ion and product ion selection, collision energy and tube lens voltage. The optimized values for the quantified compounds can be observed in Table 3. Once this optimization step was finished, calibration curves for the studied compounds were constructed at different concentration ranges. At least 5 points were considered, each point triplicated. The calibration curve equations, concentration ranges for each compound as well as the data regarding the performance of the quantification method (LODs and LOQs) are presented in Table 4. The concentration ranges used were selected according to the relative amounts of each compound found in the extracts. The linearity of these curves was always good, with R^2 values higher than 0.99 for all the studied compounds. LOQs as low as 6.2 ng/mL were reached. The reproducibility of the UPLC method was also good with RSD for the retention times lower than 2.5%.

Next, all the extracts produced using the three extraction techniques were analyzed and the amounts of antioxidants present on them determined. The summary of the obtained results from the UPLC-MS/MS experiment can be observed in Table 5. The first conclusion that can be drawn from these results is that in all cases rosmarinic and carnosic acids as well as carnosol are, by far, the main compounds present in the extracts. In fact, the rest of the compounds are present in so small amount in some extracts that could not be quantified using other more conventional detectors, such a photometric detector. However, besides the particular accurate amounts of each studied compound, the results in Table 5 also help to understand the extraction mechanism in the different extraction techniques applied. SFE was the more appropriate technique in order to obtain both carnosic acid and carnosol. The amount of these compounds were higher according to an increase in the total extraction pressure. In fact, the solubility of these two compounds in supercritical neat CO_2 was quite high. Using ethanol as entrainer in the SFE procedure allowed the attainment of good amounts of these terpenes, although the total amounts on the extractions at high pressures and neat CO_2 were higher. At this point it should be considered that the result of a SFE extraction was the sum of the results from the separator 1 (<100 bar) and separator 2 (either 300 bar or 400 bar). However, the more polar compounds were not effectively extracted using SFE. This is clearly due to the low polarity of supercritical carbon dioxide as a solvent. By using PLE, relatively high amounts of rosmarinic acid, carnosol and carnosic acid could be obtained. It is interesting to note that when using water, the less polar compounds (carnosol and carnosic) were less extracted than

Table 4
Calibration curves and concentration ranges employed for the quantification of the antioxidants, and limits of detection (LOD) and limits of quantification (LOQ) reached using the optimized UPLC-MS/MS method.

Compound	Tr (min) ± RSD (%)	Concentration range (μg/mL)	Calibration curve	R^2	LOD (ng/mL)	LOQ (ng/mL)
Gallic acid	0.5 ± 2.5	0.050–3.125	$y = 479219x + 3094$	0.9984	34.66	115.53
Chlorogenic acid	1.7 ± 1.8	0.050–6.250	$y = 6152769x - 491127$	0.9973	10.27	34.24
Caffeic acid	1.9 ± 1.7	0.05–6.250	$y = 6750308x - 55097$	0.9993	13.52	45.07
p-Coumaric acid	2.2 ± 1.0	0.050–6.250	$y = 1146328x + 166472$	0.9947	73.94	246.46
Rosmarinic acid	2.6 ± 0.6	0.098–25	$y = 2167103x + 1932441$	0.9900	4.25	14.16
Carnosol	4.6 ± 0.3	0.098–250	$y = 839822x + 3114736$	0.9981	12.81	42.71
Carnosic acid	5.1 ± 0.2	0.050–100	$y = 14038286x + 36138642$	0.9947	1.86	6.19

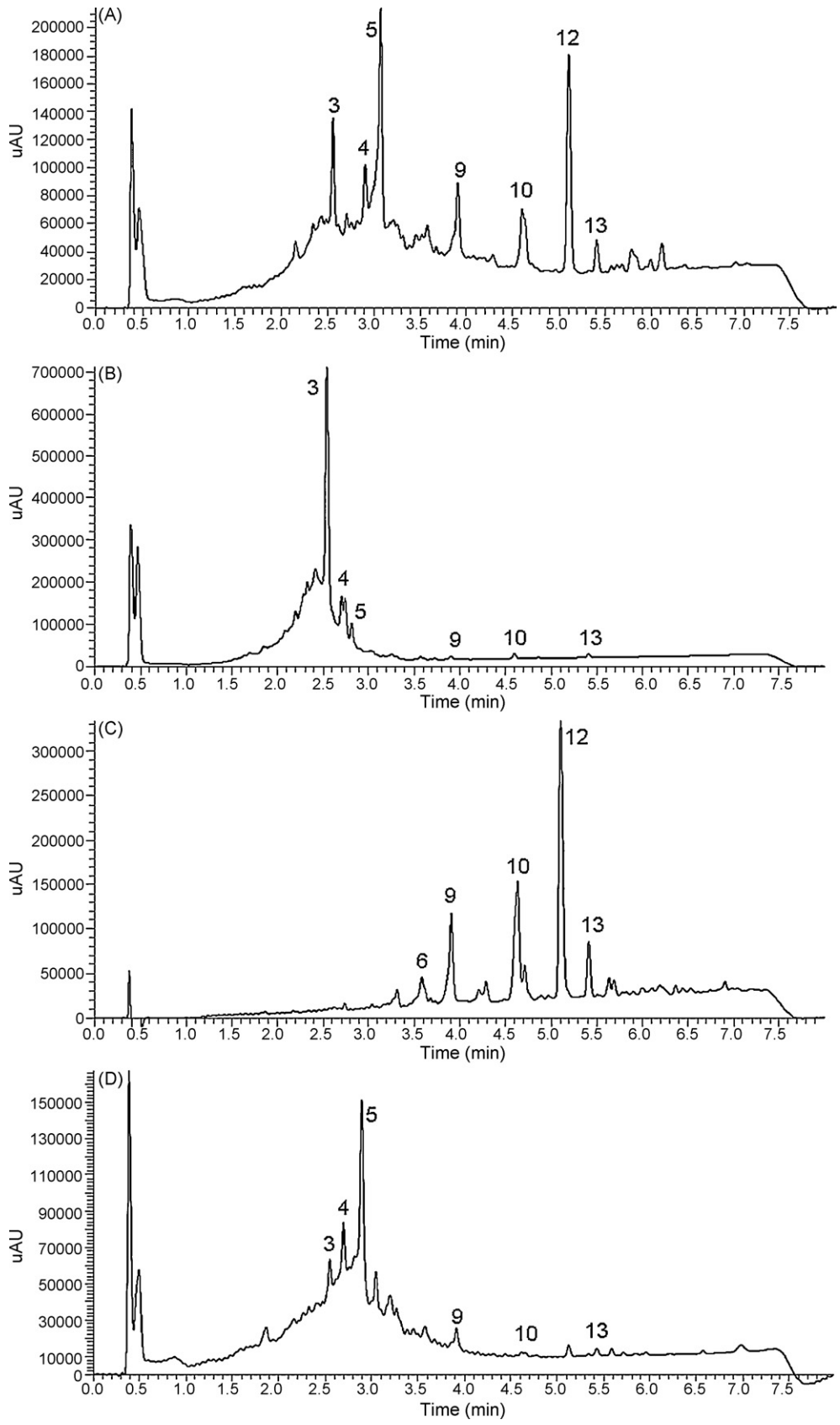

Fig. 2. Chromatograms (230 nm) corresponding to the UPLC-DAD-MS/MS analysis of the rosemary extracts obtained by (A) PLE at 200 °C using ethanol; (B) PLE at 100 °C using water; (C) SFE using supercritical CO_2 with 7% ethanol at 150 bar; (D) WEPO using water at 200 °C. Mobile phases: ACN (0.1% formic acid) and water (0.1% formic acid); column: C_{18} (50 mm × 2.1 mm, 1.9 μm d.p.). Rest of the analytical and extraction conditions in Section 2. For peaks identification see Table 2.

Table 5
Quantification of the phenolic antioxidants in the rosemary extracts indicated. Concentrations indicated as μg/mg extract ± SD. Values are the mean of three replicates.

Extraction technique	Solvent	Temp. (°C)	Time (min)	Pressure (bar)	Gallic acid (μg/mg extract)	Chlorogenic acid (μg/mg extract)	Caffeic acid (μg/mg extract)	p-Coumaric acid (μg/mg extract)	Rosmarinic acid (μg/mg extract)	Carnosol (μg/mg extract)	Carnosic acid (μg/mg extract)
PLE	Ethanol	50	20	100	n.d.	0.045 ± 0.001	0.092 ± 0.003	0.050 ± 0.003	12.368 ± 0.380	137.480 ± 5.304	76.284 ± 3.207
		100	20	100	n.d.	0.050 ± 0.003	0.224 ± 0.009	0.018 ± 0.001	16.002 ± 0.981	136.483 ± 4.291	75.542 ± 1.431
		150	20	100	<LOQ	0.063 ± 0.001	0.314 ± 0.001	0.024 ± 0.002	16.781 ± 0.643	104.269 ± 0.651	66.233 ± 0.736
		200	20	100	<LOQ	0.044 ± 0.002	0.116 ± 0.001	0.003 ± 0.001	9.119 ± 0.343	108.157 ± 2.065	52.874 ± 1.126
	Water	50	20	100	n.d.	0.115 ± 0.004	0.811 ± 0.067	0.020 ± 0.001	9.809 ± 0.895	7.798 ± 0.204	0.030 ± 0.007
		100	20	100	n.d.	0.141 ± 0.006	0.525 ± 0.027	0.020 ± 0.001	14.195 ± 0.852	45.821 ± 2.842	0.014 ± 0.003
		150	20	100	0.007 ± 0.001	0.160 ± 0.004	0.532 ± 0.013	0.027 ± 0.003	9.855 ± 0.382	35.370 ± 0.479	2.533 ± 0.122
		200	20	100	0.017 ± 0.001	0.100 ± 0.001	0.379 ± 0.007	0.033 ± 0.002	8.597 ± 1.161	46.113 ± 0.685	5.780 ± 0.149
SFE	CO_2	40	300	<100	n.d.	n.d.	0.005 ± 0.001	<LOQ	<LOQ	150.454 ± 6.855	96.713 ± 2.590
		40	300	300	n.d.	n.d.	0.010 ± 0.001	<LOQ	<LOQ	201.820 ± 4.832	94.168 ± 4.734
		40	300	400	n.d.	n.d.	0.008 ± 0.001	<LOQ	<LOQ	224.658 ± 21.260	106.467 ± 9.499
	CO_2 + 7% Ethanol	40	300	150	n.d.	n.d.	0.074 ± 0.001	0.088 ± 0.001	<LOQ	226.392 ± 1.399	151.554 ± 0.341
WEPO	Water	200	20		0.008 ± 0.002	0.045 ± 0.001	0.578 ± 0.015	0.096 ± 0.010	2.379 ± 0.151	15.925 ± 2.1280	3.760 ± 0.526

using ethanol as solvent. Interestingly, the amount of these two compounds was increased when the temperature was the highest. This is a response of the decrease of the water dielectric constant that takes place when liquid water is submitted to high temperatures. Thus, liquid hot water behaves more like an organic solvent being able to solubilize some less polar compounds. To obtain rosmarinic acid, both solvents proved to be adequate when employed at mild temperatures (ca. 100 °C). Higher temperatures could lead to some losses related to temperature-related degradation. On the other hand, although the amounts of the most polar compounds, *p*-coumaric, caffeic, chlorogenic and gallic acids in rosemary were very low, it can be clearly observed that using water the amounts of these compounds recovered was higher than those attained with ethanol. This fact supports the idea that PLE with water would be a technique more oriented to the extraction of high and medium polarity compounds, whereas PLE using ethanol would be more useful to the extraction of medium and less polar compounds. Nevertheless, the total amounts of the quantified antioxidants are not in agreement with the results obtained from the total phenol determination. Therefore, it is suggested that other compounds present on the extract would give a positive response to this assay.

The ability of the WEPO process to extract the target compounds was also studied. In this case, the amounts of antioxidants quantified were significantly lower than those obtained using PLE with water at 200 °C. This could be partially due to the fact that being WEPO a totally dynamic procedure, the mass transfer between the sample and the hot water was not as efficient as in the long static process.

These commented differences can be also clearly appreciated from the profiles shown in Fig. 2. From this figure it is possible to observe how SFE extracted less polar compounds (eluting at the end of the reversed phase analysis) whereas in the extractions performed with water, higher amounts of highly polar compounds (thus eluting first in the reserved phase separation) were obtained. On the other hand, the ethanol extract provided a mixed chromatogram in which it is possible to differentiate compounds of diverse polarity.

Considering these results together with the information regarding the antioxidant activity and extraction yield produced, the use of ethanol at very high temperatures could be the most appropriate technique to obtain bioactives from rosemary. The higher yields produced allowed attaining higher total antioxidant amounts per extraction procedure, even though using SFE, higher relative amounts could be obtained from the less polar antioxidant compounds.

4. Conclusions

The results presented on this contribution show the possibility to attain bioactive extracts from rosemary using environmentally clean extraction techniques. The three procedures employed minimized the use of organic solvents, which make them attractive to the food industry. Among them, PLE using ethanol or water as solvents at high (200 °C) or mild temperatures (100 °C), respectively, provided the best results, considering not only the higher extraction yield produced but also the amount of antioxidants extracted. The extraction temperature had an important influence on the selectivity when using a particular solvent. The WEPO process produced less extraction yield, but considering that it is still under further development, the results obtained were promising. Finally, supercritical fluid extraction using CO_2 modified with ethanol was also capable of extracting phenolic antioxidants. However, in this case, the applicability of SFE is somewhat limited given the relatively low extraction yields that this technique is able to provide.

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