



Chemical composition of bioactive pressurized extracts of Romanian aromatic plants

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ABSTRACT

In this contribution, pressurized liquid extraction (PLE) has been employed to isolate bioactive compounds from three native Romanian plants, oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*). Different PLE conditions have been tested including extraction with water, ethanol and their mixtures in a wide range of extraction temperatures (50–200 °C), and the antioxidant capacity of the extracts was measured using different assays (DPPH radical scavenging, TEAC assay and Folin–Ciocalteu assay to measure total phenols). Moreover, a complete chemical characterization by using LC–MS/MS was carried out to be able to correlate the bioactivity with the particular chemical composition of each extract and plant. The use of PLE with water as a solvent at the highest temperature tested (200 °C) always provided the highest extraction yields for the three studied plants, being maximum for oregano (>60%). Besides, oregano's pressurized water extracts at lower temperatures (50 °C) presented the highest content on total phenols (184.9 mg gallic acid/g extract) and the best antioxidant activities (EC₅₀ 6.98 µg/ml). In general, oregano extracts were the most active, followed by wild thyme extracts. The antioxidant capacity measured by DPPH assay was highly correlated with the amount of total phenols. Moreover, the use of a LC–MS/MS method allowed the identification of 30 different phenolic compounds in the different extracts, including phenolic acids, flavones, flavanones and flavonols, which have an important influence on the total antioxidant capacity of the different extracts.

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1. Introduction

At present, the increase on the demand for natural bioactive compounds that can be used as functional compounds for the food industry has led to an exhaustive search of new potential natural sources. Among them, different plant species have been already studied in detail [1–3], although there are still numerous matrices whose potential is still unknown [4].

Moreover, nowadays, a great deal of attention is being put on the extraction mechanisms commonly used to obtain these potential bioactive compounds. As the environmental concern is increasing, new greener extraction mechanisms are proposed to replace conventional extraction techniques towards more green and sustainable processes. Traditional extraction techniques often imply the use of a great amount of organic solvents, frequently toxic. Besides, they are laborious, lengthy and not very selective. In contrast, new advanced and environmentally friendly extraction techniques such as pressurized liquid extraction (PLE) and super-

critical fluid extraction (SFE) are gaining importance [5] and have been widely employed for the extraction of natural matrices [5].

In this sense, PLE has emerged as a fast extraction technique based on the extraction with liquids at high temperatures and pressures enough to keep the solvent in the liquid state during the whole extraction process. The application of these particular conditions allows the attainment of faster extraction processes, in which less amount of solvents are used, besides typically obtaining significantly higher yields compared to the traditional extraction mechanisms. Moreover, a wide variety of solvents may be employed, most notably water. In this case, the increase on temperature, while maintaining its liquid state, led to a significant decrease of the dielectric constant of water, providing solvent properties similar to those of some organic solvents such as methanol or ethanol [6]. Therefore, the use of water in PLE can be seen as a real alternative to the use of organic solvents in some applications.

Thus, the aim of the present work was to screen three different species of native Romanian plants, i.e., oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*) and wild thyme (*Thymus serpyllum*), for bioactivity using advanced extraction techniques together with different functional and chemical characterization techniques. PLE was used as a green and sustainable extraction technique while functional characterization was carried out by using differ-

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ent *in vitro* assays, including total phenols determination as well as two different antioxidant capacity assays (DPPH and TEAC). Moreover, extracts were chemically characterized by using a LC–MS/MS method to correlate the antioxidant activities with the particular chemical composition.

2. Materials and methods

2.1. Samples and chemicals

Three different plants, belonging to three botanical families which are commonly grown in Romania, were chosen for this study: oregano (*O. vulgare*), tarragon (*A. dracuncululus*) and wild thyme (*T. serpyllum*). The plant samples were obtained from a local herbalist's shop (Galati, Romania) and dried using a traditional method.

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). 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Fluka (Buchs, Switzerland). Folin–Ciocalteu phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas antioxidant standards, i.e., gallic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were supplied by Sigma–Aldrich (Steinheim, Germany). 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. Pressurized liquid extraction (PLE)

PLEs of plants were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Two different solvents (i.e., water and ethanol) and their mixtures were used in order to obtain extracts with different compositions. Extractions using either 100% water or 100% ethanol were performed at four different extraction temperatures (50, 100, 150 and 200 °C). In order to test the influence of the solvent composition, extractions using water/ethanol mixtures were performed at a fixed temperature of 100 °C. The extraction time was maintained constant for all the experiments (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 1.5 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 is as follows: (i) sample is loaded into cell, (ii) cell is filled with solvent up to a pressure of 1500 psi (1 psi = 6894.76 Pa), (iii) heat-up time is applied, (iv) static extraction takes place (i.e., 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 takes place. Between extractions, a rinse of the complete system was made in order to overcome any carry-over.

Once extractions were finished, solvents were removed. For the evaporation of the ethanol, a Rotavapor R-210 (from Buchi Labortechnik AG, Flawil, Switzerland) was used. The water extracts were lyophilized using a freeze-dryer (Labconco Corporation, MO, USA). Just before their HPLC analysis, the dried extracts were redissolved to a known concentration and filtered through 0.45 μm nylon filters (Symta, Madrid, Spain).

2.3. Determination of total phenols

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g.d.m. (dry matter) according to the Folin–Ciocalteu assay [7]. The total volume of reaction mixture was miniaturized to 1 mL. Six hundred microliters water and 10 μL of sample were mixed, to which 50 μL undiluted Folin–Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2% (w/v) Na_2CO_3 was added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 μL of the mixture was transferred into a well of the microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (BioTek) and compared to the gallic acid calibration curve (0.025–2 mg/mL) elaborated in the same manner. Data were presented as the average of duplicate analyses.

2.4. DPPH radical scavenging assay

The antioxidant capacity of all the obtained extracts was measured using the DPPH radical scavenging assay based on the protocol by Brand-Williams et al. [8] and formerly described [9]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested. Twenty five microliters of these solutions were added to 975 μL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek). DPPH–methanol solution was used 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 capacity. Measurements were done, at least, by triplicate.

2.5. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay described by Re et al. [10] with some modifications was used to measure the antioxidant capacity of the PLE extracts. ABTS radical cation ($\text{ABTS}^{\bullet+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The aqueous $\text{ABTS}^{\bullet+}$ solution was diluted with ethanol for the ethanol extracts and with 5 mM phosphate buffer (pH = 7.4) for the water and water–ethanol extracts, to an absorbance of 0.70 (± 0.02) at 734 nm. Ten microliters of sample (different concentrations) was added to 1 mL of diluted $\text{ABTS}^{\bullet+}$ radical solution. After 50 min at 30 °C, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 734 nm in a microplate spectrophotometer reader (BioTek). Trolox was used as reference standard and results were expressed as TEAC values (mmol Trolox/g extract). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were done at least in triplicate.

2.6. LC–MS/MS analyses

The LC–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 mass spectrometer via an electrospray interface. The analyti-

cal conditions employed consisted of a Hypersil C₁₈-AR column (150 mm × 4.6 mm, d.p. 3 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; 5 min, 95% B; 35 min, 40% B; 55 min, 5% B; 60 min; 5% B; 65 min, 95% B; 70 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 10 μL. The diode array detector recorded the spectra from 200 to 500 nm.

The MS analyzer was operated under ESI negative mode with the following parameters: Q1 and Q3 resolution of 0.7 Da FWHM; scan width, 0.010 Da; scan time, 0.206 s; spray voltage, 3000 V; sheath gas pressure, 35 psi; auxiliary gas pressure, 5 psi; capillary temperature, 350 °C, skimmer offset (MS/MS experiments), 30 V.

2.7. Statistical analysis

Microsoft Excel 2000 Program was employed for statistical analysis of the data with the level of significance set at 95%. One-way analysis of variance (ANOVA) was used to assess statistical differences between extractions. Differences were considered as significantly different at a value of $p < 0.05$.

3. Results and discussion

As it has been already mentioned, the particular chemical composition of plants may vary depending on a number of parameters, including geographical-related factors, growing conditions as well as genetic variability. For this reason it is interesting not only knowing the general chemical composition of a given plant species, but also the particular proportions in which these compounds may be present on plants with different geographical origins. With the aim to obtain bioactive compounds from the three studied Romanian plants (i.e., tarragon, wild thyme and oregano), different PLE conditions were tested. The goal of this screening was to use very different extraction conditions in order to have a selected number of extracts of different compositions and associated bioactivity. Thus, extracts obtained at the different studied conditions were functionally characterized according to their antioxidant capacity and chemically characterized to know their exact composition and to correlate both.

3.1. Extraction and functional characterization

As mentioned, two different solvents were selected for PLE of Romanian plants, that is, ethanol and water, that cover different polarities. Besides, four different temperatures were also employed for the two solvents (50, 100, 150 and 200 °C), covering the whole instrument's temperature working range. Based on our previous experience with natural matrices [9], the pressure was maintained during the whole extraction procedure at 1500 psi and the static extraction time was set at 20 min. This pressure was selected considering that once the extraction pressure is enough to maintain the solvent in the liquid state, its effect is not statistically significant on the outcome of the extraction [11]. Likewise, it has been statistically demonstrated that the influence of the static extraction time is not extremely high [11], and that 20 min is sufficient to ensure the complete extraction of valuable compounds from natural matrices [12]. Moreover, in order to more precisely study the influence of the solvent, different proportions of water and ethanol were combined, namely 25/75, 50/50 and 75/25. To perform these experiments, a medium temperature (100 °C) was selected.

Fig. 1 shows the results in terms of extraction yield for the different conditions tested and the three studied plants. As it can be observed, the highest yield was obtained by PLE using water at 200 °C for the three plants, being maximum for oregano, reaching values higher than 60%, whereas the lowest yields were obtained

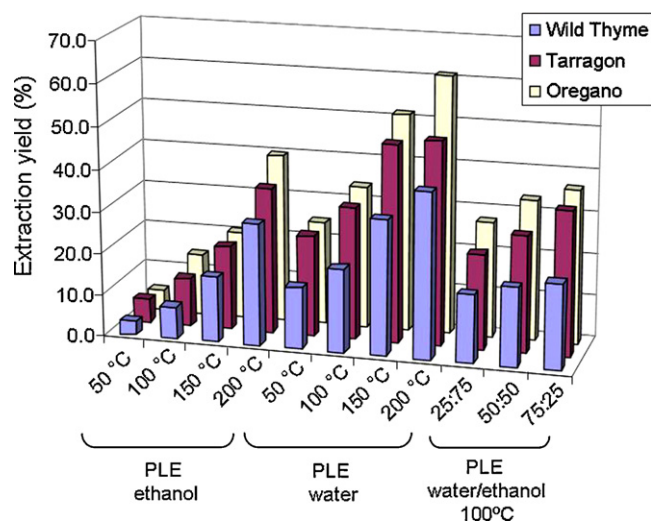


Fig. 1. Extraction yield (%) produced after the PLE of the three studied plants at the indicated conditions.

using ethanol as solvent at 50 °C (particularly the yield obtained for wild thyme, 3.2%). Considering the different extraction temperatures tested, the extraction yield was higher when increasing the temperature, independently of the solvent employed. For the same temperature, in all cases significantly higher yields were obtained with water compared to those with ethanol. In agreement with this observation, when the extraction temperature was maintained at 100 °C and the solvent composition was changed, the extraction yield increased when higher proportions of water were employed. Interestingly, similar yields were obtained with 100% water and a mixture water/ethanol 75:25. These results suggest that most of the compounds present on these plants had a relatively high polarity, and therefore, were preferentially extracted with ethanol and, above all, with water. The increase of extraction yield with the temperature corresponded to a typical increment of the mass transfer as a result of the application of higher temperature as well as to a decrease on the solvent viscosity which helps the solvent to penetrate the matrix.

The next step consisted on the functional analysis of the extracts: assays such as Folin–Ciocalteu, DPPH and TEAC were used to assess both, the total phenols and the antioxidant capacity of the extracts obtained under the screened conditions; data are presented in Table 1. In terms of total phenols, it can be seen that oregano was, by far, the richest plant in terms of total phenols followed by wild thyme and tarragon; this behavior was maintained in all the PLE conditions tested. On the other hand, the highest amount of total phenols was obtained with pressurized water for all the studied plants. However, the behavior of the different plants as a response of the increase of temperature was different. Whereas oregano extracts presented a maximum at 100 °C, 200 °C was the most efficient temperature for phenol's extraction in tarragon and wild thyme. In both cases, a higher extraction temperature meant a higher amount of total phenols extracted for the two tested solvents. When keeping the extraction temperature constant at 100 °C, it could be observed how the maximum amount of total phenols was attained using a mixture of ethanol/water 50:50 for tarragon and wild thyme, whereas for oregano 100% water provided with better results. Nevertheless, the amount of total phenols obtained from oregano with the three solvent mixtures of water/ethanol was not statistically different ($p > 0.05$). Nonetheless, looking at the results as a whole, it can be affirmed that the three plants, particularly oregano, were rich on phenols, and thus, had the potential for providing with active antioxidant extracts.

Table 1
Antioxidant capacity of the Romanian plants extracts obtained by PLE at the indicated conditions, measured using the DPPH radical scavenging and Trolox equivalents antioxidant capacity assays. Results are expressed as mean \pm SD. Analyses were performed, at least, by triplicate.

Solvent	Extraction conditions ^c	Antioxidant activity								
		Tarragon		Wild thyme		Oregano				
		EC ₅₀ ^a (μ g/ml)	TEAC ^b (mmol/g)	mg gallic acid/g	EC ₅₀ (μ g/ml)	TEAC (mmol/g)	mg gallic acid/g	EC ₅₀ (μ g/ml)	TEAC (mmol/g)	mg gallic acid/g
Ethanol	50 °C	29.53 \pm 0.34	0.46 \pm 0.01	16.80 \pm 1.88	17.48 \pm 0.73	0.65 \pm 0.06	34.57 \pm 0.75	17.10 \pm 1.47	1.15 \pm 0.04	68.30 \pm 6.91
	100 °C	25.76 \pm 0.84	0.54 \pm 0.02	33.25 \pm 3.32	15.92 \pm 0.63	1.06 \pm 0.07	58.52 \pm 5.56	11.51 \pm 1.22	1.34 \pm 0.16	102.25 \pm 3.34
	150 °C	23.24 \pm 1.02	0.64 \pm 0.04	44.42 \pm 3.04	14.70 \pm 0.71	1.11 \pm 0.05	78.72 \pm 1.97	7.30 \pm 0.68	2.37 \pm 0.21	144.25 \pm 5.42
Water	200 °C	21.32 \pm 0.76	0.67 \pm 0.02	50.40 \pm 2.75	14.31 \pm 1.39	1.08 \pm 0.07	72.20 \pm 4.22	7.40 \pm 0.19	2.02 \pm 0.04	134.40 \pm 6.64
	50 °C	24.27 \pm 0.82	1.63 \pm 0.05	44.75 \pm 3.91	13.75 \pm 1.14	2.40 \pm 0.17	79.02 \pm 6.62	6.98 \pm 0.45	3.51 \pm 0.10	184.90 \pm 21.98
	100 °C	17.42 \pm 0.28	2.09 \pm 0.05	59.52 \pm 5.51	11.76 \pm 0.25	2.82 \pm 0.05	91.07 \pm 9.25	8.55 \pm 1.01	3.31 \pm 0.18	183.10 \pm 14.43
Water/ethanol 50:50	150 °C	20.55 \pm 1.50	2.41 \pm 0.11	69.47 \pm 7.08	15.01 \pm 1.15	2.58 \pm 0.15	80.97 \pm 7.28	10.06 \pm 0.16	3.31 \pm 0.06	173.65 \pm 6.87
	200 °C	19.02 \pm 1.11	2.64 \pm 0.05	71.70 \pm 5.90	11.83 \pm 0.67	2.71 \pm 0.08	112.27 \pm 16.75	8.70 \pm 0.5	3.73 \pm 0.09	159.12 \pm 18.25
	100 °C	15.85 \pm 1.39	2.29 \pm 0.12	60.62 \pm 6.03	10.85 \pm 0.86	2.61 \pm 0.02	102.20 \pm 5.78	9.70 \pm 1.17	3.13 \pm 0.25	168.85 \pm 11.28
Water/ethanol 75:25	100 °C	17.20 \pm 1.41	2.26 \pm 0.06	67.17 \pm 2.12	10.39 \pm 0.55	3.08 \pm 0.09	119.95 \pm 3.7	7.78 \pm 0.73	2.77 \pm 0.18	160.45 \pm 14.25
	100 °C	19.77 \pm 0.39	2.09 \pm 0.07	56.40 \pm 2.02	10.46 \pm 0.66	2.92 \pm 0.03	107.77 \pm 5.94	7.04 \pm 0.49	2.98 \pm 0.29	172.92 \pm 11.09

^a DPPH radical scavenging assay.

^b Trolox equivalents antioxidant capacity assay.

^c All the extractions at 1500 psi for 20 min.

Two methods to assess the antioxidant capacity of the extracts were selected, namely DPPH radical scavenging assay and TEAC (Trolox equivalents antioxidant capacity) assay. The use of two different antioxidant capacity methods may provide a deeper insight on the chemical constituents present on the extracts as well as their different activities against different radicals. The results collected using these procedures are summarized in Table 1. It is important to consider that the results from the DPPH method were expressed as EC₅₀ [8] and therefore, the lowest the value, the highest the antioxidant capacity. As can be seen, the best results in terms of EC₅₀ were obtained for oregano. As a general trend for the three plants, an increase of extraction temperature using ethanol provided a higher antioxidant capacity, although values obtained for extractions at 150 and 200 °C (using ethanol) were not statistically different ($p > 0.05$). In the case of the PLEs using water, an increase in the antioxidant capacity was generally observed when the temperature was raised from 50 to 100 °C, then decreased and finally increased again at 200 °C. This behavior can be explained by an improved recovery of antioxidant compounds at temperatures up to 100 °C and a subsequent degradation at higher temperatures. The improvement of antioxidant capacity at 200 °C, can be due to other phenomena that can occur at very high temperatures using water as extraction solvent, such as the neoformation of antioxidant compounds derived from Maillard reaction, among others [13]. These phenomena have been demonstrated to occur, to some extent, in natural matrices containing reducing sugars and aminoacids, therefore contributing to the total antioxidant capacity of the extracts compared to those obtained at 150 °C.

Combining the information regarding the antioxidant capacity in terms of EC₅₀ and total phenols' content, it can be observed how there is a clear correlation between the two measurements (Fig. 2) indicating that the samples with a higher content on total phenols were, in general, also the most active in terms of antioxidant capacity. This behavior has previously been suggested for different natural matrices including plants, algae and vegetables [14]. As it can be observed in this figure, only in the case of oregano, some extracts possessed the same antioxidant capacity or even higher than other which, however, were richer on total phenols. In this case, as mentioned, partial degradation of total phenols could occur when extracting with water at the highest temperature while, at the same time, new antioxidants might be forming at these conditions.

As for the results of TEAC assay (Table 1), extracts followed the same trend previously mentioned for EC₅₀ values but, in this case, higher values corresponded to higher antioxidant capacity. Both methods measured the ability of an antioxidant to transfer an electron and scavenge a radical (DPPH or ABTS), thus, considering similar mechanisms, an equivalent behavior is expected.

3.2. Chemical characterization of the obtained extracts

An LC–MS method was adapted to characterize the obtained PLE extracts from the three studied plants. A quite slow gradient was employed, not chasing a fast analysis but a higher resolution of the complex profiles of the different extracts. In Fig. 3, the chromatograms corresponding to the extracts obtained by PLE using water and ethanol as solvents at 200 °C from the three studied Romanian plants are shown. As it can be appreciated, even if the six profiles were very different, a good separation of the compounds was achieved. Identification of compounds was attempted combining the information provided by the DAD and by the MS detector together with retention times and information available on the literature. Particularly useful was the combination of UV–Vis and MS spectra together with data regarding the fragmentation of the main ions detected. Using this approach, different compounds could be identified or tentatively assigned on the different samples. Identification of compounds is shown in Table 2, together with the data

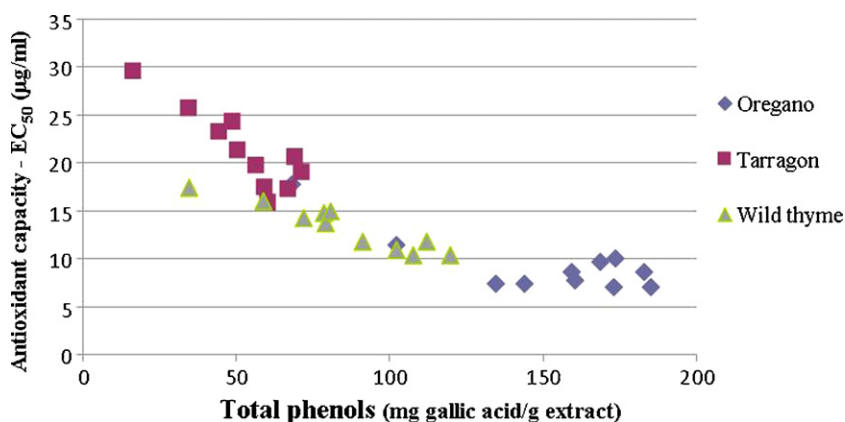


Fig. 2. Correlation between the amount of total phenols determined on the plant extracts and their corresponding activity measured using the DPPH radical scavenging assay.

collected using the two detectors (DAD and MS) in series. Besides, the plant in which each compound was found is also indicated.

3.2.1. Oregano PLE extracts

Although much interest has been put in the chemical composition of oregano essential oil obtained through the application of different techniques [15–19], including supercritical fluid extraction (SFE) [20,21], its phenolic chemical composition has not been so extensively studied [22]. In fact, few applications of PLE can be found in the literature for the extraction of phenolic antioxidants from oregano [23], although none of them compared the possible performance of different solvent compositions. As it can be observed in Fig. 3A and B, together with the information given in Table 2, the profile obtained when using water as extraction solvent

was different than with ethanol. As expected, the main differences were observed for the less polar compounds that were preferably extracted using ethanol. When a mixture of ethanol/water was employed, results were similar to those obtained only using water; these results are in agreement with those on total phenols that, for mixtures, were closer to the values obtained with water at the same temperature.

The main phenolic antioxidant present on the extracts obtained with water was rosmarinic acid (peak 21); this compound is well-known by its potent antioxidant activity [24]. Other important compounds in these extracts were luteolin-7-*O*-glucuronide (peak 15) as well as luteolin (peak 22) and different phenolic acids including syringic (peak 1), protocatechuic (peak 2), homovanillic (peak 3), chlorogenic (peak 6), hydroxybenzoic (peak 7) and caffeic (peak

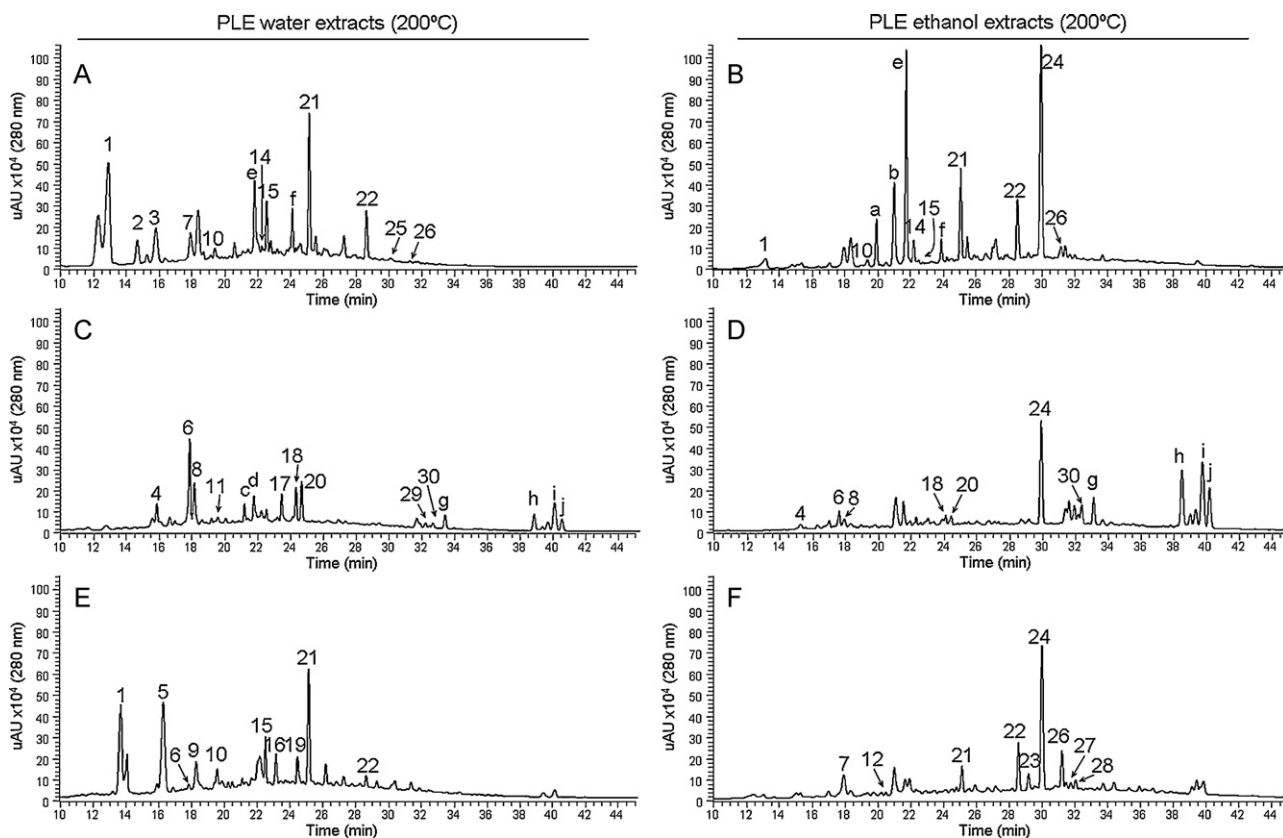


Fig. 3. LC-DAD-MS/MS chromatograms (280 nm) of the different extracts obtained using PLE at 200 °C from Romanian oregano (A and B), tarragon (C and D) and wild thyme (E and F). For peak identification and information see Tables 2 and 3.

Table 2
Compounds identified in the PLE extracts analyzed by LC–MS.

ID	Retention time (min)	Compounds identified	UV–Vis maxima (nm)	[M–H] [–]	Main fragments detected	Plant in which was detected
1	12.7	Syringic acid	280	197.1	179, 135	O, Wt
2	14.5	Protocatechuic acid	260, 293	153.1	108	O
3	15.6	Homovanillic acid	277	181.2	167, 137	O
4	15.7	3-Caffeoylquinic acid	297, 325	353.2	191, 179	T
5	17.7	Vanillic acid	277	167.2		Wt
6	17.8	Chlorogenic acid	300, 326	353.3	191	O, T, Wt
7	17.9	Hydroxybenzoic acid	282, 312s	137.1		O, Wt
8	18.0	4-caffeoylquinic acid	299, 326	353.2	191, 173	T
9	18.2	<i>p</i> -Coumaric acid	286	163.1	137	Wt
10	19.3	Caffeic acid	291, 323	179.2	135	O, Wt
11	19.4	Caftaric acid	298, 326	311.2	179	T
12	20.9	Luteolin-7- <i>O</i> -glucoside	265, 340	447.2	285	Wt
13	21.9	Rosmarinic acid isomer	291, 329	359.1	161	O
14	22.2	Protocatechuic glucoside	264, 287s	421.1	153	O
15	22.4	Luteolin-7- <i>O</i> -glucuronide	265, 347	461.1	285	O, Wt
16	23.0	Eriodictyol-7- <i>O</i> -glucuronide	283, 329s	463.2	287, 175	Wt
17	23.3	Dicafeoylquinic acid	300, 325	515.2	353, 191, 173	T
18	24.2	Dicafeoylquinic acid	299, 328	515.2	353, 191, 173	T
19	24.3	Apigenin-7- <i>O</i> -glucuronide	267, 334	445.2	269	Wt
20	24.5	Dicafeoylquinic acid	298, 327	515.3	353	T
21	25.0	Rosmarinic acid	291, 329	359.2	161	O, Wt
22	28.5	Luteolin	265, 347	285.2		O, Wt
23	29.1	Eriodictyol	287	287.2	151	Wt
24	29.9	Caffeic acid ethyl ester	299, 323	207.2	179, 161, 135	O, T, Wt
25	30.0	Naringenin	284, 330s	271.2		O
26	31.2	Apigenin	332	269.1		O, Wt
27	31.7	Cirsimaritin	338	313.2		Wt
28	32.0	Preynlaringenin	261, 321s	339.8	271	Wt
29	32.3	Isorhamnetin	286s, 360	315.2		T
30	32.4	Quercetin	287, 345s	301.2		T

s, shoulder; O, oregano; T, tarragon; Wt, wild thyme.

10) acids. For the characterization of the phenolic acids, typical UV–Vis spectra as well as their corresponding [M–H][–] ions and common fragments were found. These types of phenolic compounds are widely distributed on nature and are well known by their functional properties, among others, a potent antioxidant activity [25,26]. On the other hand, the peak corresponding to luteolin-7-*O*-glucuronide presented a molecular ion ([M–H][–]) at *m/z* 461.1. Besides, the UV–Vis spectrum matched with that corresponding to luteolin, characterized by a maximum absorbance at 340 nm. Moreover, the detection of the fragment corresponding to luteolin (*m/z* 285) corroborated its identification. Higher amount of phenolic compounds were extracted when using water at 100 °C compared to the extraction at 200 °C (see Table 1). Nevertheless, qualitatively, the main difference among these two extracts was the lack of extraction of less polar antioxidants, mainly luteolin at the lower temperature. Also at 100 °C (chromatogram not shown), apigenin-7-*O*-glucuronide could be tentatively identified since its molecular ion, as well as the fragment corresponding to apigenin, was detected, together with the match of its UV–Vis spectrum. This compound was not recovered when using water at 200 °C, probably because of too higher temperatures led to its degradation.

Concerning the ethanol extracts, their chromatographic profiles were very similar, although a higher amount of phenolics could be obtained at the highest temperature (Table 1). In these extracts, rosmarinic acid (peak 21) was also among the main components present, although luteolin (peak 22) and caffeic acid ethyl ester (peak 24) could be also extracted in high amounts. Regarding this latter compound, identification was based on the combination of the typical UV–Vis spectra of a hydroxycinnamic acid, with absorption maxima at 299 and 323 nm, together with a molecular weight ([M–H][–]) of 207.2. This information suggested the presence of a hydroxycinnamic acid derivative. Moreover, the fragmentation of this base peak provided with fragments corresponding to *m/z* 179, 161 and 135, typical of caffeic acid. Thus, combining all

this information, this peak could be tentatively assigned to caffeic acid ethyl ester, as it is shown in Fig. 4. In general, a total of 14 different compounds could be tentatively identified in the Romanian oregano extracts. Besides, as it can be observed in Fig. 3, other important peaks in the chromatograms could not be successfully assigned; information regarding their UV–Vis maxima, molecular ion and main fragments detected is shown in Table 3. For instance, peak f showed UV–Vis and MS spectra that may indicate the presence of dyhydroxykaempferol. The retention time of this peak could also confirm this tentative assignment. However, due to the absence of a clear fragment at *m/z* 259, this peak could not be successfully assigned.

3.2.2. Tarragon PLE extracts

To the best of our knowledge, the possibility of extracting antioxidant compounds using PLE from tarragon has not been explored so far. In fact, in general, only the characterization of the essential oil produced by some species of *Artemisia* has raised some attention [27–29]. As it can be observed in Fig. 3C and D, the profiles obtained for the extracts obtained with water and ethanol at 200 °C from tarragon were qualitatively quite similar, although, in general, water extracts possessed higher amount of phenols than their corresponding counterparts obtained with ethanol (see Table 1). In fact, the same compounds could be basically identified in both extracts. Nevertheless, the water extracts were mainly characterized by the presence of caffeoylquinic (peaks 4, 6 and 8) and dicafeoylquinic (peaks 17, 18 and 20) acids whereas in the ethanol extracts the major compounds were found at the end of the chromatogram, corresponding to less polar compounds (e.g., peaks h, i, j). Besides, the same hydroxycinnamic derivative compound also found in oregano, tentatively identified as caffeic acid ethyl ester (peak 24), was the main peak in these extracts. On the other hand, in water extracts, these compounds were found in less amounts or not found at all (e.g., compound 24). The presence of

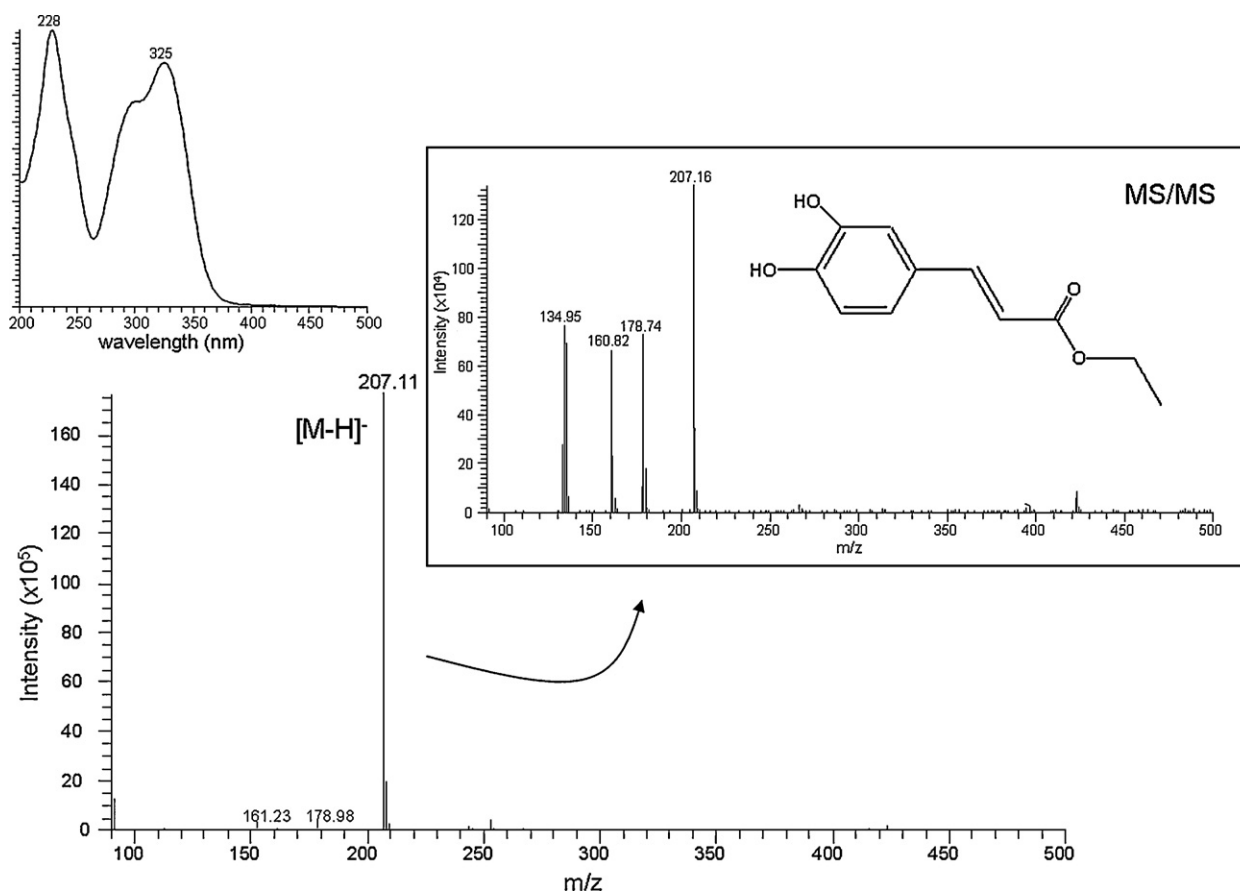


Fig. 4. UV-Vis and MS spectrum of caffeic acid ethyl ester (m/z 207.1, peak 23), as well as its fragmentation pattern and proposed chemical structure.

caffeoylquinic acids is characteristic of some species of *Artemisia* [30]. These compounds have been associated to several interesting functional properties, such as antiviral [31], analgesic [32] or antioxidant activities [33]. These acids possess a particular UV-Vis spectrum with absorption maxima at 300 and 325 nm, which detection was used in the present work as a first hint for a possible identification. Next, the information provided by the MS detector was studied. Several of these compounds presented molecular ions ($[M-H]^-$) corresponding to m/z 353 (i.e., peaks 4, 6 and 8, respectively). Among them, the main peak (peak 6) provided a fragment of m/z 191, and was tentatively assigned to chlorogenic acid. Besides, it is widely known that chlorogenic acid is the principal caffeoylquinic acid in tarragon [30]. On the other hand, compounds 4 and 8 gave fragments of m/z 179 and 173, respectively. According to this latter fragment, typical from the 4-acyl groups, peak 8 was tentatively identified as 4-caffeoylquinic acid, whereas the finding

of the fragment m/z 179 in peak 4 suggested that this compound could be 3-caffeoylquinic acid. Besides, three other peaks, eluting later on the chromatogram, presented also the typical UV-Vis spectrum of caffeoylquinic acids. For these compounds (compounds 17, 18 and 20), MS base peaks ($[M-H]^-$) of m/z 515 were detected as well as fragments of m/z 353, thus clearly indicating the presence of dicaffeoylquinic acids. Although these compounds were not fully characterized, the occurrence of fragments at m/z 173 in peaks 17 and 18 indicated the presence of 4-acyl dicaffeoylquinic acids. Examples of the assignment process as well as the structures proposed for compounds 6 and 17 are shown in Fig. 5. Besides these compounds, caftaric acid (peak 11) as well as caffeic acid ethyl ester (peak 24) and other flavonoids (isorhamnetin and quercetin, peaks 29 and 30, respectively) were identified in the tarragon extracts.

Other important peaks that could not be completely identified (peaks g, h, i and j, see Fig. 3C and D) were also detected in

Table 3

UV-Vis and MS data of the main peaks detected in the PLE extracts analyzed by LC-MS which identity could not be confirmed.

ID	Retention time (min)	UV-Vis maxima (nm)	$[M-H]^-$	Main fragments detected	Plant in which was detected
a	19.9	277	329.2	167	O
b	21.0	281	393.2	231, 123	O
c	21.1	283, 335	639.2	609, 451	T
d	21.5	294, 319	481.3	355, 193	T
e	21.7	263, 283s, 295s	437.2	153	O
f	24.0	283, 325	287.2	243, 121	O
g	33.2	276, 310	257.2		T
h	38.6	288, 331s	285.2		T
i	39.8	266	207.2		T
j	40.3	276, 310	271.3		T

s, shoulder; O, oregano; T, tarragon; Wt, wild thyme.

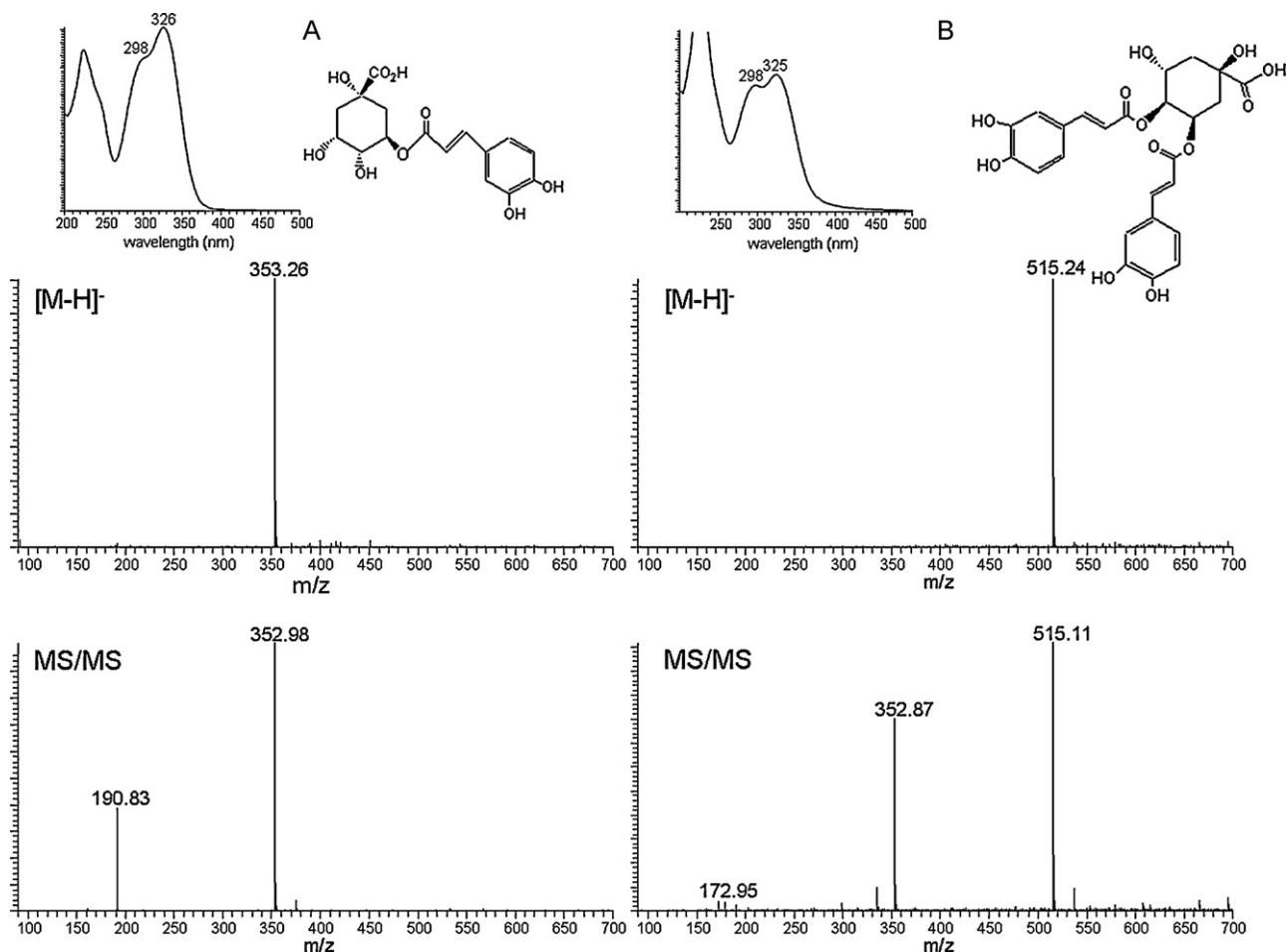


Fig. 5. Information collected for the identification of (A) chlorogenic acid (peak 5) and (B) dicaffeoylquinic acid (peak 16). UV–Vis, MS spectra, fragmentation pattern and proposed chemical structures.

the extracts produced using both solvents, although they were in higher extent in the ethanol extracts. Characteristics of these non-identified peaks are shown in Table 3.

3.2.3. Wild thyme PLE extracts

The last plant characterized was *T. serpyllum*. This plant, as well as other *Thymus* species, has been described to possess essential oils with antioxidant capacity [34,35]. However, up to now, PLE has not been applied for the extraction of phenolic antioxidants from this kind of plant. The chemical characterization of the wild thyme extracts by LC–MS revealed that those obtained with water and with water/ethanol mixtures did not differ significantly from a qualitative point of view; this is in agreement with the total phenols observed for both, water and water/ethanol extracts (Table 1), as mentioned previously for oregano extracts. However, those extracts obtained with ethanol possessed a different composition. As it can be clearly observed in Fig. 3E and F, less polar compounds dominated in the ethanol extract chromatogram whereas more polar compounds were extracted with water. Among them, rosmarinic acid (peak 21) was the main compound in the wild thyme water extracts. Besides, other polar phenolic acids were also detected, notably, syringic (peak 1), vanillic (peak 5), chlorogenic (peak 6), *p*-coumaric (peak 9) and caffeic (peak 10) acids. All these phenolic acids have an important influence on the total antioxidant capacity shown by these extracts. Moreover, other flavonoids such as luteolin-glucoside, luteolin-glucuronide, eriodictyol-glucuronide, and apigenin-glucuronide (compounds 12, 15, 16 and 19, respectively) could be identified together with

the aglycones luteolin, eriodictyol and apigenin (peaks 22, 23 and 26). The different glucuronides were clearly assigned based on the detection of their molecular ions as well as the fragments corresponding to their aglycones. Data on UV–Vis spectra were used to confirm the identification. This combination allowed, for instance, the correct assignment of the ion with m/z 463, as it can be appreciated in Fig. 6. Considering that this compound should be a flavonoid, in agreement with its retention time and UV–Vis spectrum, the detection of a fragment derived from the main peak of m/z 287 permitted the assignment of this compound as an eriodictyol derivative instead of other with similar molecular weight, such as isoquercetin. Besides, a fragment of m/z 175, typical from the glucuronide moiety, was detected, supporting also this assignment. Although the possibility of assigning positional isomers could be theoretically achieved by using MS, under the conditions employed in the present research these glycosylated flavonoids could not be unambiguously characterized. Nevertheless, their more frequent forms, containing a 7-*O*-linkage were assumed.

On the other hand, in the wild thyme ethanol extracts, rosmarinic acid was not the main identified compound, although its presence could also be confirmed. Instead, important peaks appeared later on the chromatogram, corresponding to luteolin (peak 22), apigenin (peak 26) and in less extent, eriodictyol (peak 23), cirsimaritin (peak 27) and prenylnaringenin (peak 28). This last compound was assigned thanks to the detection of a base peak at m/z 339.8 ($[M-H]^-$) together with a typical fragment of m/z 271 corresponding to the loss of the prenyl moiety. Nevertheless, the main compound in these chromatograms (see Fig. 3) was again caf-

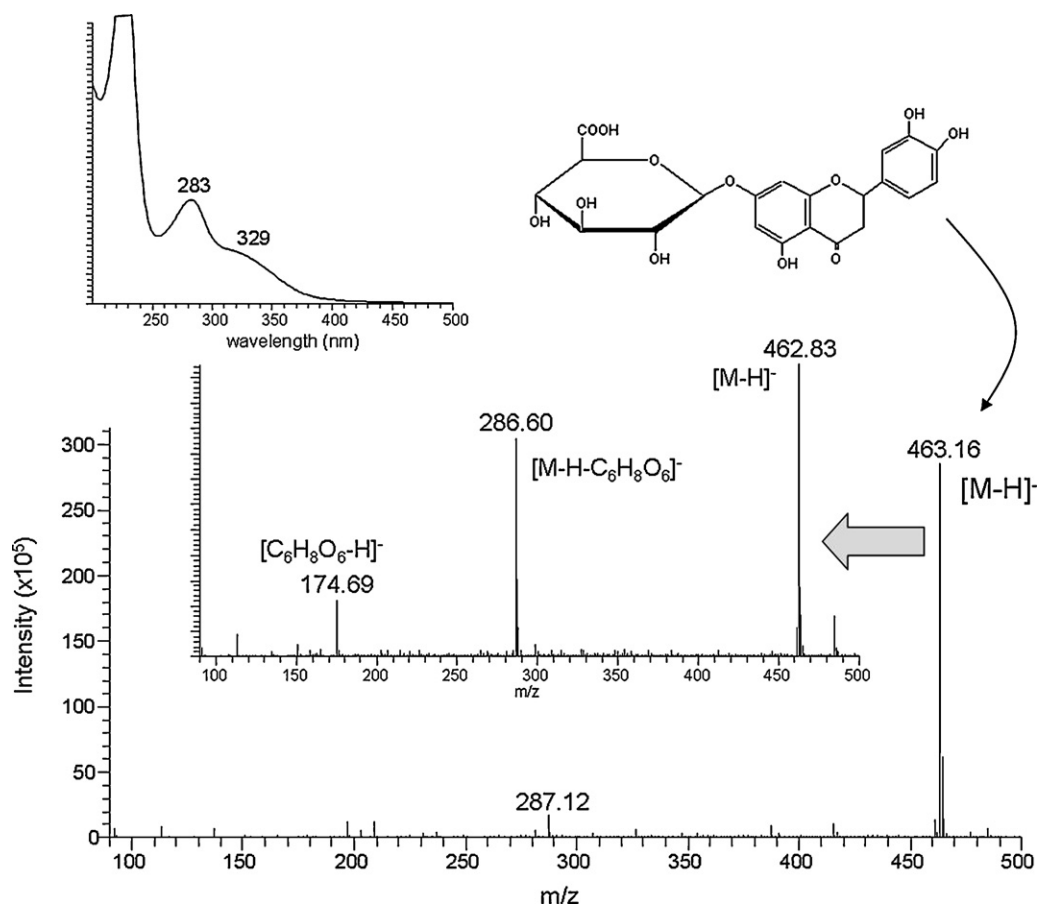


Fig. 6. UV-Vis and MS spectra of eriodictyol-7-O-glucuronide (peak 15) and fragmentation pattern and chemical structure proposed for this assignment.

feic acid ethyl ester (peak 24). This compound appeared also in the ethanol extracts of the other two Romanian plants studied.

In conclusion, a total of 17 different phenolic compounds could be tentatively identified in the wild thyme PLE extracts, which may probably have a strong influence on the total antioxidant capacity observed.

4. Conclusions

The applicability of PLE as an advanced environmentally friendly extraction technique for the extraction and characterization of native Romanian plants such as oregano, tarragon and wild thyme, has been demonstrated. Different combinations of solvents-temperatures were screened to obtain extracts with important bioactivities, extraction yields, antioxidant capacity and chromatographic profiles were studied to obtain a complete picture of the process. Results showed that higher yields were obtained with water at very high temperatures (200 °C), reaching values around 62% when using oregano as raw material. Besides, the higher antioxidant capacity was obtained using water at 50–100 °C, being oregano the most active. Data suggested a direct correlation between the amount of total phenols and the antioxidant capacity measured using DPPH radical scavenging protocol. Besides, the use of an LC-MS/MS method allowed the characterization of the phenolic compounds on PLE extracts. Thirty different compounds could be tentatively assigned by using this method, some of them described for the first time in these plants. Oregano extracts were mainly characterized by the presence of phenolic acids, mainly rosmarinic and caffeic ethyl ester acids. Extracts from tarragon were particularly rich on caffeoyl and dicaffeoylquinic acids, as well as on other flavonoids, whereas wild thyme presented the most complex

chemical profile including phenolic acids and different glycosylated flavonoids and aglycones. To the best of our knowledge, the possibility of obtaining such compounds from these species through the application of PLE-*in vitro* antioxidant assays-LC-MS/MS is shown for the first time.

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