Talanta 85 (2011) 167–176

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Storage method, drying processes and extraction procedures strongly affect the phenolic fraction of rosemary leaves: An HPLC/DAD/MS study

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ARTICLE INFO

Article history:
Received 17 August 2010
Received in revised form 11 March 2011
Accepted 20 March 2011
Available online 8 April 2011

Keywords:
Rosemary
HPLC/DAD/MS
Carnosic acid
Rosmarinic acid
Flavonoids
Chemical stability

ABSTRACT

The *Rosmarinus officinalis* L. is widely known for its numerous applications in the food field but also for the increasing interest in its pharmaceutical properties. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction and the phenolic constituents. The latter group is mainly constituted by rosmarinic acid, by a flavonoidic fraction and by some diterpenoid compounds structurally derived from the carnosic acid. The aim of our work was to optimize the extractive and analytical procedure for the determination of all the phenolic constituents. Moreover the chemical stability of the main phenols, depending on the storage condition, the different drying procedures and the extraction solvent, have been evaluated.

This method allowed to detect up to 29 different constituents at the same time in a relatively short time. The described procedure has the advantage to being able to detect and quantify several classes of compounds, among them numerous minor flavonoids, thus contributing to improving knowledge of the plant.

The findings from this study have demonstrated that storing the raw fresh material in the freezer is not appropriate for rosemary, mainly due to the rapid disappearing of the rosmarinic acid during the freezing/thawing process. Regarding the flavonoidic fraction, consistent decrements, were highlighted in the dried samples at room temperature if compared with the fresh leaf. Rosmarinic acid, appeared very sensitive also to mild drying processes. The total diterpenoidic content undergoes to little changes when the leaves are freeze dried or frozen and limited losses are observed working on dried leaves at room temperature. Nevertheless it can be taken in account that this fraction is very sensitive to the water presence during the extraction that favors the conversion of carnosic acid toward it oxidized form carnosol. From our findings, it appear evident that when evaluating the phenolic content in rosemary leaves, several factors, mainly the type of storage, the drying process and the extraction methods, should be carefully taken into account because they can induce partial losses of the antioxidant components.

1. Introduction

Rosmarinus officinalis L. (Lamiaceae) is a plant widely distributed in Europe, Asia and Africa and one of its elective growing areas is the Mediterranean basin where spontaneous plants are diffusely distributed. The plant is widely known for its numerous applications in the food field but also for the increasing interest in its pharmaceutical properties. Two groups of compounds are mainly responsible for the biological activities of the plant: the volatile fraction and the phenolic constituents. The latter group is mainly constituted by rosmarinic acid, by a flavonoidic fraction and by some diterpenoid compounds structurally derived from the carnosic acid.

In the food industry rosemary is a very frequently used herb and its oleoresins are added to several products to improve their oxidative stability and to ameliorate the organoleptic profiles [1].

The quality and value of commercial rosemary extracts are closely related to their phenolic content, particularly of carnosic and rosmarinic acids, the most abundant constituents, which are also well known for their various biological properties. According to the ESCOP (European Society Cooperative on Phytoterapy), ethanol and aqueous extracts from rosemary leaves are used as coleretic, colagogue, epatoprotective, and antioxidants, but also as light diuretic, antiulcer, antitumor and antiviral [2] products. The derived essential oil is mainly used for local applications for its balsamic, antispasmodic and anti-inflammatory activities [2].

Moreover, rosemary and its oleoresins and extracts are widely used to preserve and improve the organoleptic and functional properties of foods. A protective effect against the discoloration of

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doi:10.1016/j.talanta.2011.03.050

paprika samples over time was highlighted for rosemary extracts containing rosmarinic and carnosic acids [3].

The introduction of rosemary ethanol extracts to the products of wiener-type and liver sausages limited lipid oxidation better than direct addition of the antioxidant to both meat products [4]. A synergistic antioxidant effect between an extract from rosemary leaves and BHT and a synergistic interaction with BHA to inhibit *Escherichia coli* and *Staphylococcus aureus* growth were demonstrated. Therefore, rosemary not only enhances the antioxidant efficiency of BHA and BHT, but also the antibacterial effect of BHA, allowing a decrease from 4.4- to 17-fold in the amounts of the synthetic compounds used [5].

Currently, considerable and renewed scientific interest is directed toward the rosemary plant and its various health properties. Protection exerted by carnosol against induced oxidative stress was highlighted on the liver in rats [6,7]. Anti-angiogenic potential of rosmarinic acid relating to its antioxidant properties [8] and its ability to suppress retinal neovascularization in a mouse model of retinopathy were recently pointed out [9]. These latter findings suggest this molecule could be used in the treatment of vasoproliferative retinopathies. Recently, by *in vitro* test on hepatic stellate cells, rosmarinic acid showed antifibrogenic effects [10]. Administration of a dose of rosemary leaf extract (200 mg/kg BW) was effective to limit weight gain induced by a high-fat diet and protected against obesity-related liver steatosis in mice [11]. Supplementing the diet with supercritical fluid rosemary extract, containing 20% carnosic acid, reduced oxidative stress in aged rats [12]. Long-term dietary administration of ground rosemary at a 1% (w/w) level in the diet improved the antioxidant status of rat tissues following carbon tetrachloride intoxication [13].

In light of all this scientific evidence, it could be of considerable interest to have a method suitable to well characterize and quantify all the phenolic constituents of the leaf and to evaluate the factors that can affect the chemical stability of these components.

The aim of our work was to improve knowledge in this field by optimizing the extractive and analytical procedure, working on natural rosemary populations from Tuscany. The efforts have been focused to develop an extractive procedure to exhaustively recover and quantify the antioxidant phenolic components, mainly rosmarinic acid, carnosic acid and its analogous, together with all the flavonoids, both from fresh and dried rosemary leaves. Within this research the chemical stability of the main phenols, depending on the storage condition, the different drying procedures and the extraction solvent, have been evaluated.

2. Materials and methods

2.1. Samples

Mature foliar tissue samples were collected from plants growing at Montebenichi (Firenze). Aliquot samples of fresh leaves were dried in an oven at 105 °C, by a freeze drier and at room temperature in the dark for several days. Fresh leaves were also stored at −22 °C for some weeks before extraction.

2.2. Extraction procedure

The leaves (5 g as fresh material and 2.5 g as dried sample were derived from 10 young twigs each of 8–10 cm length) were ground in liquid nitrogen and extracted as summarized in Fig. 1.

Liquid/liquid extraction (two steps) with hexane (1:1, v/v) was applied mainly to remove part of the chlorophylls. The residual ethanol solutions were directly analyzed by HPLC/DAD/ESI.

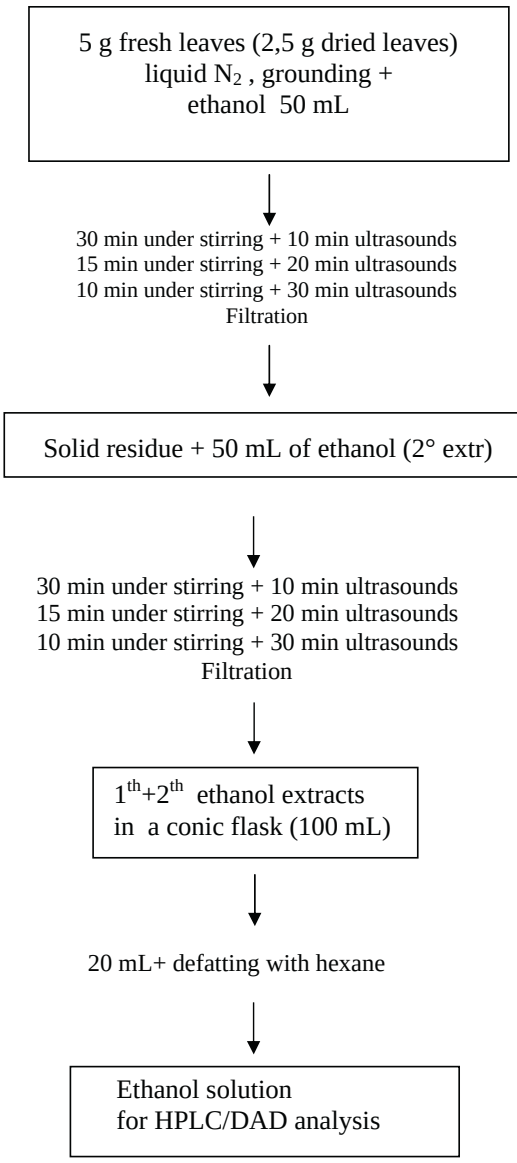


Fig. 1. Extractive scheme applied to recover the phenolic constituents of rosemary leaves. § for the extraction from dried leaves about 3% of water was added before the defatting with hexane.

2.3. HPLC/DAD/ESI analyses

The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector coupled to a HP 1100 MSD mass spectrometer with an API/electrospray interface (all from Agilent Technologies, Palo Alto, CA, USA).

A 150 mm × 3.9 mm i.d., 4 μm Fusion, RP18 column (Phenomenex, USA) equipped with a precolumn of the same phase was used. The mobile phases were (A) 0.1% formic acid/water and (B) CH_3CN. The multi-step linear solvent gradient used was: 0–15 min 15–25% B; 15–25 min, 25–35% B; 25–35 min 35–50% B; 35–40 min 50–100% B with a final plateau of 8 min at 100% B; equilibration time 10 min; flow rate 0.4 mL min^{-1} and oven temperature 26 °C; injection volume 5 μL. The UV/vis spectra were recorded in the range 200–500 nm and the chromatograms were acquired at 240, 284, 330 and 350 nm. After every four injections a wash method with 100% isopropanol was applied for several minutes to remove traces of liphophilic compounds from the column.

The mass spectra were recorded in negative and positive ion mode, setting the fragmentation energy between 80 and 180 V

Table 1
Extractive methods proposed to recover the phenolic constituents from rosemary leaves.

Selected amounts (f: fresh; d: dried)	Ratio (mg/mL)	Extractive mixture	Steps[a]	Compounds	References
1 g/15 mL (f)	67	CH_2Cl_2/ethanol 75:25	1	Carnosic acid, other diterpenoids, cirsimaritin, genkwanin	22
800 mg/100 mL (d)	8	Methanol	1		20
20 mg/mL (d)	20	DMSO	1	Phenolic acids and flavonoids	23; 24
50 mg/45 mL (d)	1.1	Ethanol/H_2O 3:7 and ultrasounds[b]	2	Rosmarinic and caffeic acids	14; 15
10 g/100 mL (d)	100	Ethanol and ultrasounds[c]	1	Rosmarinic and carnosic acids	21
50 mg/25 mL	2	Methanol and ultrasounds		Rosmarinic and carnosic acids, carnosol	17
500 mg/20 mL (f)	25	Methanol	4	Flavonoids, rosmarinic and carnosic acids	18
500 mg/20 mL (d)	25	Methanol	4		
1 g/45 mL (f)	22.2	Methanol	3	Flavonoids, rosmarinic, carnosic acids	19
500 mg/40 + 10 mL		Methanol/H_2O 3:7 and acidic water	1	Phenolic acids, rutin, quercetin, luteolin, rosmarinic acid	16

[a] Number of successive extractive steps.
[b] Ultrasounds for 10 min at 40 MHz.
[c] Ultrasounds for 45 min at 40 MHz.

and applying the same chromatographic conditions as described previously. The mass spectrometer operating conditions were: gas temperature, 350 °C; nitrogen flow rate, 9 L min^{-1}; nebulizer pressure, 30 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V. All solvents used were of HPLC grade; CH_3CN was from E. Merck (Darmstadt, Germany).

2.4. Quantitative evaluation

The quantitative evaluation of the main constituents was performed through the use of two external standards, rosmarinic acid at 330 nm and carnosic acid at 284 nm. The first compound was used at 330 nm, to quantify also all the flavonoids, while the second one at 284 nm to determine all the other diterpenoids. The calibration curve of rosmarinic acid (Sigma–Aldrich) was in a linearity range between 0.1 μg and 9.4 μg with a r^2 0.9999; the calibration curve of carnosic acid (Sigma–Aldrich) was in the linearity range of 0.05–3.4 μg with r^2 0.9998.

2.5. Statistical analyses

Data were not normally distributed (Kolmogorov–Smirnov one sample test) and were analyzed by the non-parametric Kruskal–Wallis ANOVA followed by the Mann–Whitney *U*-test for multiple comparisons using SYSTAT 12.0 software (Systat Software Inc., Richmond, California, USA). Differences were accepted when significant at the 5% level.

3. Results and discussion

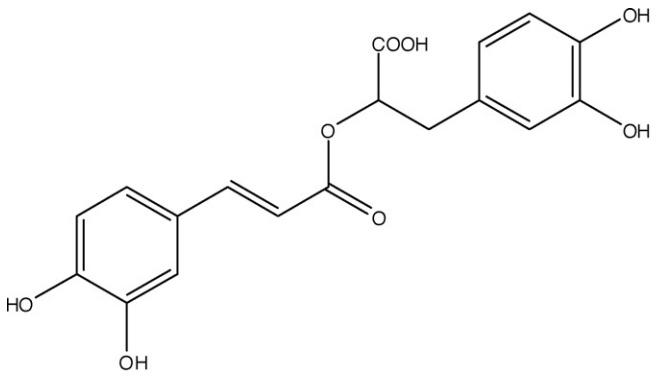
3.1. Extraction process

As summarized in Table 1, to recover the phenolic fraction from rosemary leaves, several procedures have been described proposing different times of extractions, solvents and weigh/volume ratios. Hydro alcoholic mixtures in various proportions [14–16], or methanol [17–20] and ethanol alone [21] have been proposed as suitable media to recover the main phenolic constituents from leaves. A small number of reports indicate the use of other solvents, such as CH_2Cl_2/EtOH, mainly for the diterpenoidic fraction [22], or DMSO [23,24], a solvent not easily to remove in case of concentration of the sample. Moreover, as highlighted in Table 1, the total amount of leaves, and the applied ratios between solvent and raw matrix varies widely, ranging between 20 mg and 10 g and between 1.1 and 100 mg/mL, respectively. In addition, several of these studies focus only on recovering a part of the complex phenolic fraction of the leaf. In Fig. 2 are shown some chemical structures of the main phenolic constituents of this plant. To date, a unique extractive method, suitable to simultaneously and efficiently recover all these components with high yields, has not yet been proposed.

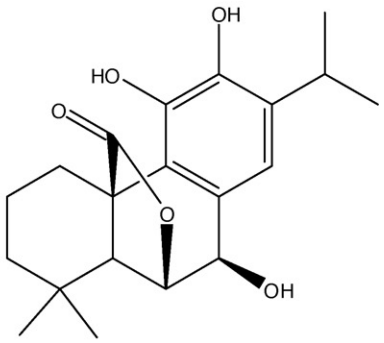
With the objective of obtaining a representative sample and after some preliminary tests (data not shown), 5 g of fresh leaves and 2.5 g of dried sample were chosen to carry out the extraction process. To select the extractive mixture, several factors were taken into account: (i) the need to use a solvent that would guarantee an adequate chemical stability of the target molecules with good extraction yields of all the components; and (ii) the willingness to use a non-toxic mixture suitable also for future applications in the food field. In light of these requirements, only ethanol and ethanol–water mixtures were chosen to carry out the extraction tests within this work. Moreover, it is worth noting that, mainly to improve the extraction yields of rosmarinic and carnosic acids, several previous works have already opted for alcoholic mixtures (Table 1).

Considering the co-presence in the leaf both of polar and non-polar phenols, it was decided to first operate with ethanol mixtures containing water but in a percentage of not over 50%. Preliminary tests on fresh leaves carried out with ethanol/water (1:1) or ethanol for the first extraction and ethanol/water (1:1) for the next step, did not give encouraging results. In fact, if compared with the yields reached using only ethanol for all the extractive steps, lower amounts of rosmarinic acid and a rapid degradation of carnosic acid toward its oxidized forms, were obtained with the use of hydro-alcoholic mixtures.

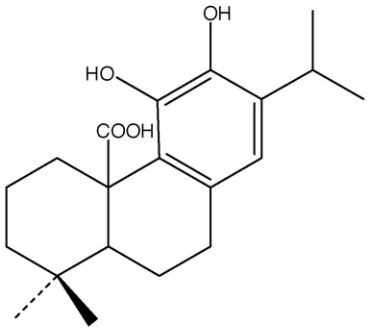
In literature it is reported that in water media the carnosic acid rapidly degraded toward the oxidized form of carnosol quinone [25] and in methanol it is converted toward carnosol [26] with a consistent decrease after only 24 h at room temperature [27]. Due to the lack of data in ethanol, some tests were done in this solvent to verify the stability over time not only of carnosic acid but also of the other phenols. To this purpose firstly the two standard, carnosic and rosmarinic acids, were dissolved in ethanol at different concentra-



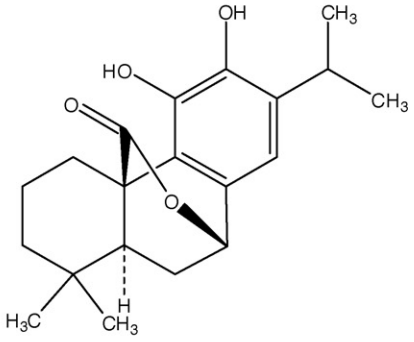
Rosmarinic Acid



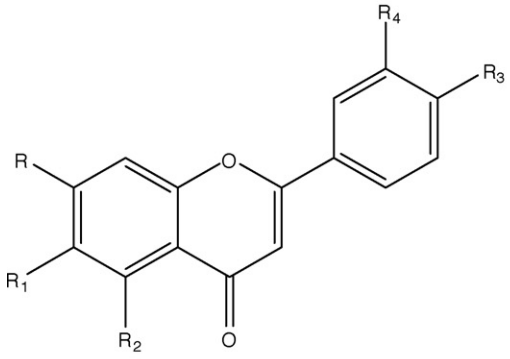
Epirosmanol



Carnosic acid



Carnosol



Esperidin-7-O-rhamnoglucoside

	R	R_1	R_2	R_3	R_4
Esperidin-rhamnoglucoside	O-glu-rha	H	OH	OCH_3	OH
Cirsimaritin	OCH_3	OCH_3	H	OH	H
Genkwanine	OCH_3	H	OH	OH	H
Luteolin O-glucoside	OH	H	OH	OH	O-glu

Fig. 2. Chemical structures of the main phenolic constituents of rosemary leaves.

tions (0.29 mg/mL and 0.94 mg/mL), and the solutions treated with ultrasound for more then 2 h, stored at room temperature or at −23 °C for different times and then analyzed by HPLC/DAD/MS. The carnosic acid remained unaltered up to 48 h at room temperature in dark so guarantying the possibility to make automated quantitative analysis by an autosampler. After 3 months at −23 °C the carnosic acid in ethanol solution was reduced of 17% obtaining carnosol as main oxidated derivative. Almost the same behavior was observed in ethanol extracts from rosemary leaves. The analyses of several samples after 3 months at a −23 °C, highlighted increments of the

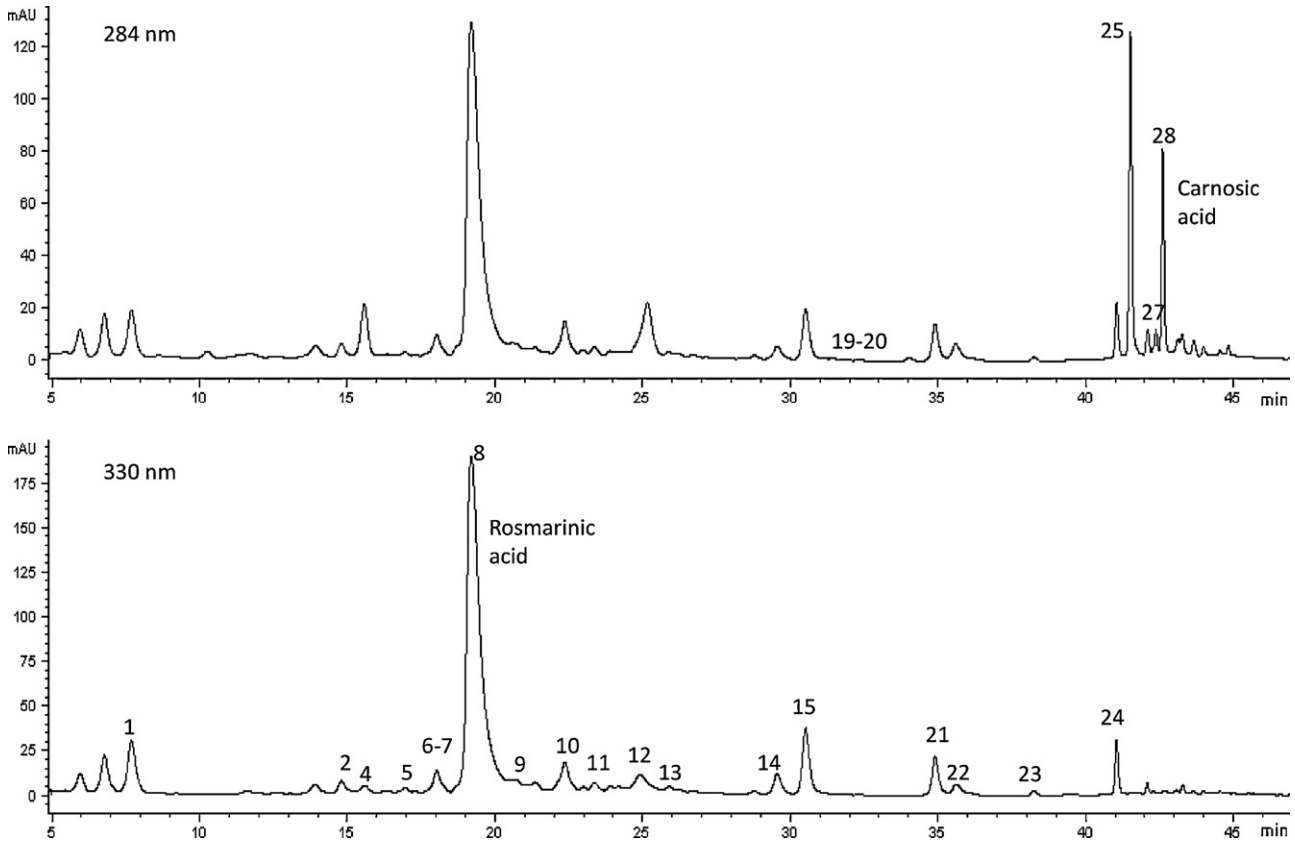


Fig. 3. HPLC profiles at 284 nm and 330 nm of an ethanol extracts from fresh leaves.

oxidized derivatives in the ranges 14–18% for carnosol; with traces for rosmanol, and only a reduction up to 20% of the initial amount of carnosic acid. The other phenolic constituents of the leaves, the rosmarinic acid and the flavonoidic fraction, remained almost unaltered in ethanol solution up to 3 months at −23 °C.

It is known that sonication, which contributes to destroying cellular structures, guarantees a better penetration of the extractive solvent within the raw matrix. Since the rosemary leaves are fibrous and with a leathery texture, to increase the yields and to optimize the extraction time, the use of an ultrasound bath was inserted in the procedure, also according to that suggested by other authors [14,21]. Using a ultrasound bath at 35 KHz and not 40 KHz as previously applied, extraction times longer then 45 min were tested maintaining the water temperature below 50 °C. The coupled use of stirring and ultrasounds during the extraction has been inserted.

Due to the non-availability of an instrument able to apply, at the same time, these two conditions, it was elaborated the extraction procedure summarized in Fig. 1. This optimized procedure includes alternatively using magnetic stirring and ultrasounds with a total time of 115 min for each extraction.

Preliminary shorter times of extraction (by about half) were also applied highlighting a consistent decrease of the extractive yields. The amounts of rosmarinic acid were about four times lower and of the total flavonoids about two times lower with respect to those obtained applying longer extraction times.

The choice to use only two consecutive extractions for each fresh or dried sample was determined by the evidence that about 20–30% of the main phenolic constituents (rosmarinic acid and carnosic acid with its analogues) remained in the leaves after the first extractive step, while less then 5% of the total phenolic amount was recovered applying a third consecutive extraction.

3.2. Identification of the phenolic constituents

The analytical HPLC method was optimized with the aim to obtain a good chromatographic separation for all the numerous components of the ethanol extract. After preliminary tests on different reverse phase columns, the choice fell on the Fusion® column. The multistep elution method was suitable to separate more then 28 different components (Fig. 3), among them the rosmarinic acid, numerous minor flavonoids, either as aglycones and glycosides, and the diterpenoidic constituents (Table 2), within a total time of 45 min.

The rosmarinic acid, easily identified by comparison with the standard, is always the main component of the phenolic fraction. Among the diterpenoids, the presence of carnosic acid was confirmed by comparison with the pure standard and a very good response of the mass detector both in negative and positive mode was highlighted. As discussed in the next paragraph, several oxidation products were obtained maintaining this standard in ethanol solution for several days; this sample was then used as reference to detect and identify the same diterpenoids in the rosemary extracts.

The mass spectra of carnosic acid and carnosol resulted diagnostic both in positive and negative ionization mode as clearly shown in Fig. 4a and b. The positive ionization allowed to obtain the $[M+H]^+$ and the $[M+Na]^+$ ion species for both the compounds and the fragments related to the loss of the carboxyl group. In negative ionization mode the most intense and diagnostic ions were the $[M-H]^-$ and the $[M-H-CO_2]^-$ ion at m/z 287, and m/z 285 for carnosic acid and carnosol respectively.

In agreement with previous data that highlighted close retentions times for the two isomers, rosmanol and epirosmanol [18], and in absence of suitable pure standards, they were indentified mainly by their UV–vis and mass spectra. Appling a negative

Table 2
List of phenolic compounds tentatively identified by their Rts, UV–vis and mass spectra and by the use of standards. Glu, glucose; gluc, glucuronic acid; rha, rhamnose.

	Compounds	Rts	mw (aglicone)	MS (−)	MS(+)	References for flavonoids
1	Caffeic acid	7.7	180	135, 179	181	
2	Flavonoid monoglicoside	13.4	478 (316)	315, 477	317, 479	
3	Apigenin rha-glu	15.5	578 (270)			23
4	Esperidin rha-glu	15.8	610 (302)	301, 609		24
5	Diosmin rha-glu	17.4	608 (300)	299, 607		19
6	Luteolin 7-O gluc	17.9	462 (286)	285, 461		28
7	Ispidulin 7-O glu	18.5	462 (300)	461		23
8	Rosmarinic acid	18.8	360	161, 197, 359, 719		
9	Flavonoid diglicoside	20.8	640 (316)	639		
10	Cirsimaritin O-glu	22.0	476 (316)	315, 475		
11	Flavonoid diglicoside	23.0	654 (316)	653		
12	Isoscutellarein 7-O-glu	23.1	462 (286)	285, 461		23; 24
13–18	Flavonoids	24–31				
19, 20	Rosmanol/epirosmanol	34.4–35.1	346	283, 345		
21	Cirsimaritin	35.2	314	313		18; 28
22	Flavonoid	35.7			315	
23	Genkwanine	38.4	284	283	285	18; 23; 24
24	Flavonoid	41.2	–	–	329, 351	
25	Carnosol	41.6	330	285, 329, 659	331, 353	
26	4′Metoxytectochrysin	42.2	298		299	18
27	Carnosic acid derivative	42.4	374		375, 397	
28	Carnosic acid	42.8	332	287, 331		
29	Methyl carnosate	43.3	346	287, 331, 287		

ionization, the mass spectrum of rosmanol/epirosmanol at higher fragmentation energy, showed the $[M-H]^-$ ion at *m/z* 345, the $[M-H-CO_2]^-$ ion at *m/z* 301 and $[M-H-CO_2-H_2O]^-$ specie at *m/z* 283. Moreover the loss of a water molecule observed only for these compounds within the diterpenoidic fraction, can be attributed to a dehydratation process involving the free OH group linked on carbon C7.

Even if in traces amount the presence of methyl carnosate was confirmed by its mass spectrum with the $[M-H]^-$ ion at *m/z* 345, the specie related to the loss of the methyl group at *m/z* 331, and the ion at *m/z* 287 attributable to the loss of the CH_3–COO group, typical also of the mass spectrum of carnosic acid. This metabolite was also detected, as impurity, in the commercial standard of carnosic acid.

According to literature [18,23,24,28] several flavonoidic derivatives, were detected in our extracts, even if in lower amount if compared to the main constituents, the diterpenoidic fraction and the rosmarinic acid. The applied methods allow to separate and

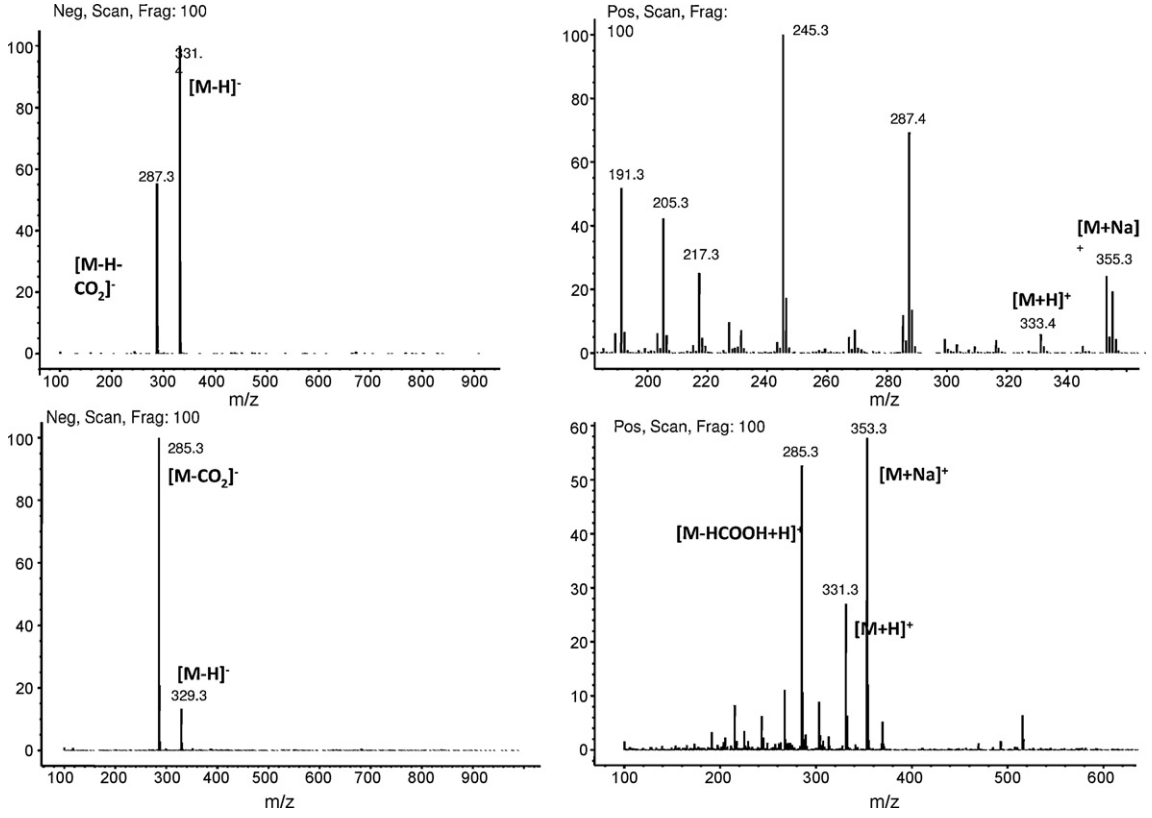


Fig. 4. Mass spectra in positive and negative ionization mode of (a) carnosic acid and (b) carnosol.

Table 3
Amount of phenols (μg/g dried weight) for fresh and frozen leaves harvested in February. The values are a mean of three different extractions of the same batch of an adult plant.

	Fresh		Frozen		
	μg/g	SD	μg/g	SD	
Flavonoid monoglu.	205.15	17.51	189.94	3.57	NS
Esperidin rha-glu	102.11	8.71	113.15	2.36	NS
Ispidulin 7-O-gluc	420.01	22.09	365.68	21.42	*
Rosmarinic acid	12349.83	1321.14	3520.84	604.67	*
Cirsimaritin glu.	573.93	47.57	473.91	41.60	*
Flavonoid diglu	116.56	5.44	82.74	3.09	*
Isoscutellarein 7-O-glu	444.51	14.15	474.43	24.27	NS
Flavonoid	109.03	7.14	94.59	2.36	*
Flavonoid	58.77	1.09	50.78	3.22	*
Flavonoid	376.66	9.30	326.51	31.24	*
Flavonoid	1088.47	57.51	828.49	56.22	*
Cirsimaritin	581.47	70.19	525.97	14.86	NS
Flavonoid	202.63	40.90	167.78	16.14	NS
Genkwanine	88.54	3.30	81.71	2.36	NS
Flavonoid	423.15	34.29	461.03	13.86	*
Total flavonoids	4790.98	280.2	4236.71	186.16	*
Carnosolo	11283.96	129.22	12894.43	872.79	*
Carnosic acid	14240.19	253.06	14562.51	338.33	NS
Total Terpenoids	25524.15	273.95	27456.94	1030.27	NS
Total phenols	42664.97	1372.8	35214.49	1419.89	*

* $P < 0.05$.

detect an higher number of flavonoidic structures with respect to those previously reported [18,23,24,28].

These molecules were recognize to belong to the class of flavones or flavonols mainly by their characteristic UV–vis spectra with two main bands between 260–270 nm and 335–345 nm.

Among these constituents the aglycones cirsimaritin and genkwanine showed mass spectra characterized only by the presence of the $[M-H]^-$ ions with 100% of intensity. Other glycosilated forms were also detected, among them ispidulin, cirsimaritin and isoscutellarein monoglucosides and some minor diglicosides, all showing diagnostic fragmentation in negative ionization mode (Table 2).

3.3. Effects of freezing and drying processes on the phenolic fraction

In this paragraph we discuss the main factors that strongly modify the phenolic amount in the ethanol extracts, particularly when obtained from fresh or frozen leaves. The following data were obtained working on two batches of rosemary leaves harvested from the same plants. The sample from February was selected to evaluate the consequences of freezing/thawing processes, while the other one from September was used to study the effects of drying processes.

Moreover, with the aim to propose a mode of quantization easy to be applied only the two main constituents, available as commercial standards, carnosic and rosmarinic acids, were selected as external standards; for all the flavonoids again was chosen the rosmarinic acid.

In the ethanol extract from frozen leaves the concentration of rosmarinic acid was dramatically lower with respect to the fresh leaves, and all the minor flavonoids were better highlighted. Frequently when a large number of fresh samples are handled, the sample plant material is frozen to guarantee its stability over time. For rosemary leaves it is easy to observe a rapid and marked browning of the surface of the leaf during thawing. In fact, even if the frozen sample had been handled within a few minutes (e.g. not more than 5 min), before the addition of the extractive solution a partial browning could not be avoided and a significant decrease of rosmarinic acid ($\chi^2 = 3.857$, $P < 0.05$) between 65% and 80% was measured (Table 3). This phenomenon must be taken into account when a quantitative determination of this phenol in rosemary leaf is done. The enzymatic browning can be related to the endogenous enzymes that, during and after the thawing, presumably oxidize the two catecolic groups of rosmarinic acid. This aspect has not yet been sufficiently emphasized in the scientific literature to date. In fact, despite these problems, freezing has been recently reported as a method to store, over time prior to chemical analysis, aromatic plants including rosemary [16]. In agreement with our results, these authors observed that the antioxidant activity of all the extracts from frozen leaves was considerably lower when compared with those from dried leaves. This evidence can be mainly related to the oxidation of rosmarinic acid.

At the same time, in the extract from frozen leaves also the total flavonoidic content decreased significantly but not more than 11.5% (Table 3). The extracts from fresh and frozen leaves did show only little differences in the terpenoidic fraction, and the relative distribution within the different constituents was almost unchanged as well (Fig. 5).

Due to the loss of phenols induced by freezing of the leaves, drying processes under mild conditions were hypothesized as alternative methods to maintain the raw sample over time. Toward this aim two procedures were tested: freeze drying and drying at room temperature for several days in the dark. The optimized extraction procedure was then applied to evaluate the phenolic content in dried and freeze-dried leaves with respect to the fresh sample, all obtained from the same batch of adult Tuscan plants (Table 4).

Observing the quantitative results, particularly those summarized in Table 3, an high variability of the phenolic content is pointed out. Nevertheless, also some recent works, focused to evaluate these compounds in fresh rosemary leaves, often highlight an high variation of the quantitative data. Almela et al. [18] have shown RSD values up to 14% for rosmarinic acid, 25.6% for carnosol and 18.6% for carnosic acid. Other authors, even applying a completely different extraction procedure such as a supercritical fluid extraction, have had similar findings, with RSDs between 3.01% and 13.5% for rosmarinic acid [29]. Differently from the fresh leaves, the data related to the dried samples, particularly for the freeze dried leaves (Table 4) have shown lower variability.

The rosmarinic acid content did not vary significantly after lyophilization, while Mann–Whitney *U*-test showed a significant variation between fresh and dried leaves ($\chi^2 = 4.257$, $P < 0.05$) and

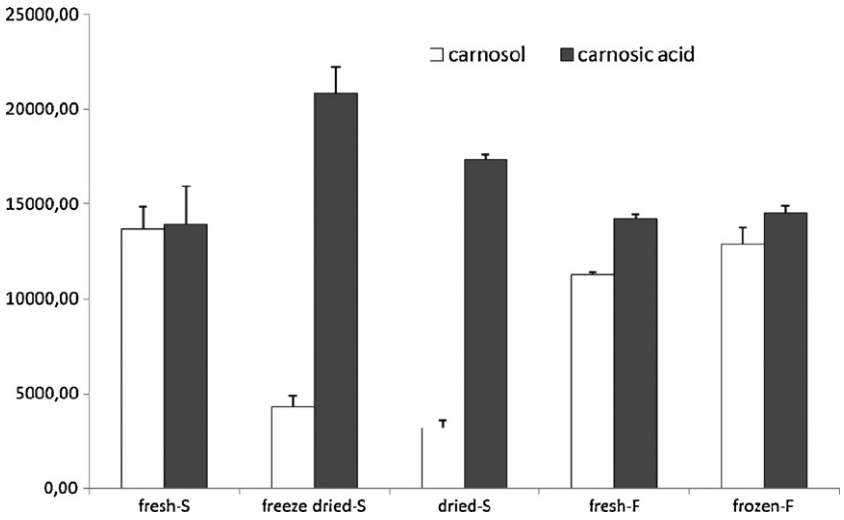


Fig. 5. Distribution of the different diterpenoids in ethanol extract from fresh, dried and frozen leaves. The data, expressed as μg/g dried leaves, are a mean of three determinations. S = leaves harvested in September; F = leaves harvested in February.

between freeze-dried and dried leaves ($\chi^2 = 3.857$, $P < 0.05$). The total flavonoids showed a significant decrease, close to 34% and 53% in the freeze-dried and dried leaves respectively, with the highest losses observed for some glycosilated forms. The total diterpenoidic content did not show significant variations between the different treatments but the contents of carnosol and carnosic acid strongly varied. The highest amount of carnosol was detected in fresh leaves and Mann–Whitney *U*-test showed significant variations between fresh and dried leaves, freeze-dried and dried leaves and fresh and freeze-dried leaves (Table 4). Fresh leaves showed the lowest content of carnosic acid and significant variations were detected between fresh and dried leaves ($\chi^2 = 3.857$, $P < 0.05$), freeze-dried and dried leaves ($\chi^2 = 3.857$, $P < 0.05$) and fresh and freeze-dried leaves ($\chi^2 = 3.857$, $P < 0.05$) as also showed in Fig. 5.

Our previous results obtained on leaves collected in different periods confirmed this trend: the carnosic acid/carnosol ratio ranged between 0.6 and 1.8 for the fresh sample, while it increased up to 4.9–8.4 for dried samples. This phenomenon can be related to the higher water content in the fresh leaves, close to 60% (w/w) in our samples. Previous data pointed out that the water content of fresh rosemary leaves ranges usually between 40–60% by weight, while the dried matrix contains only a residual humidity near 5% (w/w) [21]. The moisture of the fresh sample can modify, particularly during the first extractive step of our method, the composition of the extractive mixture reaching a concentration near 6% (v/v). In light of our results, it appeared that also these low amounts of water seems to be critical to induce the oxidation of carnosic acid toward carnosol during the extraction process.

The water content of the sample strongly influence the variability of the data: working on other rosemary dried leaves, also with very low content of phenolic constituents, the RSD values obtained from freeze dried samples were always below 5.14% (data not shown).

Furthermore, these preliminary findings pointed out that the phenolic profiles in fresh foliar tissue remain almost stable between samples collected on September and February (Table 4), with the only exceptions being the total flavonoids that showed an higher amount in the sample of September (Fig. 6). Within this class, almost all the flavonoids showed significant variation between these two sampling dates, particularly all the glycosilated forms

Table 4
Amount of phenolic constituents (μg/g dried weight) in fresh, freeze dried and dried leaves harvested in September. The values are a mean of three different extractions of the same batch of an adult plant. Different letters mean significant differences between the samples based on Mann–Whitney test.

	Freeze					
	Fresh	SD	Freeze Dried	SD	Dried	SD
Flavonoid monoglu	964.40c	40.12	589.55b	3.39	420.99a	58.39
Esperidin rha-glu	605.71	38.81	437.64	5.33	456.41	60.74
Ispidulin 7-O-gluc	1298.29c	92.59	674.46b	21.47	514.02a	7.99
Rosmarinic acid	6611.61b	1069.85	6302.35b	183.29	3214.24a	319.25
Cirsimaritin glu.	1137.07	24.77	540.90	14.54	512.74	66.57
Flavonoid diglu	1332.95c	109.86	426.54b	1.96	387.28a	0.00
Isoscutellarein 7-O-glu	597.12c	4.42	462.81b	7.13	414.59a	1.48
Flavonoid	600.16	8.49	467.51	2.66	479.45	22.65
Flavonoid	549.08c	9.93	409.90b	0.74	NQ	–
Flavonoid	649.85c	16.83	429.53b	4.43	NQ	–
Flavonoid	1563.78c	84.04	711.16b	22.65	387.28a	0.00
Cirsimaritin	1399.63b	60.55	1213.40b	59.07	1059.78a	36.08
Flavonoid	591.82	8.77	566.08	6.44	549.86	11.16
Genkwanine	730.65	28.44	675.74	33.24	638.62	13.57
Flavonoid	1291.09	79.48	1197.18	81.31	1055.94	19.26
Total flavonoids	13311.58c	509.77	8802.39b	208.01	6255.17a	75.78
Carnosol	13717.45c	1131.95	4328.34b	586.50	3190.83a	394.04
Carnosic acid	13951.31a	1977.71	20863.83c	1341.36	17307.04b	343.71
Total Terpenoids	27668.77	2810.73	25192.17	870.14	20497.86	67.28
Total phenols	47591.96c	4164.01	40296.9b	1242.02	29967.28a	400.3

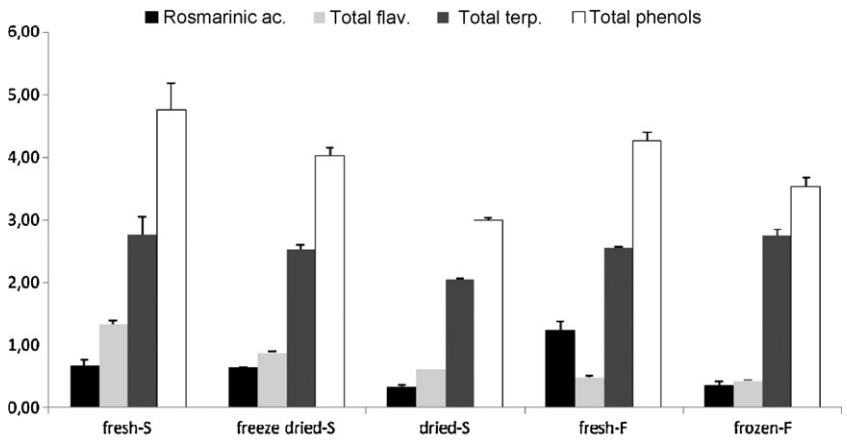


Fig. 6. Percentage values of the phenolic constituents in dried leaves after different treatments. Data are a mean of three determinations. S = leaves harvested in September; F = leaves harvested in February.

and particularly the genkwanine is reduced up to eight times in the sample from February.

The leaves harvested in September, richer in flavonoidic compounds with respect to those collected in February (see Tables 3 and 4), can be used for further researches to isolate and characterize the minor unknown flavonoids of rosemary.

4. Conclusions

When it is impossible to extract the fresh material and to limit losses of the phenolic content, the freeze dried process is often applied as the best method for storing the sample before analysis. The findings from this study have demonstrated that storing the raw fresh material in the freezer is not appropriate for rosemary, mainly due to the rapid disappearing of the rosmarinic acid presumably involving the phenoloxidase activity. Further research is needed to evaluate the roles of these endogenous enzymes in determining the oxidation of this compound during the freezing/thawing process. Regarding the flavonoidic fraction, consistent decrements, were highlighted in the dried samples at room temperature if compared with the fresh leaf. Differently from rosmarinic acid, limited losses were pointed out in the extracts from frozen leaves. Rosmarinic acid, appeared very sensitive also to mild drying processes. Regarding the total diterpenoidic content this undergoes to little changes when the leaves are freeze dried or frozen and limited losses are observed working on dried leaves at room temperature. Nevertheless it can be taken in account that this fraction is very sensitive to the water presence during the extraction that favors the conversion of carnosic acid toward it oxidized form carnosol. From our findings, it appear evident that when evaluating the phenolic content in rosemary leaves, several factors, mainly the phenoloxidases activity in the fresh and frozen leaf, the drying process and the extraction methods, should be carefully taken into account because they can induce partial losses or modification of the antioxidant components.

It has been pointed out, that the highest variation of the quantitative data is related to the treatment of fresh leaves and to the role of endogenous oxidative enzymes in presence of water, while this problem does not exist working on dried leaves. Despite the variability observed in the fresh samples, the statistic evaluation has pointed out, in any case, some macroscopic differences as statistically significant.

Moreover the proposed HPLC method has the advantage to detect up to 30 phenolic constituents belonging to different chemical classes. At the same time, it allows the quantitative evaluation of numerous compounds, among them several minor flavonoids. The method guarantees an analytical procedure suitable for detecting all the constituents that contribute to the antioxidant potency of the rosemary extracts in a relatively short time.

Acknowledgments

This work was partially supported by a contribution from ARSIA – Tuscany. Moreover, we are grateful to Ente Cassa di Risparmio di Firenze for supplying a part of the instrumentation used for this research. Finally a thank to Dr. Sandra Gallori for her technical assistance in preparing this manuscript.

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