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HPLC-DAD-ESI$^+$-MS PHYTOCHEMICAL PROFILES OF SEVERAL *ROSMARINUS OFFICINALIS* ACCESSIONS FROM SPAIN AS INFLUENCED BY DIFFERENT ENVIRONMENTAL STRESS CONDITIONS

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ABSTRACT. Rosemary, a native Mediterranean plant is a well-known source of phytochemicals with antioxidant activity attributed mainly to diterpenoids and flavonoids. The aim of the study was to establish an accurate evaluation of the rosemary metabolite profiles from several accessions under changing environmental conditions (water stress and soil salinity) comparing two sampling seasons (summer vs. spring) from four different habitats in Eastern Spain. The methodology was based on the identification and the quantitative evaluation of phytochemicals (phenolic acid derivatives, flavonoids, diterpenes and triterpenes) by HPLC coupled with diode-array detection and electrospray ionization mass spectrometry (ESI$^+$-MS). Phytochemical profiles were statistically compared by factorial ANOVA, cluster analysis, principal component analysis and univariate analysis (Pearson correlations), that allowed the discrimination between the extract composition in correlation to their habitat and stress conditions. Out of twenty-three compounds identified, the major ones were represented by diterpenoids (carnosic acid, carnosol and oxidized metabolites rosmanol, epirosmanol, rosmadial, rosmanol methyl ether) and flavonoids, which showed significant metabolic regulation induced by water stress. The main conclusion of the work is that the diterpene derivatives and their oxidized metabolites may be considered as optimal biomarkers of the environmental stress in *Rosmarinus officinalis*.

Keywords: *Rosmarinus officinalis; environmental stress; high performance liquid chromatography & mass spectrometry; metabolomic profile*

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Abbreviation list

DAD - Diode Array Detection
GC- Gas-chromatgraphy
LC- Liquid Chromatography
HPLC - High Performance Liquid Chromatography
ESI^+-MS - Electrospray ionization mass spectrometry
CA - cluster analysis
PCA - principal component analysis
MS – Mass spectrometry
ROS – Reactive Oxygen Species
AsA-GSH – Ascorbate- glutathion

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L., Lamiaceae family) is a woody herb that grows wild in the Mediterranean region, but is also largely cultivated in many areas worldwide as a tasty culinary spice. It has also well-known medicinal uses, such as antimicrobial [1-3] or neuroprotective [4, 5]. Rosemary's high medicinal interest is mainly due to its antioxidant potential brought by its high contents in terpenoids and phenolic derivatives [6-9]. Numerous scientific studies were performed on the chemical composition of rosemary. Advanced techniques, such as high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with diode array (DAD) or mass spectrometry (MS) detection, are currently used to separate and identify bioactive metabolites in rosemary extracts [10-12]. The major compounds were represented by phenolic diterpenoids (e.g. carnosic acid and carnosol, rosmanol), flavonoids (rosmarinic acid and luteolin derivatives, genkwanin or homoplantaginin) or pentacyclic triterpenoids (oleanolic, betulinic and ursolic acids) [13-16] besides monoterpenes (cineole, camphor and α-pinene). Ultrasound or microwave-assisted procedures have improved the extraction efficiency of rosemary components [15, 17,18]. An HPLC/DAD/MS study [19] showed that drying temperature, storage conditions and extraction procedures can affect the chemical composition of leaf extracts, especially the high antioxidant derivatives e.g. rosmarinic acid and carnosic acid [20, 21].

The phytochemical profile of rosemary was related to its phenological stage [22], geographic location [18, 23], seasonal variation [7] and abiotic stresses, such as drought [24], salinity [25] or photoxidation [26]. Under stressful conditions, including irrigation with effluents that contain high levels of salts and heavy metals, rosemary accumulates reactive oxygen species, which leads to the activation of antioxidant defence mechanisms [13]. Using accessions of rosemary, grown in different field trials and sites in Greece, it was shown that the phenolic chemical profile and the antioxidant behaviour of carnosic acid were affected by stress [27].

Environmental stress has a strong influence on the biosynthesis of bioactive compounds, by generation in excess of ROS, a group of free radicals, reactive molecules and ions derived from oxygen. ROS are normal by-products of cellular metabolisms but under conditions of abiotic and biotic stress the balance between their formation and scavenging is lost, and levels of ROS increase. As they are highly reactive and toxic, when in excess, they produce oxidative stress, affecting cells´ physiology and eventually leading to cellular death. To mitigate oxidative stress, organisms activate their enzymatic and chemic or non-enzymatic defence mechanisms. In plants, the first category is represented by several enzymes, mainly by those involved in the ascorbate–glutathione cycle (AsA-GSH) in chloroplasts, cytosol, mitochondria, apoplast, and peroxisomes. Non-enzymatic antioxidants, include diverse chemical compounds, such as ascorbic acid and E vitamin, carotenoids, reduced glutathione or phenolic compounds, especially the subclass of flavonoids [28]. The antioxidant enzymes represent 'the first line of defence against ROS', but under severe stress their activity may be insufficient for maintaining the ROS homeostasis. At this stage, the biosynthesis of secondary ROS scavenging systems (non-enzymatic compounds) is triggered [29]. As such, plants naturally growing in harsh environmental situations, should have efficient mechanisms to reduce the oxidative damage associated with situations of drought, increased salinity or other types of environmental stress. The role of phenolic compounds in plants is well-known [30, 31]. Out of the wide array of their biological functions, phenolics and especially flavonoids, participate in plant responses to practically all types of abiotic stress: UV radiation, intense light, extreme temperatures, mineral nutrient imbalance, anoxia, ozone exposure, drought, salinity, heavy metals and herbicides [30, 31-35].

In this study, we have analysed samples of rosemary collected in the wild, from different types of environments in South East of Spain. All habitats selected suffer the rigor of Mediterranean climate characterized by dry summers; the combination of drought, high temperatures, risk of salinity and high solar radiation induces a multiple stress, and plants tend to reduce their vegetative activity during this time. There are several well-known strategies developed by Mediterranean plants to with stand the restrictions of the summer stress, such as the syndrome of *Sclerophyllous* leaves or reduction of vegetative activity by the loss of leaves in summer. On the other hand, apart from these anatomic and physiologic adaptations, biochemical responses are triggered, such as osmotic adjustment or activation of antioxidant systems, both chemic and enzymatic, as response to the oxidative stress generated by the activation of ROS, with direct deleterious effects by oxidation of proteins, membrane lipids and nucleic acids. Previously, the content of total phenolics and flavonoids has been determined by spectrophotometric methods in different

plant species, including *R. officinalis*, from these Mediterranean habitats, and a significantly positive correlation between the level of these compounds and soil water deficit was established [36].

The present study aimed to establish phytochemical profiles of four *Rosmarinus officinalis* accessions, as influenced by different environmental growth conditions (two sampling seasons: in spring under favourable climatic conditions vs summer dry conditions**)** in Spain. The hypothesis of work is that the concentrations of main antioxidants will be correlated with environmental parameters, especially those related to the water deficit. The rosemary extracts were analysed by a combined, simultaneous HPLC-DAD and LC-ESI$^+$-MS procedure, in order to evaluate the phytochemicals' fingerprinting and the quantitative analysis of main compounds, followed by statistical analysis. The impact of the environmental stress was established by focusing on the variations of phenolic compounds and terpenoids.

RESULTS AND DISCUSSION

Phytochemicals profile and identification by LC-ESI$^+$-MS

Fig. 1 shows the general fingerprint of thirty-six superposed LC-ESI$^+$-MS chromatograms (peak area *versus* retention time) of rosemary extracts from the sampling groups. Based on their retention times, m/z values and main fragments twenty-three compounds were separated and tentatively assigned.

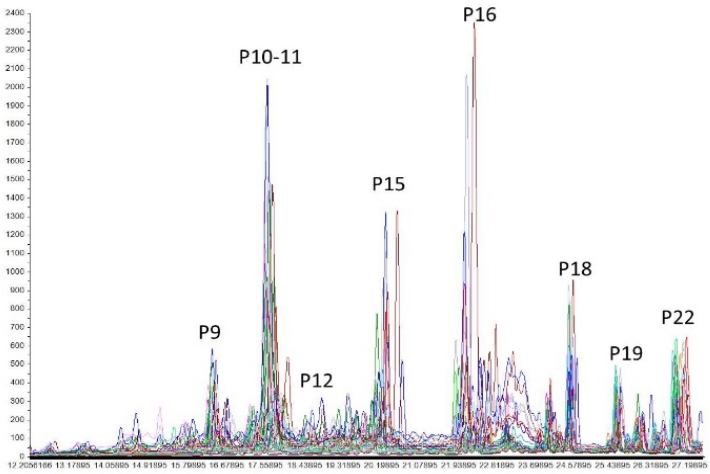

Figure 1. A general view of thirty six superposed LC-ESI$^+$-MS chromatograms (peak area *versus* retention time) obtained from eight sampling groups. The major compounds were marked as P9, P10-11, P12, P15, P16, P18, P19 and P22.

Table 1 includes the retention times (t_R), the specific *m*/*z* $[M+H]^+$ values, the main fragments derived from the MS analysis, and tentative identification, as compared with the literature data and data bases (Phenol Explorer, KEGG). Three categories of phytochemicals were identified: (1) diterpenoid ethers and other conjugates, (2) phenolic acid derivatives, and (3) flavonoids (luteolin derivatives).

Table 1. LC-ESI$^+$-MS data provided for rosemary extracts: retention time t_R (min)), m/z values used for ESI (+)MS identification, the main fragments and the identified compounds, in agreement with international data bases.

Name	t_R (min)	m/z $[M+H]^+$	Main MS Fragments	Tentative identification
P1	2.91	199.0510	181; 137	Syringic acid
P2	3.90	193.0553	129; 95	p-Coumaric acid ethyl ester
P3	12.02	465.0884	303; 287	Quercetin 3-*O*-glucoside
P4	12.64	523.1320	361; 325	Rosmarinic acid 3-*O*-glucoside
P5	13.49	479.1130	464; 317;302	Nepetrin (6-methoxy-luteolin 7-*O*-glucoside)
P6	14.29	611.1535	303	Hesperidin (Luteolin 7,3'-diglucoside)
P7	14.65	463.1089	285; 163	Homoplantaginin
P8	15.96	463.0798	287	Luteolin3'-glucuronide
P9	16.40	361.0772	199;181;163;137	Rosmarinic acid
P10	17.33	505.0905	401; 287	Luteolin 3'-(3''-acetylglucuronide) Isomer I
P11	17.74	505.0905	445; 287	Luteolin 3'-(4''-acetylglucuronide) Isomer II
P12	18.58	347.1707	303; 285	Rosmanol
P13	19.36	347.1707	285	Epirosmanol (isomer 1)
P14	19.73	285.0611	270	Genkwanin
P15	20.24	331.1863	287	Carnosol
P16	22.06	303.0427	229; 153	Quercetin
P17	24.03	317.1965	287	Rosmaridiphenol
P18	24.54	345.1707	317; 301; 285	Rosmadial
P19	25.66	473.1869	457	Benthamic acid
P20	26.15	361.1966	333; 287	Rosmanol methyl ether
P21	26.63	347.1863	333; 287	12-Methoxy carnosic acid
P22	27.12	333.1864	287	Carnosic acid
P23	27.59	455.1867	-	Micromeric acid

Nine compounds were selected as being major, based on the chromatogram peak intensity higher than 200 and signal/noise ratio of minimum 3 (Fig. 1). These compounds were tentatively identified as follows, P9 (rosmarinic acid), P10 and P11 (two isomers of luteolin acetyl glucuronide), P12 (rosmanol), P15 (carnosol), P16 (quercetin), P18 (rosmadial), P19 (benthamic acid) and P22 (carnosic acid) (Table 1). Fig. 2 shows the chemical structures of the main compounds.

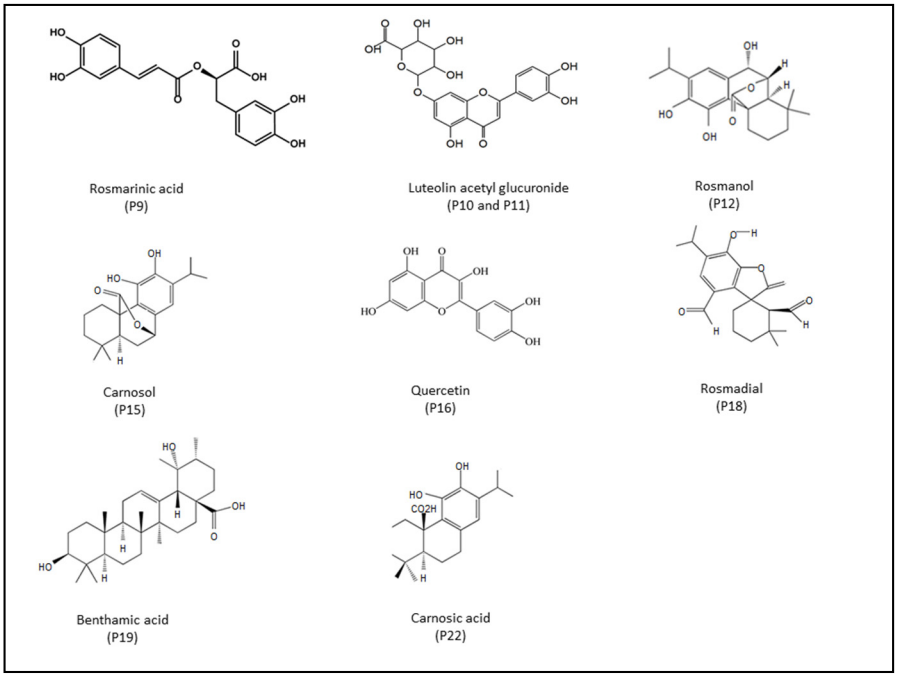

Figure 2. Chemical structure of the major compounds identified in the rosemary samples.

The first major class of compounds from diterpene family were carnosic acid (P22, m/z=333.1864), carnosol (P15, m/z=331.1863), methylcarnosate (P21, m/z=347.1863). Rosmanol (P12, *m/z* 347.1707) and its isomer epirosmanol (P13, *m/z*=347.1707) were formed by oxidative degradation of carnosic acid. Rosmaridiphenol (P17, *m/z* 317.1965, with a fragment of 287), rosmadial (P18, *m/z* 345.1707) and rosmanol methylether (P20, *m/z* 361.1965) were also identified. All these compounds have been previously reported also by other authors [37]. In addition, some minor triterpenes, namely benthamic (P19, *m/z* 473.1869) and micromeric acid (P23, *m/z* 455.1867) were also detected.

The phenolic acid derivatives were represented by rosmarinic acid (P9, *m*/*z* 361.0772), a caffeic acid ester of salvianic acid A and rosmarinic acid 3-*O*-glucoside (P4, *m*/*z* 523.1320S). Syringic acid (P1) and p-coumaric ethyl ester (P2), were also identified, but only as minor components.

Flavonoids such as glucuronic acid derivatives of luteolin, represented by luteolin 3'*O*-(3"*O*-acetyl) β-D-glucuronide (P10-isomer I-, *m/z* 505.0905) and luteolin 3'*O*-(4"*O*-acetyl) β-D-glucuronide (P11-isomer II, *m/z* 505.0905) were also identified. The MS analysis revealed their specific fragmentation and discrimination by fragments of *m*/*z* 410 and 445, respectively. Other minor luteolin derivatives were identified, e.g. nepetrin (P5, *m/z* 479.1130), hesperidin (P6, *m/z* 611.1535), homoplantagin (P7, *m/z* 463.0798), luteolin 3'-glucuronide (P8, *m/z* 463.0798) and genkwanin (P14, *m/z* 285.0611). Flavonols were also identified, including quercetin (P16, *m/z* 303.0427) and quercetin 3-*O*-glucoside (P3, *m/z* 465.0884), in agreement with other reported data [14, 17].

The major fragment, with *m*/*z* = 287, corresponded to glucuronic acid loss, as indicated by previous data found in the literature and databases. For nepetrin (P5) and quercetin 3-*O*-glucoside (P3), the fragment ions with *m*/*z* 317 and 302 were assigned to fragments arising from glucose loss. Similarly, hesperidin (P6) presented a major fragment ion at *m*/*z* 302 due to rutinoside loss.

Quantitative analysis by HPLC-DAD

Based on the preliminary profile evaluations, the flavonoid derivatives (P10, P11 and P16) were the major components of extracts, therefore, we used rutin as a representative flavonoid for the calibration curve. The mean concentrations of all 23 compounds were calculated from the calibration curve equation and expressed as micrograms rutin equivalent/g dry leaf.

Table 2 presents the mean values and standard deviations (x ± SD) of the 23 compounds, as determined by HPLC-DAD, after calibration with rutin. A large variability was observed between these compositions, inside an accession group but especially between the different accession groups, as can be seen for luteolin glucuronide isomers (P10 and P11), rosmarinic acid (P9), carnosic acid derivatives (P15 and P22), rosmanol (P12) and rosmadial (P18), quercetin (P16), and benthamic acid (P19).

Although some of the compounds show significant differences between areas; P7 homoplantagin and P23 micromeric acid, the greatest differences can be found in samples from different seasons. The phenolic acids rosmarinic (P9) and it derivate P4 rosmarinic acid 3-*O*-glucoside increase significantly in summer. Most of the flavonoids also increase significantly in summer time: P5 Nepetrin (6-methoxy-luteolin 7-*O*-glucoside), P6 Hesperidin (Luteolin 7,3'-diglucoside), P8 Luteolin3'-glucuronide, P10 and P11 Isomers I and II of Luteolin 3'-(3"-acetylglucuronide), P14 Genkwanin and P16 Quercetin.

Table 2- Mean values ±standard deviation of the quantified phytochemicals (P1 to P23) in the different areas (A) and sampling date (B- summer and spring). Significant differences by multiple comparison (Two way anova) at P < 0.05 : *, **. ***, significant at P<0.05, 0.01 and 0.001 respectively.

	Gypsum top		Gypsum bottom		Semiarid		Dune		A.	B.
Plot/ season	Spring	Summer	Spring	Summer	Spring	Summer	Spring	Summer		
P1	100 ±12	95 ±60	61 ±76	161 ±35	49 ±42	89 ±49	104 ±48	108 ±60	NS	NS
P2	117 ±54	152 ±18	115 ±66	143 ±20	139 ±21	107 ±64	131 ±19	120 ±69	NS	NS
P3	66 ±53	274 ±115	301 ±69	201 ±59	65 ±32	147 ±93	173 ±65	298 ±65	NS	NS
P4	83 ±42	303 ±243	119 ±83	298 ±95	77 ±72	304 ±56	178 ±99	219 ±96	NS	**
P5	77 ±36	329 ±185	105 ±55	334 ±92	101 ±43	286 ±58	237 ±92	286 ±94	NS	***
P6	84 ±40	378 ±373	70 ±21	432 ±98	79 ±54	168 ±54	328 ±99	388 ±89	NS	**
P7	62 ±12	275 ±171	450 ±97	478 ±78	149 ±86	562 ±67	608 ±98	873 ±97	*	NS
P8	164 ±87	723 ±306	731 ±76	948 ±87	417 ±78	749 ±65	549 ±76	672 ±65	NS	*
P9	165 ±54	1561 ±564	688 ±76	1391 ±372	516 ±68	1522 ±95	1565 ±78	1551 ±87	NS	***
P10	464 ±24	1118 ±521	807 ±369	1159 ±339	716 ±95	1240 ±543	996 ±294	1759 ±150	NS	*
P11	331 ±20	4730 ±337	1865 ±142	4145 ±165	831 ±90	4870 ±297	3681 ±255	4695 ±514	NS	**
P12	399 ±85	1418 ±102	1048 ±785	849 ±691	725 ±96	1844 ±120	1141 ±139	349 ±219	NS	NS
P13	146 ±15	693 ±485	455 ±203	890 ±419	496 ±25	1040 ±341	875 ±350	1336 ±695	*	**
P14	182 ±17	936 ±495	374 ±191	1122 ±335	361 ±22	911 ±481	912 ±488	1330 ±599	NS	***
P15	989 ±90	2483 ±180	982 ±44	1971 ±805	988 ±92	2104 ±974	2424 ±111	2645 ±157	NS	NS
P16	258 ±98	3399 ±392	322 ±144	2178 ±722	728 ±82	3924 ±401	2171 ±157	1839 ±226	NS	*
P17	507 ±64	747 ±319	539 ±72	773 ±323	639 ±73	765 ±122	482 ±192	515 ±276	NS	NS
P18	956 ±82	2094 ±606	496 ±140	1475 ±327	890 ±20	2062 ±237	579 ±243	1573 ±521	*	**
P19	998 ±92	727 ±311	1121 ±213	1042 ±321	985 ±91	1259 ±147	1072 ±371	785 ±76	NS	NS
P20	747 ±87	800 ±332	743 ±96	768 ±93	834 ±94	883 ±287	527 ±76	798 ±87	NS	NS
P21	821 ±95	492 ±301	584 ±83	800 ±95	73 7±94	718 ±115	449 ±86	667 ±89	NS	NS
P22	2296 ±427	2089 ±955	2854 ±343	1703 ±446	999 ±93	1551 ±464	1949 ±856	1799 ±345	NS	*
P23	425 ±23	394 ±177	437 ±89	566 ±83	512 ±90	635 ±236	397 ±32	410 ±20	*	NS

The water stress (summer drought) induced a significant increase of diterpenoids rosmadial (P18) in all plots, whereas carnosic acid (P22) had higher content in spring. Differences in compounds are more significant in the two plots subjected to greater water stress, that are one from the gypsum area, on top of the hill and the semiarid area.

In the dune area, these differences were not as significant. This pattern can be explained by a constant environmental stress in this habitat, independent on the season: similar salinity and low water retention of the sandy substrate.

When comparing the two plots in the gypsum area, the mean concentrations of these compounds are related to water stress. In spring, due to humidity and rains, the site at the bottom of the hill became more stressful for plants because the gypsum soil concentration (salt stress) was higher than at the top. Generally, non-significant effects of altitude were detected in summer time.

The concentrations of minor components ranged between 90 and 130 micrograms rutin eq./g dry leaf. Such components were represented by rosmarinic acid derivatives (P13, P17, P20), micromeric acid (P23), methoxycarnosate (P21) luteolin derivatives (P8, P14), glycosylated flavonoid derivatives (P3-P7) and phenolic acids (P1 and P2).

Comparing the concentrations of different phytochemical classes, the most significant differences were observed for flavonoids and rosmarinic acid derivatives, positively correlated with water stress.

Significant increases were noticed in summer compared with spring for flavonoid glycosides (P4-P6, P13, P17) in the gypsum zone and in the semiarid area but no differences were observed for triterpenes and diterpenes. The levels of the some compounds increased in summer in the dune sand, especially epirosmanol (P13) and genkwanin (P14). These data suggest that specific synthesis of flavonoids and terpenes, as well their oxidation is up-regulated by the environmental stress, in agreement with other data [38]. The most significant effects on rosemary metabolites from different accession groups studied here were mainly related to water stress (summer *vs* spring) and, to a lesser extent, to salt stress (sea *vs* gypsum).

The potent antioxidant properties of *R. officinalis* extract is attributed to its diterpene, carnosic acid, that under drought conditions scavenges free radical within the cloroplast giving highly oxidized diterpenes such as carnosol, rosmanol and isorosmanol [7, 13]. The antioxidant protection mechanism by carnosic acid is especially relevant in rosemary [13, 26]. In this context, our data reflected similar findings: not only an up-regulation of flavonoid synthesis is induced by water stress, but also a post-synthesis oxidation of carnosic acid to its metabolites (carnosol, methoxy carnosate).

Statistical correlations and significance of phytochemicals' modifications induced by environmental stress

The Cluster analysis (CA) allowed the identification of the similarities and discriminations between the different accession groups of rosemary. The Euclidian distance among the accession groups shows good discriminations between the profiles of samples A1, B1, C1, D1 collected in summer (water stress - dry season) and samples D2, B2, A2, B2 collected in spring (humid season).

Table 3. Pearson correlation factors (R) and their significance (S), considering the differences between the mean values (x ± SD) of phytochemicals' total concentrations found in accession groups. The significant correlations are bolded.

		A1	**B1**	**A2**	**B2**	**C1**	**C2**	**D1**	**D2**
A1	R	1	**0.9663**	0.2890	0.5726	**0.97551**	0.5568	**0.9043**	**0.9326**
	S	--	7.2E-14	0.1809	0.0043	2.6E-15	0.0057	3.2E-09	9.2E-11
B1	R	**0.9663**	1	0.2996	0.6264	**0.94429**	0.56064	**0.96137**	**0.9493**
	S	7.2E-14	--	0.1648	0.0013	1.3E-11	0.0053	3.0E-13	4.9E-12
A2	R	0.2890	0.2996	1	0.7804	0.2134	0.8909	0.2629	0.3310
	S	0.1809	0.1648	--	1.1E-05	0.3281	1.2E-08	0.2255	0.1228
B2	R	0.5726	0.6264	0.7804	1	0.5029	0.8016	0.5918	0.6777
	S	0.0043	0.0013	1.1E-05	--	0.0144	4.2E-06	0.0029	3.8E-04
C1	R	**0.9755**	**0.9442**	0.2134	0.5029	1	0.4902	0.8618	**0.9049**
	S	2.6E-15	1.E-11	0.3281	0.0144	--	0.0175	1.2E-07	3.0E-09
C2	R	0.5568	0.5606	0.8909	0.8016	0.4902	1	0.5376	0.6282
	S	0.0057	0.0059	1.2E-08	4.2E-06	0.0177	--	0.0081	0.0013
D1	R	**0.9043**	**0.9613**	0.2629	0.5918	0.8618	0.5376	1	**0.9243**
	S	3.2E-09	3.0E-13	0.2255	0.00293	1.2E-07	0.0081	--	2.8E-10
D2	R	**0.9326**	**0.9493**	0.33101	0.67773	**0.90496**	0.62812	**0.9247**	1
	S	9.2E-11	4.9E-12	0.1228	3.8E-04	3.0E-09	0.0013	2.8E-10	--

The metabolites responsible for statistical discrimination were P11 (luteolin 3'*O*-(4"*O*-acetyl) β-D-glucuronide), P22 (carnosic acid), P15 (carnosol), P16 (quercetin), P9 (rosmarinic acid), and P19 (benthamic acid).

Table 3 presents the Pearson correlation factors (R) and their statistical significance (S), considering the mean total concentrations of all twenty-three phytochemical found in rosemary accession groups. Significant correlations (R > 0.90) were noticed among "summer" accession groups A1-B1-C1-D1 but no significant correlations inside "spring" groups A2, B2 and C2. No correlations between the profiles of phytochemicals in spring season versus summer season in gypsum and semi-arid areas (e.g. A1 vs A2, B1 vs B2, C1 vsC2) were noticed, excepting the saline dune with significant positive correlations (D1 *vs* D2).

Metabolic relevance of phytochemicals' profile and their stress-regulated pathways

According to the qualitative and quantitative data, the specific biosynthetic pathways for phenolics and terpenoids in rosemary were affected by the environmental stress factors (water stress/salinity/altitude).

Mainly the water stress (soil aridity) during the dry summer season and the salt stress, to a lesser extent, were key factors that up-regulated the synthesis of phenolics, especially the flavonoids pathways and their glycosylation, for the protection of cell membranes against dryness. Water stress was signalised as the factor inducing significant changes in the metabolites profiles in this species [13] and salinity increased total amount of antioxidants in rosemary plants grown under increasing concentratiosn of NaCl [39].

The diterpene synthesis was also affected, especially by the oxidative degradation of carnosic acid to rosmanol and its derivatives, induced and amplified by dryness and salinity, in agreement with previous published data on rosemary [7, 13]. Antioxidant properties of carnosic acid in *in vitro* sytems were used in food technology and medicine [40]. It has been recently establishing that oxidation of carnosic acid is an efficient ROS scavenger mechanism and fulfils an important antixodant role in this species in *planta* [26]. The oxidized metabolites of carnosic acid, such as carnosol, acid carnosol, rosmanol, and epirosmanol are also strong antioxidants. As such, carnosic acid oxidation is a "cascade-type process", generating different secondary antioxidants, and constitutes a very efficient anti-oxidant mechanism in labiates. To confirm the impact of environmental stress on phenolics and diterpene metabolism, a principal component analysis (PCA) was carried out (Fig. 3).

The PCA biplot shows the relationships and variance between the environmental factors (altitude, rainfall, temperature and soil humidity) and the concentrations of the nine major compounds presented in Table 2. The three main components with eigenvalues equal to or higher than 1 explain a significant cumulative variance of 86.46%. The first component, that explain 64.77 % of the variance is positively correlated with water availability (rainfall and soil humidity). The second component which explain an additional 15.50% is related to altitude. These findings are in agreement with previous observations [36] where positive correlations were found between total phenolics and flavonoids and water stress. It is therefore strongly supported the idea that the phenolics' synthesis is intensified by water stress and may contribute to the drought tolerance in *R. officinalis*, as it has been reported in many other plant species [41].

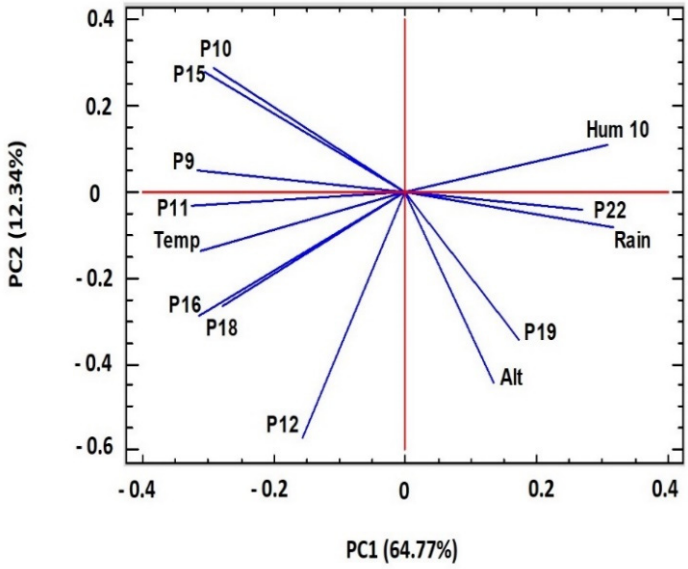

Figure 3. Biplot by the principal component analysis showing the relationships and variance between the environmental factors (altitude, rainfall, temperature and soil humidity) and the concentrations of the nine major compounds identified expressed as micrograms rutin/g dry leaf. For compound identification and quantification see Table 1.

CONCLUSIONS

Using an advanced methodology, HPLC-DAD-ESI+-MS, the phytochemical profiles of several *Rosmarinus officinalis* accessions from four different habitats in Spain, at two seasons (summer vs. spring), as influenced by different environmental stress (water stress and soil salinity), were established.

There were separated and quantified 23 molecules, nine being major: rosmarinic acid, two isomers of luteolin acetyl glucuronide, rosmanol, carnosol, quercetin, rosmadial, benthamic acid, carnosic acid. According to the qualitative and quantitative data, the specific biosynthetic pathways for these phenolics and terpenoids were affected by the environmental stress factors (water stress/salinity/altitude).

The diterpene derivatives and their oxidized metabolites were more sensitive than flavonoids to environmental stress and can be considered good biomarkers of water stress in wild *R. officinalis* grown in areas affected by the Mediterranean climate. By Cluster Analysis and Principal Component Analysis, the differences between the accession groups were determined,

being dependent on the environmental conditions. These data underline the key role of carnosic acid and its up-regulated biosynthesis by water and its degradation to oxidized derivatives, as an adaptation to water stress.

EXPERIMENTAL SECTION

Environmental conditions and sample harvesting

Rosemary leaves were harvested from different areas in the province of Valencia (Spain) growing in distinct environmental growth conditions: a plot in a sea sand dune; a plot in a semiarid area on limestone substrate at 200 m altitude, and two plots on a hill in a gypsum area at an altitude of about 700 m. To assess the climatic conditions from each area, data on mean temperatures from the previous month and accumulated rainfall were collected on a daily basis from the nearest agroclimatological stations, located in Benifaió (less than 6 km from the dune zone), Bétera (10 km from the semiarid zone) and Chulilla (18 km from the gypsum area). Soil water content was monitored through several sensors for soil moisture installed to 10 cm depth, connected to data loggers. For a more extensive description of the soil and climatic characteristics of the experimental zones, see [36].

The dune area is a stressful environment given its proximity to the sea, but plants were also affected by a constant water stress due to the low water retention by sand and the moderately saline shallow water table. In the semiarid zone, the plants were grown on a thin soil over a stony bedrock, the main stress factor being the water deficit in the dry season. In the gypsum area, it was a combination of salt and water stress (since gypsum soils are found always under arid climatic conditions). In this area, different environmental conditions were found at the top (with low gypsum, drier soil) and bottom areas of a hill (more gypsum, more humid, even during the summer season). In order to investigate the effect of different levels of water stress, the samples were collected from the same plants, during two seasons: summer, after a month without rainfall, and spring, after a rainy period.

Young shoots of flowering rosemary plants were sampled separately from five individuals from each locality, cooled on ice and transported to the laboratory, where leaves were separated from branches and dried in an oven at 45ºC for 72 h until constant weight. The harvested rosemary samples are indicated in Table 4.

Table 4. The rosemary accessions coding according to habitat and sampling season. Temperature values and accumulated rainfall were recorded by the nearest agro-climatological stations and correspond to the month before sampling date. Soil humidity was measured by electronic sensors installed at 10 cm depth.

Habitat	Code (m)	Sampling date	Number of samples	Mean T (ºC)	Cumulated rainfall (mm)	Soil humidity cm^3/cm^3
Gypsum Top 710 m	A1	Summer (I)	5	24.8	7	0.111
	A2	Spring (II)	5	12.0	113	0.194
Gypsum Bottom 690 m	B1	Summer (I)	4	24.8	7	0.139
	B2	Spring (II)	4	12.0	113	0.258
Semiarid 220 m	C1	Summer(I)	4	25.9	3	0.008
	C2	Spring (II)	5	14.1	35	0.242
Sand sea dune	D1	Summer (I)	4	21,0	1	0.073
	D2	Spring (II	5	13.5	50	0.138

Extraction protocols

Aliquots of rosemary dried leaves (2 g) were mixed with aq. methanol 95% (20 ml) containing 1% HCl (aq.), stirred and kept in an ultrasonic bath (XUB5 model, at operating frequency of 32 KHz and power supply of 100 W), for 24 h at 25°C. After centrifugation (2000 rpm), the supernatant was filtered through a 0.25-µm nylon filter. All the extractions were done in duplicate for each sample.

UV-VIS spectrometry

As a preliminary evaluation of extracts, the UV absorption profiles were determined by recording the spectra from 200 to 400 nm (using a UV/Vis Lambda 25 Perkin Elmer spectrophotometer), looking to the peaks at 280 and 340 nm, as markers of the phenolic acid and flavonoids, respectively.

HPLC-DAD and LC-ESI^+-MS analysis

A simultaneous evaluation (qualitative and quantitative) of phytochemicals was done using an Agilent 1200 HPLC Series system (Agilent Technologies, Santa Clara, CA. USA). The extract components were separated in a Zorbax Eclipse XDBC18 column (4.6 x 150 mm; 5 µm particle), at 25°C. The mobile phase consisted of a mixture of water:acetonitrile:acetic acid (99:0.9:0.1, v/v/v) (solvent A) and acetonitrile:acetic acid (99.9:0.1, v/v) (solvent B). The linear gradient for solvent B was as follows: 0-2 min.: 5% B,

2- 18 min. (from 5 to 40% B), 18 - 40 min (from 40 to 90% B) 20-24 min (isocratic 90% B), 24-27.5 min. (from 90 to 5% B). The flow rate was 0.5 mL/min and the injection volume of the sample dissolved in methanol was 5 μL. Before injecting, all the samples were filtered through a PTFE filter (13 mm i.d., 0.22 μm). The HPLC system was equipped with a diode array (DAD-G1315D) detection at specific wavelengths (280 and 340 nm). The system was coupled online with a single quadrupole mass spectrometer (MS) (Agilent Technologies 6110 system, Santa Clara, USA), in the ESI$^+$ mode. The MS settings were: dry gas N_2 at a flow rate of 8 L/min; drying temperature at 350°C, nebulizer pressure at 65 psi, capillary voltage of 3000 V, scan range of *m/z* from 150 to 1000.

The HPLC-DAD analysis was applied for the optimization of the phytochemicals separation and for the quantitative evaluation, based on chromatograms recorded at 340 nm.

For an accurate identification of the metabolites, the HPLC-DAD separation protocol was optimized using pure standards of phenolic acids (p-coumaric, caffeic and syringic acid) absorbing at 280 nm, and flavonoids (quercetin and rutin), carnosic acid and betulinic acid of 95-99% purity (purchased from Sigma Aldrich), at 340 nm. All pure standards were dissolved in methanol and injected individually and as a mixture in the HPLC column and their specific retention times (t_R) and UV absorption spectra were recorded.

For the quantification of metabolites, the optimized HPLC-DAD separation protocol was applied using pure rutin (quercetin 3-*O*-rutinoside) standard to build a calibration curve, based on five different concentrations (10, 5, 2.5, 1.25 and 0.625 micrograms rutin/mL). The calibration curve was represented by a regression equation y=32.846+273.7; R^2=0.9983 used to calculate the concentrations of metabolites separated by HPLC-DAD (expressed as micrograms rutin equivalents/ gram of dry sample).

The LC-ESI$^+$-MS analyses aimed to fingerprint and identify the metabolites based on their m/z values and fragmentation features, compared with literature reports and databases (Phenol Explorer, KEGG) finalized by tentative identification consistent with published data.

Statistical Analysis

The qualitative data derived from LC-ESI+-MS data were processed by non-targeted statistical analysis (Cluster Analysis and Principal Component Analysis), to discriminate the profile differences between the sampling groups depending on the environmental conditions. The mean values of identified compounds were compared by the Pearson correlation test, the significance of differences being established at 3E-9.

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