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Variation of the Chemical Profile and Antioxidant Behavior of *Rosmarinus officinalis* L. and *Salvia fruticosa* Miller Grown in Greece

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In this study, the essential oil and the phenolic composition along with the antioxidant activity of *R. officinalis* L. and *S. fruticosa* Miller, collected in Zakynthos island (Ionian Sea, Greece), were investigated. The essential oil composition of the plants was characterized by the presence of 1,8-cineole. Mean values of the antioxidant activities of rosemary and sage essential oils indicated slight differences. The antioxidant activity of sage oil was correlated with the oxygenated sesquiterpenes and diterpenes concentrations. Concerning the methanolic extracts, a close relationship between the phenolic content and the development stage during vegetative cycle of these plants was observed. The identified flavonoids, except rutin, seemed to increase with the advancement of developmental stages, while phenolic acids followed an opposite pattern. The antioxidant activity was correlated with the amount of total phenolic content.

KEYWORDS: Seasonal variation; essential oil; antioxidant activity; phenolics; *Rosmarinus officinalis* L.; *Salvia fruticosa* Miller; HPLC; GC-MS

INTRODUCTION

Rosmarinus officinalis L. (Lamiaceae) is an evergreen sclerophyll plant that is well-adapted to the limitations of the Mediterranean climate. In most of the cases, it is found near the coast (*1, 2*). The chemical composition and the antioxidant activity of rosemary oil have been studied by many workers (*2–5*). Two major types of rosemary oil have been reported. The first one contains more than 40% of 1,8-cineole (oils from Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy, and France), and the second one (oils from France, Spain, Italy, Greece, and Bulgaria) contains approximately equal ratios of 1,8-cineole, α-pinene, and camphor. There are few other types of rosemary oil reported in the literature, rich in verbenone and borneol (Sardinia ecotype) or in myrcene in oils from Argentina, Portugal, and Spain (*6*). However, the main components of the oil are not responsible for its remarkable antioxidant activity (*7*).

The extracts of *R. officinalis* were first marketed as sources of natural antioxidants (*8*). It has been an important spice and medicinal herb since early times, and it has received increasing attention due to its antimicrobial, anti-inflammatory, and antioxidative constituents (*9*). Rosemary contains a large number of compounds responsible for its antioxidant activity, such as rosmarinic acid, carnosic acid, and carnosol; the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity of the rosemary extracts is related to the amount of rosmarinic acid (*9*). The aqueous extracts of rosemary showed an increased $DPPH^{\bullet}$, radical-scavenging activity in comparison with those of sage and oregano and similar with the latter in terms of their scavenging action against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) radicals (*10*). To the best of our knowledge, most attention has been paid to its phenolic content and antioxidative properties during a single vegetative stage, while differences in antioxidant capacity and phenolic content during different harvesting periods have not been thoroughly investigated.

Salvia fruticosa Miller (Lamiaceae), commonly known as Greek sage, mainly occurs in the macquis and phrygana ecosystems of Southern Greece and the Ionian and Aegean islands, at altitudes varying between 0 and 1000−1350 m (*11*). The species is widespread in the Mediterranean basin as the climatic conditions favor its growth. Many studies on essential oil composition have reported 1,8-cineole as the main component, followed by camphor α-thujone, β-thujone, and β-caryophyllene. Besides the high content of oxygenated monoterpenes, the species is also known to contain biologically active sesquiterpenes and diterpenes (*12, 13*). However, the antioxidant potential of the oil has not been thoroughly studied.

S. fruticosa is one of the most commercially exploited sage plants. However, the extracts of *S. fruticosa*, which may also be rich in rosmarinic acid, have not received much attention in comparison to *S. officinalis* extracts (*8, 10, 14, 15*). Furthermore, no research has been reported on the variations that may occur

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Table 1. Collection Data[a] for the Plants Used in the Study

plants	vegetative stage	month and year	temperature (°C)[b]	humidity (%)[b]	rainfall (mm)[b]
R. officinalis and *S. fruticosa* (Lamiaceae)	before flowering	February 2005	7.9	63.1	86.6
	flowering	May 2005	17.8	66.7	11.4
	late fruiting	August 2005	24.1	63.1	0.0
	before flowering	February 2006	9.3	64.6	101.8
	flowering	May 2006	17.9	63.4	4.6
	late fruiting	August 2006	25.4	66.0	6.8

[a] Latitude, 37° 42′ 59N; longitude, 20° 50′ 36E; altitude, 20 m (for *R. officinalis*). Latitude, 37° 43′ 11N; longitude, 20° 48′ 59E; altitude, 186 m (for S. *fruticosa*). [b] Seasonal average values.

in the chemical profile and antioxidant activity of its extracts during its vegetative cycle.

Increased market demand for *R. officinalis* and *S. fruticosa* has led to several investigations of secondary metabolite levels in plants from different areas of the world. Research of these plants revealed different antioxidant capacities and chemical profiles. This differentiation can be attributed to genotype variety, age of the plant, geographical and ecological conditions, stage of plant development, proportion of plant tissue analyzed, time of collection, method of drying, and analysis. In most of these cases, variation of the chemical constituents present in the essential oils and extracts, as a whole, was not investigated. Furthermore, when this was performed, it was only focused on different plant populations.

The aim of this study is to assess the variation of yield and composition of the essential oil and extract of *R. officinalis* and *S. fruticosa*. Despite the well-known potentials of these plants throughout the Mediterranean area, knowledge of the site under investigation in Greece is scarce. Plants were obtained at three different development stages from two consecutive years and collected from the same stands to limit the influence of factors previously described. In continuation of our investigation, efforts were made to elucidate the relationship between the plant development stages and their antioxidant profiles and activities.

MATERIALS AND METHODS

Plant Materials. The aerial parts of *R. officinalis* and *S. fruticosa* were collected at the end of (i) February, (ii) May, and (iii) August of 2005 and 2006, in Zakynthos (a Greek island located on the Ionian Sea). *R. officinalis* was collected from one large shrub, while *S. fruticosa* was collected from 15 plants of the same population. The part of the plants used for extraction and further analysis almost exclusively consisted of leaves (stem parts and flowers accounted for less than 5% of the total weight). Full details are provided in **Table 1**. The freshly cut plants were dried in a dry and shady place at ambient temperature for 1 month, packed in paper bags under an N_2 atmosphere, and stored at ambient temperature. Crushed plant materials were analyzed within 3 months of collection.

Chemicals and Reagents. All solvents and reagents were of the highest purity needed for each application. Folin−Ciocalteu reagent, potassium persulfate (p.a.), methanol (p.a.), and ethanol (p.a.) were purchased from Merck (Darmstadt, Germany). DPPH$^{\bullet}$ (98%) and ABTS (∼98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid (37%, p.a.) was purchased from Panreac (Barcelona, Spain). Methanol and glacial acetic acid of high-performance liquid chromatography (HPLC) grade were obtained from Fisher Scientific Co. (Leicestershire, United Kingdom) and Panreac (Barcelona, Spain), respectively. Dichloromethane (p.a.) was purchased from Laboratory-Scan (Dublin, Ireland). Authentic standards, such as *p*-coumaric acid, rosmarinic acid, and quercitrin, were purchased from Sigma-Aldrich (Steinheim, Germany). Ferulic acid, vanillic acid, and luteolin were from Alfa Aesar (Karlsruhe, Germany), and caffeic acid was purchased from Merck (Schuchardt, Germany). Gallic acid was from Serva Inc. (NY). Rutin and 3,4-dihydroxybenzoic acid were purchased from Alexis biochemicals (Lausen, Switzerland), and L-ascorbic acid was from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) was a kind donation of the National Agricultural Research Foundation (N.AG.RE.F, Greece). The identification of the chemical constituents of the essential oils was performed using as reference compounds the following chemicals: α-pinene, β-pinene, borneol, γ-terpinene, *p*-cymene, 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor, and *trans*-pinocarveol from Fluka (Buchs, Germany); β-myrcene, α-terpinene, linalyl acetate, and β-caryophyllene from Sigma-Aldrich (Steinheim, Germany); and linalool, fenchyl alcohol, and caryophyllene oxide from Acros Organics (Geel, Belgium).

Preparation of the Samples. Essential oils were obtained as follows (*16, 17*): Thirty grams of plant material of each species was subjected to hydrodistillation in a Clevenger apparatus for 3 h. In all cases, deionized water was used. The resulting essential oils were dried over anhydrous sodium sulfate, and after filtration, they were stored at −4 °C until further analysis. The yields (% v/w dry plant material) of the essential oils are presented in **Tables 2** and **3**.

Methanolic extracts were obtained as follows (*17, 18*): Forty milliliters of aqueous methanol (70:30 v/v) was added to 0.5 g of plant material in a 50 mL spherical flask. Then, 10 mL of 6 M HCl was added carefully, and the mixture was stirred and sonicated for 15 min. The mixture was then bubbled for 40−60 s with N_2 and refluxed in a water bath at 90 °C for 2 h. After the mixture was cooled in the dark, it was filtered and made up to 50 mL with methanol. Extracts were purged with nitrogen and kept in a freezer at −20 °C until analyzed. For the HPLC analysis, the extracts were further filtrated through a 0.45 μm membrane filter (Millex-HV).

GC/MS Analysis. GC/MS analysis of the essential oils was performed using a Fisons 8000 series gas chromatograph (model 8060) coupled to a Fisons MD 800 quadrupole mass spectrometer (Fisons Instruments, Manchester, United Kingdom). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Separation of the compounds was performed on a CP-Sil 8 (30 m × 0.32 mm; film thickness, 0.25 μm; Chrompack) and on a DB-Wax capillary column (30 m × 0.25 mm; film thickness, 0.25 μm; J&W). Diluted samples (1/100 in dichloromethane, v/v) of 1 μL were injected manually in split mode (split ratio, 1/30). The oven temperature was programmed from 40 to 250 °C at a rate of 4 °C/min and held at 250 °C for 5 min. The injector, ion source, and interface temperatures were set at 230, 200, and 270 °C, respectively. The mass spectrometer was operated in electron impact mode with the electron energy set at 70 eV and a scan range of 30−400 *m/z*. Oil constituents were identified by comparing (i) linear retention indices based on a homologous series of even-numbered *n*-alkanes (C8−C24) (Niles, IL) with those of standard compounds and by comparison with literature data (*19*) and (ii) MS data with those of reference compounds and by MS data obtained from Wiley (*20*) and NIST (*21*) libraries.

HPLC Analysis. HPLC analysis of the phenolic constituents was performed using a Jasco HPLC System (Tokyo, Japan), consisting of a quaternary gradient pump (PU-2089 plus), a Rheodyne model 7725i injection valve with a 20 μL fixed loop, and a diode array detector (DAD; Jasco MD-910). Separations were performed on a Waters Sherisorb ODS2 (C_{18}) column (5 μm particle size, 250 mm × 4.6 mm i.d.), operating at ambient temperature (20 °C) with a flow rate of 1 mL/min. The mobile phase was acidified water containing 2.15% glacial acetic acid, pH 2.7 (solvent A), and methanol (solvent B). Phenolic compounds present in the aromatic plant extracts were analyzed according to the gradient elution program used for the determination of phenolic compounds in medicinal plants (*22*), with some modifica-

Table 2. Seasonal Variations of the Chemical Composition of the Essential Oil from *S. fruticosa*, Obtained by Gas Chromatography−Mass Spectrometry (GC/MS)

	relative retention index (RRI)[a]		% composition[b]						
			2005			2006			
compounds	CP-Sil 8	DB-Wax	February	May	August	February	May	August	fit[c]
α-pinene	940	1017	3.3	4.0	3.2	3.2	3.8	3.4	A
camphene	951	1053	1.6	0.8	0.3	1.1	0.8	0.7	B
β-pinene	975	1093	2.7	2.0	5.0	3.5	2.5	3.2	A
β-myrcene	991	1157	3.2	3.5	3.7	3.5	4.6	3.4	A
α-phellandrene	1001	1206	0.2	0.1	0.2	0.1	0.2	0.1	B
α-terpinene	1012	1174	0.2	0.1	0.2	0.2	0.2	0.2	A
p-cymene	1020	1266	0.6	1.0	0.4	0.8	0.4	0.4	A
1,8-cineole	1026	1210	46.0	47.9	52.9	52.5	58.9	56.9	A
γ-terpinene	1053	1243	0.2	0.2	0.3	0.2	0.2	0.2	A
cis-sabinene hydrate	1061	ND	tr	0.1	0.2	0.2	0.1	0.1	B
α- terpinolene	1083	1279	0.1	0.1	0.1	0.1	0.1	0.1	B
trans-sabinene hydrate	1091	1473	tr	0.1	0.1	0.1	0.1	0.1	B
linalool	1096	1554	0.2	0.3	1.5	tr	tr	1.4	A
cis-thujone	1098	1421	1.3	1.2	2.2	3.2	1.4	3.0	A
trans-thujone	1109	1440	1.7	1.1	3.0	3.1	1.8	2.6	A
camphor	1135	1514	5.8 ab,A	2.0 ac	0.7 bc,B	3.0 A	1.8	2.6 B	A
trans-pinocamphone	1152	ND	0.2	0.3	0.2	0.3	0.2	0.2	B
borneol	1158	1708	0.8	0.3	0.1	0.9	0.1	0.9	A
δ-terpineol	1161	ND	1.5	1.3	1.3	0.5	0.7	0.4	B
cis-pinocamphone	1166	ND	0.1	0.1	0.2	0.4	0.1	0.4	B
terpinen-4-ol	1171	1605	0.5	0.5	0.5	0.3	0.3	0.2	B
α-terpineol	1185	1704	4.3	4.0	3.4	3.9	2.8	3.9	B
linalyl acetate	1258	1559	0.1	0.2	2.3	0.2	0.1	2.7	A
α-terpenyl acetate	1346	ND	0.4	0.6	2.6	0.8	0.1	1.5	B
β-caryophyllene	1414	1594	5.1	3.4	2.0	2.4	3.3	1.0	A
aromadendrene	1434	ND	0.5	0.6	0.4	0.1	0.1	0.1	B
α-humulene	1449	1668	2.0	1.3	1.2	0.8	1.1	0.7	B
γ-muurolene	1474	1688	0.1	0.2	0.1	0.1	tr	0.1	B
viridiflorene	1492	ND	0.2	0.2	0.2	0.1	0.1	0.1	B
trans-calamene	1520	ND	0.2	0.2	tr	tr	tr	0.1	B
δ-cadinene	1521	1757	0.2	0.2	0.2	0.1	0.1	0.1	B
caryophyllene oxide	1576	1984	0.9	0.6	0.7	0.9	0.1	0.6	A
viridiflorol	1586	2091	6.3	7.0 A	4.0	5.0	2.1 A	3.1	B
cis- artennuic alcohol	1590	ND	0.4	0.2	0.2	ND	ND	ND	B
humulene epoxide	1602	ND	0.7	0.5	0.5	0.5	0.1	0.6	B
caryophylla-4(14),8(15)-dien-5-ol	1631	ND	0.4	0.3	0.1	0.3	0.1	0.2	B
manool	2050	ND	2.5	3.6 aA	1.2 a	1.5	0.5 A	1.1	B
others			2.6	3.0	1.4	2.4	0.4	tr	
monoterpene									
hydrocarbons			12.1	11.8	13.5	12.7	12.8	11.7	
oxygenated			63.1	60.2	71.5	70.3	68.5	75.5	
sesquiterpene									
hydrocarbons			8.9	6.8	4.3	3.7	4.9	2.4	
oxygenated			8.8	8.7 A	5.6	6.7	2.4 A	4.5	
diterpene									
oxygenated			2.5	3.6 aA	1.2 a	1.5	0.5 A	1.1	
alcohols-esters			0.1	0.1	0.1	0.1	tr	0.2	
unknown			1.7	1.9	0.6	1.3	0.2	0.9	
total identified constituents			97.2	93.1	96.8	96.3	89.3	96.3	
oil yield[d]			1.8 ± 0.1 a	1.6 ± 0.1 b,A	3.2 ± 0.3 a,b	1.8 ± 0.1 c,d	2.7 ± 0.2 c,A	2.9 ± 0.2 d	

[a] Relative retention indices to C8−C24 *n*-alkanes on the CP-SIL 8 and DB-WAX. [b] The percentage composition was calculated from the chromatograms obtained on the CP-Sil 8 column. Normalized peak area %. tr, <0.1%; ND, not detected. [c] A, MS data and retention index in agreement with those of authentic compound; B, MS data and retention index in agreement with those in literature. [d] v/w %, volume oil to weight of dry plant (in g). All data represent the mean values of three independent replicates. Values with the same lowercase letter within columns are statistically ($p < 0.05$ and $p < 0.01$) different between the months for each year. Values with the same uppercase letter within columns are statistically ($p < 0.05$ and $p < 0.01$) different between the years for each month.

tions: 0−15 min, 5% solvent B; 15−40 min, 30% solvent B; 40−50 min, 35% solvent B; 50−60 min, 45% solvent B; 60−70 min, 50% solvent B; 70−90 min, 55% solvent B; 90−100 min, 100% solvent B; and post-time 10 min before next injection.

Identification of the individual phenolics was based on comparison of the retention times and the UV spectrum, obtained by DAD of unknown peaks to those of authentic compounds. Detection for flavanols was effected at 280 nm, for flavanones and hydroxybenzoic acids at 290 nm, except for vanillic acid, which shows a maximum at 260 nm, for hydroxycinnamic acids at 330 nm, for flavones at 360 nm, and for flavonols at 380 nm. Quantitation was achieved using an external standard, and the phenolics were expressed as mg/g dry plant. Standard curves were prepared from each standard compound. Because of the limited availability of commercial standards, HPLC could not be used for the identification and quantitation of all peaks. However, their chemical categories could be identified from their chromatographic behavior and UV spectra. The same categories of phenolics usually exhibit similar chromatographic behavior and UV spectra characteristics (*23*). Therefore, the total amounts of unidentified phenolic acids were quantified and expressed as caffeic acid equivalents (CAE, mg/g dry plant), whereas the unidentified flavonoids and their glycosides were quantified and expressed as quercetin equivalents (QE, mg/g dry plant).

Table 3. Seasonal Variations of the Chemical Composition of the Essential Oil from *R. officinalis*, Obtained by GC/MS

			% composition[b]						
	RRI[a]		2005			2006			
compounds	CP-Sil 8	DB-Wax	February	May	August	February	May	August	fit[c]
tricyclene	930	1006	0.1	0.1	0.1	0.1	0.1	0.1	B
α-thujene	936	1021	0.0	0.1	0.0	0.1	0.0	0.0	B
α-pinene	940	1017	8.4	9.7	8.4	9.1	9.9	7.9	A
camphene	951	1053	2.4	3.5	2.7	2.8	2.8	2.2	B
β-pinene	975	1093	0.8	3.1	1.5	1.5	1.2	1.2	A
7-octen-4-ol	983	1458	0.2	ND	0.3	0.3	ND	0.4	B
3-octanone	976	1253	0.2	ND	0.3	0.2	ND	0.2	B
β-myrcene	991	1157	0.9	1.0	1.1	0.9	1.0	1.0	A
α-phellandrene	1001	1206	0.1	0.2	0.2	0.2	0.2	0.2	B
δ−2-carene	1006	ND	0.2	0.2	0.2	0.1	0.2	0.2	B
α-terpinene	1012	1174	0.3	0.8	0.4	0.5	0.6	0.5	A
p-cymene	1020	1266	3.1	1.7	2.9	2.5	2.2	2.7	A
1,8-cineole	1026	1210	54.2	48.3	55.5	50.6 ab	58.4 b	58.7 a	A
(*Z*)-b-ocimene	1035	ND	0.1	0.4	0.2	0.3	0.4	0.3	B
(*E*)-b-ocimene	1046	ND	tr	tr	tr	0.1	tr	tr	B
γ-terpinene	1053	1243	0.3	1.5	0.4	0.6	0.7	0.4	A
cis-sabinene hydrate	1061	ND	tr	0.1	tr	0.2	tr	tr	B
α- terpinolene	1083	1279	0.2	0.3	0.2	0.2	0.2	0.2	B
trans-sabinene hydrate	1091	1473	tr	0.1	0.1	0.1	tr	tr	B
linalool	1096	1554	0.6	0.6	0.8	0.6	0.6	0.9	A
fenchyl alcohol	1106	1588	0.1	0.1	0.1	0.1	0.1	0.1	A
trans-pinocarveol	1131	ND	tr	tr	tr	tr	tr	tr	A
camphor	1135	1514	0.2	0.2	0.2	0.3	0.2	0.2	A
camphene hydrate	1140	ND	0.1	0.1	0.1	0.1	0.1	0.1	B
borneol	1158	1708	10.4	9.4	9.5	8.9	8.8	10.2	A
δ-terpineol	1161	ND	ND	ND	0.7	0.8	0.9	0.9	B
terpinen-4-ol	1171	1605	1.4	1.1	1.4	1.1	1.0	1.4	B
α-terpineol	1185	1704	5.9 A	5.0	5.7	4.3 A	4.5	5.2	B
bornyl acetate	1283	ND	0.7	4.3	0.8	1.8	1.7	0.8	B
exo-2-hydroxycineole acetate	1340	ND	tr	0.1	tr	0.1	0.1	0.1	C
β-caryophyllene	1414	1594	1.1 a	4.4 abA	0.7 b	1.6	1.7 cA	0.9 c	A
α-humulene	1449	1668	tr	0.2	tr	0.1	0.1	tr	B
caryophyllene oxide	1576	1984	0.3	0.4	0.4	0.5	0.2	0.3	A
unknown 1	1630	ND	0.3	0.3	0.3	0.2	0.1	0.2	C
unknown 2	1653	ND	1.2	0.8	1.0	0.8	0.5	0.8	C
monoterpene									
hydrocarbons			17.0	22.7	18.2	18.8	19.6	16.8	
oxygenated			73.8	69.3	75.1	68.9 ab	76.4 a	78.6 b	
sesquiterpene									
hydrocarbons			1.1 a	4.6 Aab	0.7 b	1.7	1.8 Ac	0.9 c	
oxygenated			0.3	0.4	0.4	0.5	0.2	0.3	
alcohols-ketones			0.4	0.0	0.6	0.5	0.0	0.6	
unknown			1.5	1.1	1.3	1.1	0.6	1.0	
total identified constituents			93.8	98	96.2	91.7	98.5	98.3	
oil yield[d]			1.8 ± 0.2 a,b	2.8 ± 0.2 a,A	2.7 ± 0.3 b	2.2 ± 0.2 c	3.3 ± 0.2 c,d,A	2.6 ± 0.3 d	

[a] Relative retention indices to C8−C24 *n*-alkanes on the CP-SIL 8 and DB-WAX. [b] The percentage composition was calculated from the chromatograms obtained on the CP-Sil 8 column. Normalized peak area %. tr, <0.1%; ND, not detected. [c] A, MS data and retention index in agreement with those of authentic compound; B, MS data and retention index in agreement with those in literature; C, MS data in agreement with those in NIST and WILEY libraries. [d] v/w %, volume oil to weight of dry plant (in g). All data represent the mean values of three independent replicates. Values with the same lowercase letter within columns are statistically ($p < 0.05$ and $p < 0.01$) different between the months for each year. Values with the same uppercase letter within columns are statistically ($p < 0.05$ and $p < 0.01$) different between the years for each month.

Total Phenolic Content Determination. The total phenolic content was estimated using the Folin−Ciocalteu colorimetric method (*24*) and gallic acid as a standard. Briefly, 0.5 mL of diluted extract (1:10 v/v) was transferred in a test tube containing 2.25 mL of distilled water. Subsequently, 0.25 mL of Folin−Ciocalteu reagent was added. The mixture was stirred for 1 min and was allowed to stand for 8 min. Then, 2.0 mL of an aqueous solution of Na_2CO_3 (7.5% w/v) was added, and the mixture was incubated for 120 min at 25 °C. The absorbance relative to that of blank prepared using distilled water was measured at 765 nm using a double beam UV−vis spectrophotometer (Jasco V-530, Tokyo, Japan) and compared to a gallic acid calibration curve. The results were expressed as milligrams of gallic acid equivalents (GAE) per g dry plant and are presented as the mean value of a triplicate analysis.

Total Antioxidant Capacity Estimation. Total antioxidant capacities of the selected plant extracts were determined by using DPPH and ABTS cation radical-scavenging assays. For the essential oils, only the DPPH assay was employed. The antioxidant activities of the main phenolic compounds of the extracts were measured (rutin, quercitrin, vanillic acid, caffeic acid, ferulic acid, and rosmarinic acid). For the DPPH assay, separately, the antioxidant potentials of two more reference compounds (ascorbic acid and BHT) were measured. Measurements were obtained by a double-beam UV−vis spectrophotometer set at a wavelength appropriate to each assay. All determinations were performed in triplicate.

The DPPH assay for the methanolic extracts was conducted as follows (*25*): A stock solution of $DPPH^{\bullet}$ (10^{-4} M) was prepared in aqueous methanol (70:30 v/v), and 3 mL of this solution was added to 1 mL of sample. The mixture was stirred vigorously and allowed to stand at room temperature in the dark for 30 min. A decrease in absorbance at 517 nm was measured against a blank (aqueous methanol solution). A mixture consisting of 1 mL of aqueous methanol (70:30

v/v) and 3 mL of DPPH$^{\bullet}$ solution was used as a control. The radical stock solutions were freshly prepared every day, stored in a flask covered with aluminum foil, and kept in the dark. The radical-scavenging activities of the samples, expressed as percentage inhibition of DPPH$^{\bullet}$, were calculated according to the formula:

$$\% \text{ inhibition} = [(A_B - A_A)/A_B] \times 100 \quad (1)$$

where A_B and A_A are the absorbance values of the control and of the test sample, respectively. The extract concentration providing 50% inhibition (IC_{50}, mg/L) was calculated from the graph-plotted inhibition percentage against extract concentration (200, 100, 80, 50, 30, 20, 10, and 5 mg/L).

The DPPH assay for the essential oil was based on the method of Molyneux (*26*), after a slight modification: Briefly, a stock solution of DPPH$^{\bullet}$ (10^{-4} M) was prepared in ethanol. Two milliliters of sample was added to 2 mL of the DPPH$^{\bullet}$ solution. The mixture was stirred vigorously and allowed to stand at room temperature in the dark for 30 min. A decrease in absorbance at 517 nm was measured against a blank (ethanol solution) using the same spectrophotometer. A mixture consisting of 2 mL of ethanol and 2 mL of DPPH$^{\bullet}$ solution was used as a control. The radical stock solutions were freshly prepared daily. The oil concentration providing 50% inhibition (IC_{50}, g/L) was calculated from the graph of inhibition percentage plotted against oil concentration (0.5, 1.2, 2, 2.8, 4, 6.5, 12, 20, and 25 g/L).

ABTS$^{\bullet+}$ was prepared by mixing an ABTS stock solution (7 mM in ethanol) with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12−16 h until reaching a stable oxidative state (*27*). The radical was stable for more than 2 days when stored in the dark. On the day of the analysis, the ABTS$^{\bullet+}$ was diluted with ethanol (1:25, v/v) to an absorbance reading of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C. For the spectrophotometric assay, 2 mL of the ABTS$^{\bullet+}$ solution and 20 μL of standard (ferulic acid; final concentration, 0.10−2.00 mmol/L) or plant extract were mixed, and the absorbance at 734 nm was recorded at 1, 5, and 10 min after initial mixing against a blank (ethanol solution). The % inhibition (measure of antioxidant capacity) was calculated according to the formula:

$$\% \text{ inhibition} = [(A_c - A)/A_c] \times 100 \quad (2)$$

where A_c and A are the absorbance values of the control (ABTS solution) and the test sample, respectively. The standard calibration curve was constructed by plotting % inhibition (at 1, 5, and 10 min) against the concentration of ferulic acid. The antioxidant capacities of the plant extracts were calculated by using the calibration curve and expressed as mmol of ferulic acid equivalents (FAE) per g dry plant.

Statistical Analysis. All statistical analyses were carried out by using STATGRAPHICS Plus 3.0 for Windows (StatPoint, Inc., VA). Data on the extract yield, total antioxidant capacity, total phenolic content as well as data obtained from essential oil and HPLC analyses were subjected to analysis of variance (ANOVA). Significant differences between means were determined by least significant difference (LSD) at levels of $p < 0.05$ and 0.01. Correlation of total antioxidant capacity and chemical composition was carried out using the regression program in Statistica for Windows (version 6.0) (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Essential Oil Analysis. Tables 2 and **3** list the linear retention indices, percentage composition, and yield of the essential oil of *S. fruticosa* and *R. officinalis*, respectively. The yield of the essential oil obtained from the aerial part of sage (**Table 2**) varied from 1.6 to 3.2% v/w (dry weight). The lowest yields were observed during May (flowering period) of 2005 and during February (before flowering period) for both years. It seems that oil yield during flowering period is particularly susceptible to environmental conditions (light, nutrient availability, and day length) (*16, 28*).

Seventy-four constituents that represent more than 90% (except for May of 2006) of the total essential oil of sage were identified. Half of them, in concentrations less than 0.1%, are referred to as "others" (**Table 2**). The essential oil was characterized by a high percentage of oxygenated monoterpenes (60.2−75.5%) followed by monoterpene hydrocarbons (11.7−13.5%) and oxygenated sesquiterpenes (2.4−8.8%). The major essential oil constituents were 1,8-cineole (46−58.9%), followed by viridiflorol (2.1−7%), camphor (0.7−5.8%), β-caryophyllene (1−5.1%), α-terpineol (2.8−4.3%), β-pinene (2−5%), β-myrcene (3.2−4.6%), and α-pinene (3.2−4.0%).

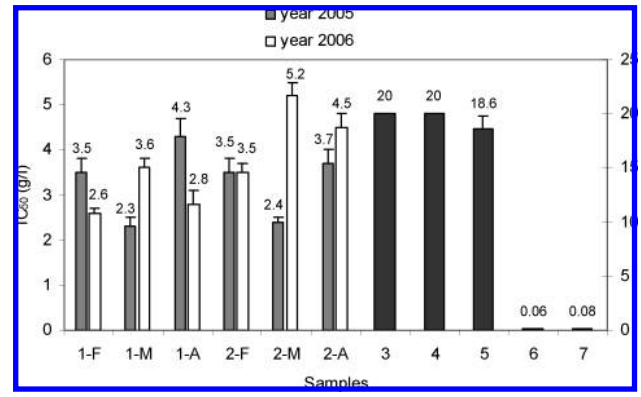

Figure 1. Radical scavenging activities of the plant essential oils against DPPH$^{\bullet}$ radical. Values are expressed as means ± standard deviations. Samples are as follows: 1, *R. officinalis*; 2, *S. fruticosa*; 3, 1,8-cineole; 4, α-pinene; 5, caryophyllene oxide; 6, ascorbic acid; and 7, BHT; its numbered sample is followed by uppercase letters F, M, and A, which represent the initials of the examined periods February, May, and August, respectively.

There was no influence of the year nor the season on the percentage concentration of the main components except for camphor, viridiflorol, and manool. The camphor concentration was higher during February (before flowering) and varied significantly with the seasons. Viridiflorol, which is the main constituent of the oxygenated sesquiterpene group, and manool, the only oxygenated diterpene identified in the investigated samples, varied significantly between the flowering periods (May 2005 and 2006), whereas in February and August for both years, no significant variation was observed (*28*). This variation was also reflected on the total amount of oxygenated sesquiterpene and diterpene groups. *cis*- and *trans*-Thujones, which are the main constituents of *S. fruticosa* population of other geographic origins (*29, 30*), were found in low concentrations (1.2−3.2 and 1.1−3.1%, respectively).

The antioxidant activities of sage oil and some of its components (1,8-cineole, α-pinene, and β-caryophyllene) were determined by DPPH assay (**Figure 1**). Free radical-scavenging activity of the oil for all seasons tested was much higher (2.4−5.2 g/L) than that of 1,8-cineole (not active), α-pinene (not active), and β-caryophyllene (18.6 g/L). When compared to ascorbic acid (0.06 g/L) and BHT (0.08 g/L), all samples and pure compounds were less effective than these antioxidants. ANOVA indicated that the antioxidant activity of the oil differed significantly during the flowering periods of both years. It was obvious that the antioxidant activity of the essential oil of *S. fruticosa* could not be attributed to the major compound nor to the rest of the compounds tested (*7*). However, a significant linear correlation was found between the oxygenated sesquiterpene ($r = -0.9125$, $p < 0.05$) and the oxygenated diterpenene concentration ($r = -0.9060$, $p < 0.05$) with the free radical-scavenging activity. This is a possible explanation of the high antioxidant activity of our samples. These results are in accordance with literature data (*31*) reporting that the oxygenated

terpenes are active compounds, with allylic alcohols (like viridiflorol and manool) being the most active ones.

The yield of the essential oil obtained from the leaves of *R. officinalis* (**Table 3**) varied from 1.8 to 3.3% v/w (dry weight). The lowest essential oil yield was observed in February for both years. Although rosemary oil production is referred to in the literature as being constant throughout the year (*1*), our results indicated that oil yield was greater during the flowering period.

Thirty-five constituents were identified. These compounds represent 91.7−98.5% of the total essential oil of rosemary (**Table 3**). Oxygenated monoterpenes (68.9−78.6%), which consisted mainly of 1,8-cineole (48.3−58.7%), borneol (8.8−10.4%), and α-terpineol (4.3−5.9%), were the major terpenes found in the rosemary essential oil. Monoterpene hydrocarbons constituted 16.8−22.7% of the oil. α-Pinene (8.4−9.9%), *p*-cymene (1.7−3.1%), and camphene (2.2−3.5%) were the major compounds of this group. A significant difference was observed in the percentage concentration of 1,8-cineole among the different development stages during the year 2006, a fact that was reflected on the total amount of the oxygenated monoterpene group. Furthermore, the concentration of α-terpineol differed significantly between February 2005 and 2006, while a significant difference was observed in the concentration of *β*-caryophyllene during May (2005 and 2006). The present results on *R. officinalis* essential oil composition indicated that the profile of plant volatiles remains steady during the years investigated. The observed variabilities on the percentage concentration of some compounds could be the result of environmental factors that affected the plant during its vegetative cycle.

The IC_{50} values of rosemary oil are illustrated in **Figure 1**. Although these values were similar to those of sage, this can not be attributed to the presence of oxygenated sesquiterpenes, as their concentration was low. The synergistic action of minor compounds may explain the results obtained from the DPPH assay. Statistical analysis of data showed a significant difference between the flowering and the fruiting period of year 2005.

Yield, Total Phenolic Content, and Total Antioxidant Capacity of Methanolic Extracts. The yield data (% v/w) and the data of the total phenolic content, expressed as GAE, for *R. officinalis* and *S. fruticosa* extracts are presented in **Table 4**. The antioxidant capacities determined by using DPPH radical and ABTS cation radical-scavenging assays are illustrated in **Figure 2A**,**B**.

With respect to extraction yields, both samples of the Lamiaceae family exhibited similar values ranging from 17.30 to 26.30% for rosemary and from 19.40 to 29.20% for sage. The results obtained for both plant extracts are in accordance with literature data (*10, 32, 33*). However, that is not the case for sage extracts from Crete (*34*). This difference can be explained in terms of extraction methods employed, geographical coordinates, climate, and ecological conditions. Considering the extraction yields of each plant separately, some variations were observed among the samples. The extraction yield of sage was significantly higher during February 2005 (29.20%) and August 2006 (26.10%). However, no significant difference was observed between May and August of 2005. The extraction yields of rosemary displayed a similar trend of variation, except for May and August 2006, in which their values did not differ significantly. The observed seasonal variations can be partially attributed to climate conditions (**Table 1**) that affect the biosynthetic pathway of different compounds in the plants (*35*) and subsequently may differentiate the amount of the extractable components in the final test system.

The total phenolic content between the selected aromatic plant extracts also varied slightly and ranged from 50.00 to 133.90 mg GAE/g dry plant for rosemary and from 63.70 to 144.00 mg GAE/g dry plant for sage (**Table 4**). Both plant extracts contained high amounts of phenolics with values comparable to those reported in previous studies (*4, 10, 14, 32, 34*). Possible differences can be attributed to the choice of parts tested, drying and extraction technique employed, methods of analysis applied, time and location of sampling, and genotypic differences (*4, 14*). The seasonal profiles of the samples, in terms of total phenolic content, showed significant differences in both years examined (**Table 4**). In the case of rosemary extracts and for the year 2005, the highest amount of phenolics was observed during before flowering period (133.90 mg GAE/g dry plant), while throughout the year 2006, the highest amount was found during the late fruiting period (106.40 mg GAE/g dry plant). The flowering period was characterized by the lowest concentration of total phenols for both years. Extracts from sage, year 2005, showed the highest total phenolic content during the late fruiting period (115.00 mg GAE/g dry plant), which was almost the same with that observed during before the flowering period (114.25 mg GAE/g dry plant). The lowest amount was observed during the flowering stage (63.70 mg GAE/g dry plant). The total phenolic content for year 2006 was significantly higher during the late fruiting period (144.00 mg GAE/g dry plant) and significantly lower during before the flowering period (90.40 mg GAE/g dry plant). Summarizing, it can be observed that both plants tend to have a similar variation of their total phenolic content for each year separately examined, whereas this variation differs from year to year. More precisely, both plants exhibited the highest phenolic content during before the flowering period for 2005, while for 2006, the highest total phenolic content was observed during the late fruiting period. This can lead us to the conclusion that a combination of environmental parameters, such as temperature, humidity, and rainfall, may accelerate or retard the accumulation of phenolic compounds present in these plants and, thus, change not only the amount of total phenolics but the phenolic profile of the extract as well. These changes in chemical composition were better elucidated by the HPLC analysis that was conducted during this study.

The total antioxidant capacities of plant extracts are presented in **Figure 2A**,**B**. Both panels show that the extracts possessed similar capabilities of scavenging action against the two radicals used. The IC_{50} values for rosemary extracts ranged from 12.40 to 43.90 mg/L, while the corresponding values for sage extracts ranged from 21.30 to 46.15 mg/L. The $ABTS^{\bullet+}$ values of rosemary extracts ranged between 0.95 and 4.40 mmol FAE/g dry plant. This range was slightly higher than the one observed in sage extracts. These results are in agreement with literature data (*15, 32, 34*). However, all plant extracts exhibited a significantly lower antioxidant activity than that of the pure phenolic compounds and the reference standards when the DPPH method was employed, whereas plant extracts and reference compounds displayed comparable activities when the ABTS method was used (**Figure 2B**).

Total antioxidant capacities of each aromatic plant extract examined varied with the year (**Figure 2A**,**B**). From the estimated IC_{50} values (**Figure 2A**) and for the year 2005, it can be seen that the extracts from rosemary demonstrated the strongest antioxidant activity during February (12.40 mg/L), whereas the lowest antioxidant activity was observed during May (43.20 mg/L). However, the value of this period did not differ significantly from the one found during May 2006 (43.90 mg/L). For 2006, the strongest antioxidant activity was observed

Table 4. Extract Yield, Total Phenolic Content, and HPLC Qualitative−Quantitative Data for the Examined Plant Extracts

	year 2005						year 2006					
	February		May		August		February		May		August	
phenolic compounds	concn [a]	RSD[b] (%)	concn	RSD (%)	concn	RSD (%)	concn	RSD (%)	concn	RSD (%)	concn	RSD (%)
R. officinalis												
3,4 di-HBA[c]	7.25 ± 0.40	5.50	1.70 ± 0.30	17.60	3.35 ± 0.07	2.10	1.45 ± 0.02 a	1.40	1.10 ± 0.01 a	0.91	2.10 ± 0.03	1.43
caffeic acid	1.50 ± 0.06 a	4.00	0.80 ± 0.07	8.75	1.65 ± 0.12 a	7.30	0.85 ± 0.03	3.50	0.40 ± 0.01	2.50	1.30 ± 0.01	0.80
vanillic acid	5.40 ± 0.10	1.85	tr[d]		0.30 ± 0.00	0.30	1.00 ± 0.01	1.00	tr		1.50 ± 0.05	3.30
p-coumaric acid	ND[e]		ND		ND		0.20 ± 0.00 a	0.50	0.20 ± 0.01 a	5.50	0.65 ± 0.03	4.60
ferulic acid	4.20 ± 0.05	1.20	1.60 ± 0.03	1.90	3.70 ± 0.22	5.95	2.95 ± 0.15	5.10	0.75 ± 0.01	1.30	3.35 ± 0.17	5.10
rosmarinic acid	1.70 ± 0.08	4.70	0.60 ± 0.03	5.00	0.95 ± 0.05	5.30	0.50 ± 0.01	2.00	0.20 ± 0.00	0.50	1.30 ± 0.07	5.40
quercitrin	4.10 ± 0.20	5.10	9.60 ± 0.20	2.10	2.60 ± 0.05	1.90	2.45 ± 0.01	0.41	3.10 ± 0.02	0.65	0.80 ± 0.01	1.25
rutin	2.75 ± 0.14	5.00	tr		5.45 ± 0.12 A	2.20	1.75 ± 0.02	1.15	0.60 ± 0.05	8.30	5.80 ± 0.29 A	5.00
luteolin	1.45 ± 0.04	2.75	2.75 ± 0.04	1.45	1.10 ± 0.04	3.60	2.20 ± 0.02	0.91	3.80 ± 0.01	0.26	1.15 ± 0.01	0.90
unidentified flavonoids[f]	3.40 ± 0.05	1.50	1.20 ± 0.07	5.80	1.70 ± 0.05 A	2.95	2.25 ± 0.03	1.30	7.40 ± 0.65	8.80	1.55 ± 0.02 A	1.30
unidentified phenolic acids[g]	1.50 ± 0.05 a	3.30	0.95 ± 0.08 A	8.40	1.55 ± 0.07 a	4.50	tr		1.00 ± 0.05 A	5.00	3.70 ± 0.01	0.30
total HPLC phenols	33.25 ± 0.90	2.70	19.25 ± 0.70 A	3.60	22.30 ± 0.45 B	2.00	15.50 ± 0.28	1.81	18.65 ± 0.81 A	4.30	23.10 ± 0.24 B	1.00
extract yield[h]	26.30 ± 0.20		20.40 ± 0.17 a		21.80 ± 0.13 a		18.90 ± 0.10 b		17.30 ± 1.00 b		24.20 ± 0.60	
total phenolic content[i]	133.90 ± 1.60		122.70 ± 2.07 a		123.20 ± 2.07 a		58.85 ± 5.75		50.00 ± 5.45		106.40 ± 4.11	
S. fruticosa												
3,4 di-HBA[c]	4.30 ± 0.02	0.46	0.60 ± 0.04	6.70	5.75 ± 0.31	5.40	ND		1.25 ± 0.03	2.40	2.40 ± 0.04	1.70
caffeic acid	0.60 ± 0.03	5.00	0.35 ± 0.01	2.85	1.70 ± 0.03	1.75	1.20 ± 0.02	1.70	1.60 ± 0.21	13.10	2.40 ± 0.11	4.60
vanillic acid	2.70 ± 0.13	4.80	tr		2.10 ± 0.13	6.20	tr		0.20 ± 0.00	0.50	1.40 ± 0.02	1.40
p-coumaric acid	0.45 ± 0.00	0.22	0.25 ± 0.01	4.00	0.60 ± 0.01 A	1.70	0.25 ± 0.00	0.40	0.55 ± 0.10 a	18.20	0.60 ± 0.01 a,A	1.60
ferulic acid	3.50 ± 0.30 A	8.60	0.40 ± 0.00	0.25	1.60 ± 0.24	15.00	3.60 ± 0.06 A	1.70	1.90 ± 0.03	1.60	5.65 ± 0.16	2.80
rosmarinic acid	2.20 ± 0.03	1.40	0.15 ± 0.00	0.70	0.85 ± 0.06 A	7.00	0.55 ± 0.01	1.80	0.30 ± 0.01	3.30	0.90 ± 0.01 A	1.10
quercitrin	4.35 ± 0.50	11.50	6.40 ± 0.26 A	4.10	1.40 ± 0.02	1.40	2.65 ± 0.03	1.10	6.25 ± 0.09 A	1.45	2.50 ± 0.20	8.00
rutin	ND		ND		ND		ND		ND		ND	
luteolin	2.05 ± 0.12	5.85	6.55 ± 0.05	0.80	1.20 ± 0.01	0.80	1.10 ± 0.08 a	7.30	4.35 ± 0.28	6.45	0.90 ± 0.05 a	5.50
unidentified flavonoids[f]	ND		ND		ND		ND		ND		ND	
unidentified phenolic acids[g]	2.45 ± 0.07 a	2.85	0.40 ± 0.00	0.25	2.25 ± 0.15 a,A	6.70	ND		1.05 ± 0.05	4.80	2.30 ± 0.03 A	1.30
total HPLC phenols	22.70 ± 0.32	1.40	15.05 ± 0.20	1.30	17.50 ± 0.30	1.70	9.30 ± 0.14	1. 50	17.40 ± 0.24	1.40	19.00 ± 0.63	3.30
extract Yield[h]	29.20 ± 0.3		25.20 ± 0.11 a		24.30 ± 0.20 a		19.40 ± 0.08		22.30 ± 0.80		26.10 ± 0.14	
total phenolic content[i]	114.25 ± 7.35 a		63.70 ± 2.14		115.00 ± 3.00 a		90.40 ± 1.52		112.10 ± 1.28		144.00 ± 7.04	

[a] Each value (mg/g dry plant) is the mean of two replications ± standard deviations. [b] RSD = (standard deviation/mean) × 100. [c] HBA, hydroxybenzoic acid. [d] tr, traces. [e] ND, not detected. [f] Quantified and expressed as CAE. [g] Quantified and expressed as QE. [h] % v/w, volume of extract per weight of plant material (in g). [i] The otal phenolic content was determined according to Folin−Ciocalteu assay, in mg GAE per g dry plant. Values with the same lowercase letter within each row are not significantly ($p > 0.05$) different between the months for the same year. Values with the same uppercase letter within each row are not significantly ($p > 0.05$) different between the years for each month.

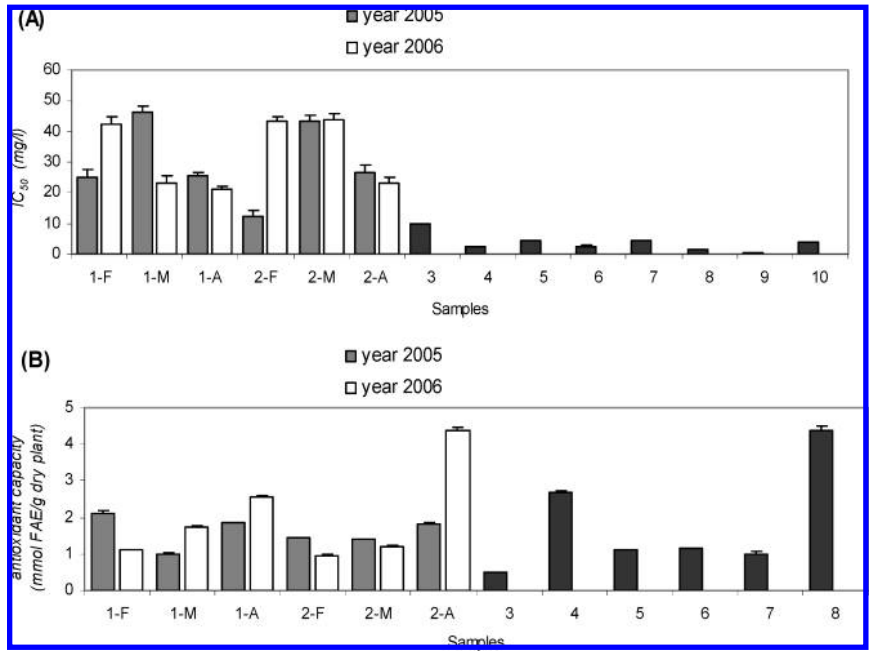

Figure 2. Radical scavenging activities of the plant extracts against (A) $DPPH^{\bullet}$ and (B) $ABTS^{\bullet+}$ radicals. Values are expressed as means ± standard deviations. Samples are as follows: 1, *S. fruticosa*; 2, *R. officinalis*; 3, vanillic acid; 4, caffeic acid; 5, ferulic acid; 6, rosmarinic acid; 7, rutin; 8, quercitrin; 9, ascorbic acid; and 10, BHT; each numbered sample is followed by the uppercase letters F, M, and A, which represent the initials of the examined periods February, May, and August, respectively.

during August (22.90 mg/L). Considering the sage extracts for the year 2005, the lowest IC_{50} values were observed during February (25.30 mg/L) and were similar to those found during August. For 2006, sage extracts exhibited significantly stronger antioxidant activity in August (21.30 mg/L), while the lowest antioxidant activity was observed during February (42.05 mg/L). Both plants displayed a similar pattern of antioxidant capacities variation when the ABTS assay was employed (**Figure 2B**).

It is known that the degree of capability of the plant extracts examined to deactivate free radicals such as $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ seems to be affected by the amount of the phenolic compounds. All of the above results indicate that the plant extracts with a high phenolic content showed a tendency to have high antioxidant activity, except for rosemary harvested during May 2005 and February 2006. A similar trend was also observed between total phenolic content and extraction yield, which was more obvious in rosemary extracts. The participation of the active constituents present in the investigated plant extracts, such as phenolic acids and flavonoids, can be further investigated through HPLC analysis. An attempt to establish a significantly strong correlation of total phenols (determined by Folin−Ciocalteu and HPLC) with extract yield and $ABTS^{\bullet+}$ radical-scavenging data was unsuccessful (**Figure 3A**,**C**). Nevertheless, a strong relationship was obtained between total phenols determined by Folin−Ciocalteu and HPLC with $DPPH^{\bullet}$ total antioxidant capacity data (**Figure 3B**). Corroborating results were also found in the literature (*10, 32*). One can assume that synergistic actions taking place between the phenolic compounds found in natural extracts might influence the differences in the antioxidant ability of plant extracts. Maillard and Berset (*36*) reported a similar action between *p*-coumaric and ferulic acids, whereas Meyer et al. (*37*) found interactive effects between flavonoids and phenolic acids.

Qualitative and Quantitative Determination. Phenolic compounds identified in the aromatic plant extracts are summarized in **Table 4**. Linear regression analysis was also used to evaluate the calibration curve of each analyte as a function of its concentration. The limit of detection (LOD) and limit of quantification (LOQ) were estimated as $3SD_b$/slope and $10SD_b$/slope of the calibration curve, respectively, where SD_b was the standard deviation of the intercept (b) (95% confidence limits). Regression analysis of the peak area ratio (y) vs concentration (x) is shown in **Table 5**.

Major phenolic components in the aromatic plants tested include flavonoids, such as quercitrin and luteolin, and phenolic acids, such as rosmarinic, caffeic, and ferulic acids. With respect to flavonoids, both extracts contained mainly quercitrin and luteolin with values ranging from 0.80 to 9.60 and from 1.10 to 3.80 mg/g dry plant, respectively, for rosemary, whereas for sage, these values ranged from 1.40 to 6.40 and from 1.10 to 6.55 mg/g dry plant, respectively. Other researchers, as well, reported the presence of these compounds (*14, 15, 34*). With respect to phenolic acids, it seems that their presence had a significant contribution to the total antioxidant capacity of both plant extracts. In rosemary extracts, rosmarinic and caffeic acid ranged from 0.20 to 1.70 and 0.40 to 1.65 mg/g dry plant, respectively, while in sage extracts, these values ranged from 0.15 to 2.20 and 0.35 to 2.40 mg/g dry plant, respectively. Although previous works have reported a similar chemical profile (*14, 15, 34*), the concentration of rosmarinic acid was rather higher in most of these studies when compared to our results. The loss of rosmarinic acid in our samples can be attributed to biological degradation processes (*38*). Compounds with characteristic spectra of ferulic acid and 3,4-dihydroxybenzoic acid were also detected in high amounts, but their contribution to the total antioxidant activity of the plant extracts was minimal (**Figure 2A**,**B**). Furthermore, some other phenolic compounds were also determined quantitatively but not identified because of lack of reference standards. These phenolic compounds, which were grouped as unidentified flavonoids and unidentified phenolic acids (**Table 4**), were present in high amounts in all harvesting periods, affecting, thus, the antioxidant potential of the plant extracts tested.

The seasonal profiles of the quantified flavonoids showed significant differences among the samples harvested in February,

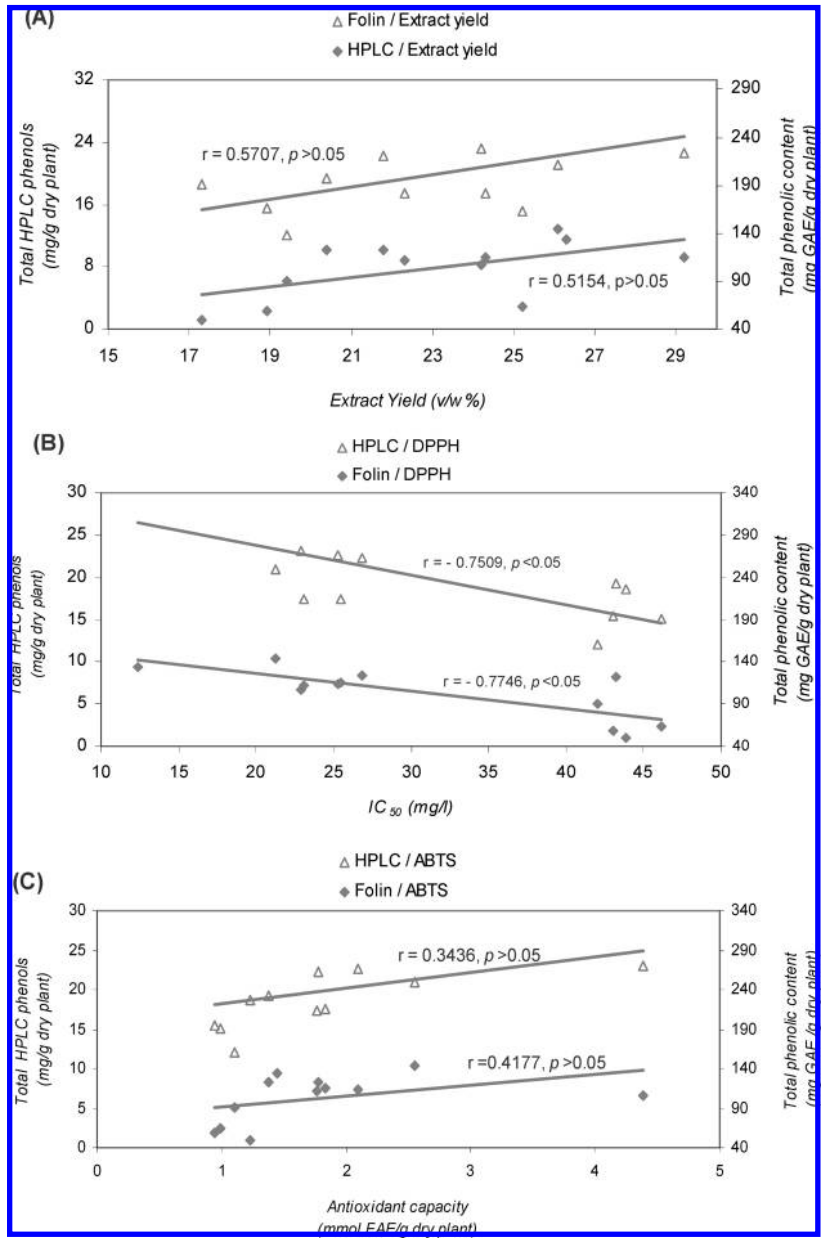

Figure 3. Correlation of total phenols determined by Folin−Ciocalteu and HPLC (A) with extract yield, (B) with DPPH$^{\bullet}$ radical scavenging data, and (C) with ABTS$^{\bullet+}$ radical scavenging data for the investigated plant extracts.

Table 5. Linear Calibration Curves for the HPLC Analysis of the Main Phenolic Compounds

	$y = ax + b$				
phenolic compounds	slope (a ± SD[a])	intercept (b ± SD)	R^2	LOD[b] (mg/L)	LOQ[c] (mg/L)
caffeic acid	$8.5 \times 10^{-4} \pm 7.0 \times 10^{-5}$	$-4.5 \times 10^{-4} \pm 7.1 \times 10^{-5}$	0.9922	0.25	0.83
ferulic acid	$9.0 \times 10^{-4} \pm 1.0 \times 10^{-5}$	$+0.001 \pm 2.8 \times 10^{-6}$	0.9980	0.94	3.14
rosmarinic acid	$2.2 \times 10^{-3} \pm 2.5 \times 10^{-5}$	$-2.5 \times 10^{-4} \pm 7.1 \times 10^{-5}$	0.9951	0.09	0.31
rutin	$8.0 \times 10^{-4} \pm 1.0 \times 10^{-5}$	$-5.0 \times 10^{-5} \pm 6.4 \times 10^{-5}$	0.9918	0.24	0.80
querctrin	$3.0 \times 10^{-4} \pm 1.4 \times 10^{-4}$	$+5.5 \times 10^{-5} \pm 7.1 \times 10^{-6}$	0.9938	0.35	1.18
luteolin	$1.5 \times 10^{-4} \pm 7.0 \times 10^{-5}$	$-1.1 \times 10^{-4} \pm 7.1 \times 10^{-6}$	0.9897	0.14	0.47

[a] Standard deviation. [b] Limit of detection. [c] Limit of quantification.

May, and August of 2005 and 2006. Quercitrin and luteolin contents in all samples increased with advancing development stages (**Table 4**). The concentration of quercitrin and luteolin in rosemary extracts reached the highest level during the flowering period (May) for both years (9.60 and 2.75 mg/g dry plant for quercitrin and luteolin, respectively, in 2005 and 3.10 and 3.80 mg/g dry plant for quercitrin and luteolin, respectively, in 2006) and the lowest in the later vegetative stages. Sage extracts showed similar trends, possessing the highest values during the flowering period and the lowest during the late fruiting. That is not the case for rutin, a phenolic compound identified only in rosemary extracts. The rutin content decreased with advancement of plant development, and its highest level for both years was achieved during late fruiting (5.45 mg/g dry plant in August 2005 and 5.80 mg/g dry plant in August 2006). To our knowledge, there are no data in the literature concerning

the seasonal variation of flavonoid concentration in rosemary and sage extracts. However, samples of *Hypericum perforatum* grown in Turkey showed a similar pattern of variation for these flavonoids (*39*).

The caffeic acid and rosmarinic acid contents of both plant extracts varied significantly between the different harvesting periods (**Table 4**). Regarding rosemary extracts, the lowest concentration of caffeic acid was observed during May 2005 and 2006 (0.80 and 0.40 mg/g dry plant, respectively), whereas the highest concentration was achieved during August (late fruiting) (1.65 and 1.30 mg/g dry plant, respectively). Regarding sage extracts, a similar pattern was observed. In the case of rosmarinic acid, both extracts exhibitied similar trends during 2006, showing lower values in May and higher values in August. Nevertheless, the results for 2005 did not follow the same pattern. The highest rosmarinic acid content for both plant extracts was observed in February (before flowering). In contrast to the other compounds examined, ferulic acid and 3,4-dihydroxybenzoic acid present in extracts showed an inconsistent behavior in both rosemary and sage extracts.

Differences in antioxidant capacity and chemical composition correlated to different harvesting periods for the selected aromatic plants were investigated. These plants did not show great variations in terms of their essential oil composition. This led us to assume that the chemical profile between the same populations is more or less defined by their genetic profile. The antioxidant activity of the essential oils varied slightly through the harvesting periods, and it is not directly associated with its main chemical components. For the sage oil, the oxygenated sesquiterpene and diterpene percentage correlated well with the measured IC_{50} values, whereas for rosemary oil, the synergistic action of minor compounds seems to be responsible for its antioxidant activity. The phenolic contents of the plant extracts mainly include quercitrin, luteolin, rosmarinic acid, and caffeic acid. The identified flavonoids (quercitrin and luteolin) and phenolic acids (rosmarinic acid and caffeic acid) were closely related to the development stage during the vegetative cycle of both plants. The highest concentration of the flavonoids was observed during the flowering stage, whereas the fruiting stage, in which the antioxidant activity was high for both plants, was characterized by high phenolic acid content. The antioxidant activity and total phenolic content varied among the periods tested and did not correlate with total phenols determined by HPLC. Moreover, among the factors that are reported to influence the biosynthesis of these compounds in plants (*10*), environmental conditions seem to affect their quantity. Results of the present study revealed that the essential oil and the extract of *S. fruticosa* and *R. officinalis* are characterized by small variations throughout the investigated years. However, they possess significant antioxidant properties. Hence, both plants can be considered as effective sources of natural antioxidants during their vegetative cycle.

ABBREVIATIONS USED

GAE, gallic acid equivalents; FAE, ferulic acid equivalents; CAE, caffeic acid equivalents; QE, quercetin equivalents; IC_{50}, inhibitory concentration of substrate (mg/L) that causes 50% loss of the DPPH activity (color); GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; DAD, diode array detection; RRI, relative retention index.

SAFETY

Ethanol is a highly flammable and slightly polluting substance; methanol is toxic and very flammable. Always wear safety glasses. You should not breathe in the vapor, so use a fume cupboard if available. If this is not possible, ensure that the area in which you work is very well-ventilated. For $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, avoid contact with the skin and eyes. Wear suitable protective clothing and eye/face protection. For all of the other reagents, suitable gloves should be worn and contact with the skin and eyes should be avoided.

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