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Investigation of the Antioxidant Behavior of Air- and Freeze-Dried Aromatic Plant Materials in Relation to Their Phenolic Content and Vegetative Cycle

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The total phenolic and flavonoid content of the aerial parts of five aromatic plants harvested at different periods was estimated, and their antioxidant capacity was evaluated. Major phenolic compounds present in their extracts were determined by RP-HPLC. The results demonstrated different amounts of total phenolic compounds and various degrees of antioxidant activity depending on the plant species, the time of harvest, and the drying method employed. Extracts from air-dried *Mentha viridis* L., *Origanum majorana* L., and *Rosmarinus officinalis* L. demonstrated the greatest efficacy during the flowering stage, in which the identified flavonoids were found in significantly higher amounts, whereas phenolic acids were found in their lowest concentration. Extracts from air-dried *Laurus nobilis* L. and *Foeniculum vulgare* Mill were less efficient in terms of antioxidant activity, with the highest values being observed during the early fruiting stage. This stage was characterized by the lowest flavonoid content and high phenolic acid content, except for *L. nobilis* L. extracts. Overall, the amount of identified phenolic acids did not vary considerably within the investigated year. The total phenolic concentration in all plant extracts decreased significantly when freeze-dried rather than air-dried samples were used. The HPLC analysis further supported the above for most of the phenolic compounds present in the extracts, except for hydroxybenzoic acids, which were better retained during the freeze-drying process.

KEYWORDS: Aromatic plants; rosemary; spearmint; fennel; marjoram; Greek bay; antioxidant activity; phenolics; freeze-drying; air drying; RP-HPLC

INTRODUCTION

For many years, aromatic plants have been used for a large number of purposes including medicine, nutrition, flavorings, beverages, dyeing, repellents, fragrances, cosmetics, charms, smoking, and industrial uses. Many of them have been considered to be excellent sources of different classes of natural antioxidants, such as phenolic compounds, and a great number of these plants have been recognized to have medicinal properties and to possess a variety of antioxidant effects (*1–4*). The latter makes the diverse group of phenolic compounds an interesting target in the search for health-beneficial phytochemicals and also offers the possibility to use them or extracts rich in them in foods or medicinal materials as an alternative to synthetic antioxidants, the use of which is restricted because of their toxicity (*5*).

Some of these plants, such as sage, oregano, and thyme, have been studied extensively and resulted in the development of natural antioxidant formulations for food, cosmetic, and other purposes (*6–8*). However, among the various medicinal and culinary herbs, the chemical and biological characteristics of some endemic species depend largely on their development stage and the climate conditions during their harvest. The literature for these species is rather scarce. Thus, the assessment of their chemical composition and antioxidant capacity can be considered as an interesting and useful task, particularly in revealing some of the most important factors that influence the phenolic and flavonoid content and subsequently the antioxidant activities of the aromatic plant extracts.

Rosemary (*R. officinalis* L.) has been an important spice and medicinal herb since earliest times. It is receiving increasing attention because of its antimicrobial, antiinflammatory, and antioxidative constituents (*9, 10*). Rosemary contains a large number of compounds responsible for its antioxidant activity, such as carnosic acid and rosmarinic acid. A previous paper reported that the DPPH radical scavenging activity of the rosemary extracts depended on the amount of rosmarinic acid (*10*). Aqueous extracts of rosemary showed increased $DPPH^{\bullet}$ radical scavenging activity in comparison with those of sage and oregano and similar activity compared with oregano in terms of their scavenging action against $ABTS^{\bullet+}$ radicals (*11*). Rosemary has been thoroughly studied; however, the focus was mainly on its phenolic content and antioxidative properties

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Table 1. Collection Data for the Examined Aromatic Plant Materials[a]

plant material	vegetative stage	harvest season[b]	environmental conditions		
			temperature (° C)[c]	humidity (%)[d]	rainfall (mm)[e]
Rosmarinus officinalis (Lamiaceae)	before flowering	Feb	12.4	89.4	2.1
	flowering	May	18.2	60.2	4.6
	late fruiting	Aug	30.3	50.8	0.0
	vegetative	Nov	17.1	90.3	8.2
Mentha viridis (Lamiaceae)	before flowering	Feb	12.4	89.4	2.1
	flowering	May	18.2	60.2	4.6
	late fruiting	Aug	30.3	50.8	0.0
	vegetative	Nov	17.1	90.3	8.2
Origanum majorana (Lamiaceae)	before flowering	Feb	12.4	89.4	2.1
	flowering	May	18.2	60.2	4.6
	late fruiting	Aug	30.3	50.8	0.0
	vegetative	Nov	17.1	90.3	8.2
Laurus nobilis (Lauraceae)	full flowering	Feb	12.4	89.4	2.1
	early fruiting	May	18.2	60.2	4.6
	Late fruiting	Aug	30.3	50.8	0.0
	in bud	Nov	17.1	90.3	8.2
Foeniculum vulgare (Apiaceae)	late fruiting	Feb	12.4	89.4	2.1
	before flowering	May	18.2	60.2	4.6
	early fruiting	Nov	17.1	90.3	8.2

[a] Location, Patra (Greece); altitude, 350 m (for all plants examined). [b] Year 2007. [c] Seasonal average values. [d] Seasonal average values. [e] Seasonal average values.

during a single vegetative stage, whereas possible variations on these characteristics during its vegetative cycle are limited.

The leaves of *Laurus nobilis* L. (Greek bay) are used as a valuable spice and flavoring agent in the culinary and food industry (*12*). This plant does not have important uses in traditional medicine but recently has been the subject of scientific research. Data about the radical scavenging activity of the leaves and phenolic constituents of bay laurel are very scarce (*12, 13*).

Foeniculum vulgare Mill is a plant known and used by humans since antiquity. This plant, commonly known as fennel, is a small genus of annual, biennial, or perennial herbs distributed in central Europe and Mediterranean regions, and it is widely cultivated throughout the temperate and tropical regions of the world for its aromatic fruits, which are used as a culinary spice (*14*). Although the chemical constituents and antimicrobial properties of the fruit volatile oil of *F. vulgare* are well studied (*15–17*) and antioxidant studies of its extracts have been undertaken (*17–19*), no work has been reported on the variations that may occur regarding its chemical profile and antioxidant activity during the vegetative cycle.

Origanum majorana L. (marjoram) is a herbaceous plant native to southern Europe and the Mediterranean. Typically, products identified as marjoram are the dried leaves and flower tops of *O. majorana*. Traditionally, it is used as a folk remedy against asthma, headache, and rheumatism. It contains phenolic terpenoids, flavonoids, tannins, phenolic glycosides, and sitosterol (*20*). However, the antioxidant and antitumor activities of marjoram have recently been determined (*21, 22*), and little it is known about the active compounds of marjoram responsible for its antioxidant activity, except for its essential oil (*23, 24*).

Mint (*Mentha viridis* L.) is widely used as a source of essential oils for flavoring. More recently, mint has been used as a valuable source of the potent antioxidant rosmarinic acid (*25*). It belongs to the family Lamiaceae, which is a rich source of polyphenolic compounds, and hence could possess strong antioxidant properties. There are few reports on the antioxidant property and total phenolic content of *M. viridis* (*3, 7, 26, 27*), but the seasonal variations of these parameters have not been studied.

For all of the aromatic plants under study, the influence of drying on their phenolic composition and antioxidant activity has not been thoroughly investigated, except for spearmint, for which a little information is given on the effect of drying on its volatile compounds and its sensory characteristics (*28*) and the effects of heat stress on the accumulation of rosmarinic acid and other secondary metabolites (*29*).

The purpose of this study was to evaluate the antioxidant behavior and phenolic content of five commonly consumed aromatic plants of Greek origin with respect to the drying process applied and the period of harvest.

MATERIALS AND METHODS

Plant Materials. A total of five fresh plant materials (**Table 1**) from Patra (Greece) were harvested in the first half of February, May, August, and November of 2007. A sample of *F. vulgare* was not collected during the August period. Immediately after harvest, a portion of each plant was air-dried in a shady, well-ventilated room at ambient temperature for 5 days. Then it was packed in paper bags under N_2 and stored for up to 6 months. Another portion was kept in a freezer at −20 °C for 2 days, freeze-dried for 6 h at −60 °C using a freeze-dryer (UNICRYO-MC2L, Munich, Germany), and stored at −20 °C. The whole aerial parts of the plants (leaves, branches, and flowers) were used for extraction and analysis in the present study. The scientific names, sources, vegetative stages, seasons of harvest, and environmental conditions are given in **Table 1**.

Chemicals and Reagents. Folin−Ciocalteu reagent, 2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ, ≥99%), aluminum chloride (>98%), potassium persulfate, iron chloride hexahydrate (p.a.), sodium acetate trihydrate (p.a.), sodium nitrite (p.a., ≥99%), methanol (p.a.), and ethanol (p.a.) were purchased from Merck (Darmstadt, Germany). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$, 98%) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ∼98%) were from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide (p.a.) and hydrochloric acid (37%, p.a.) were purchased from Panreac (Barcelona, Spain), and sodium carbonate anhydrous was obtained from Carlo Erba Reagenti (Rodano, Italy). Methanol and glacial acetic acid

were of HPLC grade and were purchased from Fisher Scientific Co. (Leicestershire, U.K.) and Panreac, respectively. All solvents and reagents were of the highest purity. Authentic chromatography standards were purchased from Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), ICN Biomedicals Inc. (Aurora, OH), Merck (Schuchardt, Germany), Fluka AG (Buchs, Switzerland), Serva Inc. (New York), and Alexis Biochemicals (Lausen, Switzerland). L-Ascorbic acid was from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and *p*-hydroxybenzoic acid were a kind donation of the National Agricultural Research Foundation (N.AG.RE.F., Greece).

Preparation of Air-Dried and Freeze-Dried Extracts. Both air-dried and freeze-dried plant materials were extracted using the same procedure. The extraction method used for plant samples was as follows (*17*): 40 mL of aqueous methanol (70:30 v/v) was mixed into 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. After sonication, the mixture was bubbled for 40−60 s with N_2 and refluxed in a water bath at 90 °C for 2 h. After cooling in the dark, it was filtered and made up to 50 mL with methanol, purged with nitrogen, and kept in a freezer at −20 °C until analyzed. For HPLC analysis, the mixtrure was further filtered through a 0.45 μm membrane filter (Millex-HV).

Determination of Total Phenolic and Flavonoid Content. Total phenolic content was estimated using the Folin−Ciocalteu colorimetric method (*30*) 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, to which 250 μL of Folin−Ciocalteu reagent was added. The mixture was stirred for 1 min and 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 at 25 °C for 120 min. 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 quantitated using a gallic acid calibration curve. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW) and are presented as means of triplicate analyses.

For the total flavonoid content estimation, a colorimetric assay was employed, using (−)-epicatechin as a reference compound (*31*). One milliliter of diluted extract (1:10 v/v) was added to a 10 mL test tube containing 4 mL of distilled water. Immediately, 0.3 mL of 5% $NaNO_2$ was added to each test tube, and after 5 and 6 min, 0.3 mL of 10% $AlCl_3$ and 2 mL of 1 M NaOH, respectively, were added. Each reaction tube was then immediately diluted with 2.4 mL of distilled water, mixed, and allowed to stand for 5 min. Absorbance of the mixture upon the development of pink color was measured against blank at 510 nm. The amount of total flavonoids was expressed as milligrams of (−)-epicatechin equivalents (ECE) per gram of DW and is presented as the mean of triplicate analyses.

Estimation of Total Antioxidant Capacity. Total antioxidant capacities of the selected aromatic plant extracts were determined by using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP), and ABTS cation radical scavenging assays. For each antioxidant assay employed, two flavonoids (rutin and quercetin) and five phenolic acids (gallic acid, vanillic acid, caffeic acid, ferulic acid, and rosmarinic acid) were used as reference standards. Assay results were obtained by using a double-beam UV−vis spectrophotometer set at an appropriate wavelength for each assay. All determinations were performed in triplicate.

For the DPPH assay (*32*) 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 then shaken vigorously and allowed to stand at room temperature in the dark. After 30 min, the decrease in absorbance at 517 nm was measured against a blank (aqueous methanol solution), by using a double-beam UV−vis spectrophotometer. A mixture consisting of 1 mL of aqueous methanol (70:30 v/v) and 3 mL of $DPPH^{\bullet}$ solution was used as 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 following formula: % inhibition $= [(A_B - A_A)/A_B]) \times 100$, 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 plotting inhibition percentage against extract concentration (200, 100, 80, 50, 30, 20, 10, and 5 mg/L).

For the ABTS assay, $ABTS^{\bullet+}$ 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 it reached a stable oxidative state (*33*). The radical was stable for >2 days when stored in the dark. On the day of 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 percent inhibition (measure of antioxidant capacity) was calculated according to the formula

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

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 percent inhibition (at 1, 5, and 10 min) against concentration of ferulic acid, and the antioxidant capacities of the plant extracts were calculated by using the calibration curve and expressed as millimoles of ferulic acid equivalents (FAE) per gram of DW.

The FRAP assay was conducted according to the method of Benzie and Strain (*34*). Prior to analysis, the methanolic extracts (1 mL) were transferred into 10 mL volumetric flasks and diluted with the same solvent, whereas 0.9 mL of FRAP working reagent (25 mL of acetate buffer, 300 mM, pH 3.6 + 2.5 mL of TPTZ, 10 mM TPTZ in 40 mM HCl + 2.5 mL of $FeCl_3 \cdot 6H_2O$, 20 mM) was mixed with 90 μL of distilled water and warmed to 37 °C in a water bath. The reagent blank reading was recorded at 595 nm, followed by adding 30 μL of the diluted extract (1:10 v/v). The absorbance was taken at 1, 4, and 30 min, against blank solution, containing 30 μL of solvent. A standard curve was prepared using different concentrations of $FeSO_4 \cdot 7H_2O$ (0.2−2 mmol/L), and the iron(III) to iron(II) reducing capacity of the extracts was calculated with reference to the reaction signal given by a Fe^{2+} solution of known concentration. The results were corrected for dilution and expressed in millimoles of Fe^{2+} per gram of DW.

HPLC Analysis. HPLC analysis was performed using a Jasco HPLC system (Tokyo, Japan), consisting of a quaternary gradient pump (Jasco PU-2089 plus, Tokyo, Japan), a Rheodyne model 7725i (Cotati, CA) injection valve with a 20 μL fixed loop, and a diode array detector (Jasco MD-910). Separations were performed on a Waters Spherisorb ODS2 (C_{18}) column (5 μm particle size, 4.6 × 250 mm i.d.) (Ireland), 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 absolute methanol (solvent B). Phenolic compounds in the aromatic plant extracts were analyzed according to the gradient elution program used for the determination of phenolic compounds in medicinal plants (*35*), with some modifications: 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; post-time, 10 min before next injection.

Identification of the individual phenolics was based on a comparison of the retention times and the UV spectrum obtained by diode array (DAD) of unkown peaks to those of reference authentic chromatography standards. Detection was performed at 280 nm for flavanols, at 290 nm for flavanones and hydroxybenzoic acids (except for vanillic acid, which shows a maximum at 260 nm), at 330 nm for hydroxycinnamic acids, at 360 nm for flavones, and at 380 nm for flavonols.

Quantification was achieved by comparison with an external standard of known phenolic compounds and expressed as milligrams per gram of DW. Standard curves were made from each standard. Because of the limited commercial standards, we could not use HPLC to identify and quantify all peaks of the six aromatic plant extracts. However, their chemical categories could be identified from their chromatographic

Table 2. Total Phenols, Total Flavonoids, and Flavonoid Concentration for the Examined Plant Extracts[a]

		identified flavonoids					
sample[b]		rutin	quercetin	luteolin	unidentified flavonoids[c]	total phenols[d]	total flavonoids[e]
1F	a	0.20 ± 0.00A	1.50 ± 0.02	1.35 ± 0.02aA	nd	66.53 ± 1.03	6.60 ± 0.32
	f	0.30 ± 0.01aA	0.70 ± 0.01	1.10 ± 0.07A	nd	58.30 ± 1.18	4.30 ± 0.38
1M	a	0.65 ± 0.02b	6.00 ± 0.10	2.50 ± 0.07	nd	81.10 ± 0.92	11.50 ± 0.38
	f	0.20 ± 0.00a	1.25 ± 0.08	0.63 ± 0.01	nd	60.90 ± 0.12	1.30 ± 0.05
1A	a	0.45 ± 0.00b	nd[f]	0.40 ± 0.02B	nd	63.40 ± 8.81	tr[g]
	f	nd	0.20 ± 0.01a	0.20 ± 0.00bB	nd	42.40 ± 0.69	tr
1N	a	nd	0.43 ± 0.10A	1.13 ± 0.09a	nd	120.25 ± 0.12	25.10 ± 1.44
	f	nd	0.40 ± 0.01aA	0.25 ± 0.01b	nd	63.60 ± 0.28	tr
2F	a	0.85 ± 0.40c	4.45 ± 0.07b	1.90 ± 0.03	nd	102.50 ± 5.49	26.10 ± 0.38
	f	0.40 ± 0.00d	0.80 ± 0.06c	0.50 ± 0.00	8.20 ± 0.04a	57.95 ± 0.75	10.80 ± 0.87
2M	a	3.90 ± 0.01	5.20 ± 0.05bB	2.20 ± 0.02	22.00 ± 0.14	138.00 ± 2.10a	27.80 ± 1.13a
	f	1.43 ± 0.08	6.45 ± 0.56B	1.10 ± 0.03c	17.50 ± 0.08	67.70 ± 0.11	16.05 ± 0.41
2A	a	0.70 ± 0.01c	0.35 ± 0.06	nd	11.00 ± 0.09	114.90 ± 2.02	24.05 ± 0.91
	f	0.20 ± 0.00d	0.75 ± 0.03c	2.40 ± 0.03	12.35 ± 0.05b	50.70 ± 0.26	5.35 ± 0.06b
2N	a	nd	1.00 ± 0.01	1.20 ± 0.04C	2.20 ± 0.03	131.70 ± 1.05a	27.75 ± 0.95a
	f	0.80 ± 0.01	2.35 ± 0.02	1.10 ± 0.06cC	10.75 ± 0.07ab	115.10 ± 2.34	5.75 ± 0.08b
3F	a	nd	0.50 ± 0.01d	nd	7.60 ± 0.23cA	101.63 ± 0.54	28.80 ± 1.18
	f	nd	2.30 ± 0.00	nd	7.62 ± 0.91A	70.10 ± 0.43	21.30 ± 0.47
3M	a	nd	1.30 ± 0.01	3.30 ± 0.05	8.75 ± 0.50c	125.80 ± 5.85b	14.50 ± 0.97c
	f	nd	tr	nd	1.50 ± 0.02	96.55 ± 1.19	13.10 ± 0.39d
3A	a	nd	0.20 ± 0.00	nd	1.40 ± 0.05	72.40 ± 1.75	16.50 ± 0.71c
	f	nd	nd	nd	0.30 ± 0.01	64.50 ± 0.21	0.40 ± 0.03
3N	a	nd	0.45 ± 0.02d	nd	3.65 ± 0.05	125.70 ± 2.74b	14.80 ± 1.15c
	f	nd	0.30 ± 0.01	nd	2.95 ± 0.04	70.10 ± 2.31	13.20 ± 2.55d
4F	a	nd	nd	1.90 ± 0.03	nd	51.30 ± 1.72	tr
	f	nd	nd	4.50 ± 0.05	nd	41.20 ± 0.78c	tr
4M	a	nd	nd	3.10 ± 0.05D	4.40 ± 0.02	80.30 ± 0.87	2.90 ± 0.18
	f	nd	nd	3.08 ± 0.04D	1.20 ± 0.25	41.15 ± 2.01c	tr
4A	a	nd	nd	0.20 ± 0.01	tr	22.90 ± 1.81A	tr
	f	nd	nd	1.15 ± 0.08	tr	21.50 ± 0.37A	tr
4N	a	nd	nd	1.30 ± 0.56	1.00 ± 0.05	42.60 ± 0.10	tr
	f	nd	nd	0.60 ± 0.02	tr	26.70 ± 0.83	tr
5F	a	0.90 ± 0.00e	1.55 ± 0.04e	nd	0.95 ± 0.00	31.10 ± 1.01dB	tr
	f	nd	nd	nd	nd	28.50 ± 1.96B	tr
5M	a	2.00 ± 0.01	1.60 ± 0.04e	nd	0.70 ± 0.02	28.70 ± 0.62d	tr
	f	nd	nd	nd	nd	35.20 ± 0.06	tr
5N	a	0.80 ± 0.10e	0.15 ± 0.01	nd	1.90 ± 0.05	69.80 ± 1.82	tr
	f	tr	nd	nd	nd	49.25 ± 1.05	tr

[a] Values (mg/g of DW) are expressed as mean ± standard deviation. Values with the same lower case letter within each column are not significantly ($p > 0.05$) different between the months for each plant. Values with the same upper case letter within each column are not significantly ($p > 0.05$) different between the two methods of drying for each plant. [b] (1) *Rosmarinus officinalis*, (2) *Mentha viridis*, (3) *Origanum majorana*, (4) *Laurus nobilis*, (5) *Foeniculum vulgare*; each numbered sample from 1 to 5 is followed by the upper case letter F, M, A, or N, which represents the initial of the examined period (February, May, August, and November, respectively), whereas the lower case letters a and f represent air-dried and freeze-dried samples, respectively. [c] Quantified and expressed as quercetin equivalents (QE). [d] Total phenols expressed as gallic acid equivalents (GAE)/g of DW. [e] Total flavonoids expressed as (−)-epicatechin equivalents (ECE)/g of DW. [f] Not detected. [g] Traces.

behavior and UV spectra. The same categories of phenolics usually have similar chromatographic behaviors and UV spectra characteristics (*36*). Therefore, the total amounts of unknown/unconfirmed phenolic acids were quantified and expressed as caffeic acid equivalents (CAE, mg/g of DW), whereas the unknown/unconfirmed flavonoids and their glycosides were quantified and expressed as quercetin equivalents (QE, mg/g of DW).

Statistical Analysis. All statistical analyses were carried out by using Statistica for Windows (version 6.0). Data on the total antioxidant capacities and total phenolic and flavonoid content as well as phenolic concentration determined by HPLC analysis were subjected to analysis of variance (ANOVA), and significant differences between means were determined by least significant difference (LSD) at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Content. The total phenolic and flavonoid content of the five aromatic plant extracts examined varied. With respect to the drying method applied, the total phenolic content of the air-dried samples ranged from 22.90 to 138.00 mg of GAE/g of DW, whereas their total flavonoid content ranged from 2.90 to 28.80 mg of ECE/g of DW. As far as the freeze-dried samples is concerned, total phenolics ranged from 21.50 to 115.10 mg of GAE/g of DW and their respective flavonoids from traces to 21.30 mg of ECE/g of DW (**Table 2**). With respect to season, the smaller differences in the total phenolic and flavonoid content were observed among *R. officinalis* (63.40−120.25 mg of GAE/g of DW and traces−25.10 mg of ECE/g of DW), *M. viridis* (102.50−138.00 mg of GAE/g of DW and 24.05−27.80 mg of ECE/g of DW), and *O. majorana* (72.40−125.80 mg of GAE/g of DW and 14.50−28.80 mg of ECE/g of DW) extracts. These members of the Lamiaceae family showed, however, higher values than the corresponding ones observed in *L. nobilis* (22.90−80.30 mg of GAE/g of DW and traces−2.90 mg of ECE/g of DW) and *F. vulgare* (28.70−69.80 mg of GAE/g of DW and traces) extracts. Similar results were observed for the freeze-dried samples. The results of the investigated plants of the Lamiaceae family are in agreement with most of the studies that have been conducted with regard the total phenolic and flavonoid content of many aromatic plants belonging to this family (*3, 6, 11, 37*). It was found that their total phenolic and flavonoid content decreased in the following order: *M. viridis* > *O. majorana* > *R. officinalis*. The findings of these studies demonstrated that the above-mentioned plants overall contain high amounts of phenolics. However, they varied significantly in terms of the

levels of these phenolics. This significant variation was probably due to genotype and environmental differences, choice of part tested, and methods applied. A far as *L. nobilis* and *F. vulgare* are concerned, a previously reported study (*6*) further confirmed our finding.

The total phenolic and flavonoid content in the selected aromatic plants was also found to vary with harvest period. In addition, their content was strongly dependent on the drying method used.

Table 2 sets out the mean and standard deviation values for the total phenolic and flavonoid content (mg/g of DW) in the aromatic plants harvested in different periods and summarizes the results of the LSD test for comparison of means. The total phenolic and flavonoid content in air-dried *R. officinalis* extracts was significantly higher during the vegetative stage (120.25 mg of GAE/g of DW and 26.10 mg of ECE/g of DW, respectively) and significantly lower than that during the late fruiting stage (63.40 mg of GAE/g of DW and traces, respectively). For the freeze-dried samples, a similar pattern of total phenolic variation was observed, whereas their total flavonoid content was higher before the flowering stage (4.30 mg of ECE/g of DW). The total phenolic and flavonoid content of extracts from *M. viridis* and *O. majorana*, obtained from air-dried and freeze-dried plant material, differed significantly in February (before flowering stage) and August (late fruiting stage), whereas no significance difference was observed between May (flowering stage) and November (vegetative stage) for the air-dried ones. Both air-dried plant extracts were rich in phenolics (138.00 and 125.80 mg of GAE/g of DW, respectively) during flowering stage (May), whereas during the same period mint extracts also displayed the highest flavonoid content (27.80 mg of ECE/g of DW). Furthermore, for extracts obtained from freeze-dried samples the highest amount of phenolics and flavonoids was observed during the same stage, with the exception of those of *M. viridis*, for which the highest total phenolic content was observed during the vegetative stage (115.10 mg of ECE/g of DW). In the case of *L. nobilis* and *F. vulgare* extracts, a significant difference in the total phenolic content for each investigated period was found. Total flavonoids were present in traces in all periods tested, with the exception of *L. nobilis* extract of May (early fruiting stage) (2.90 mg of ECE/g of DW). In the *L. nobilis* extracts, total phenols reached their maximum level during the early fruiting stage (80.30 mg of GAE/g of DW), whereas the lowest level was found during the late fruiting stage (22.90 mg of GAE/g of DW). Their values differed significantly from the ones observed during the full flowering (51.30 mg of GAE/g of DW) and in bud stage (28.70 mg of GAE/g of DW). In the *F. vulgare* extracts, total phenolic concentration was significantly higher during November (early fruiting stage) (69.80 mg of GAE/g of DW). Extracts of *L. nobilis* and *F. vulgare* obtained from freeze-dried plant materials demonstrated a similar seasonal variation with respect to total phenolic and flavonoid content. Previous studies have shown that plant development stage may have implications in the biosynthetic pathway of phenolic compounds in plants and thus in their total phenolic and flavonoid content (*38*). This outcome can be further supported by our findings, in which collection of samples at different harvesting periods (different plant development stages) resulted in different amounts of total phenols and flavonoids in the extracts. However, this differentiation may be attributed not only to the development stage of each plant tested but also to environmental factors. As can be seen from **Table 1**, each harvesting period is characterized by different climate conditions (temperature, humidity, and rainfall). This difference might have played a vital role in the accumulation of phenolic compounds and, subsequently, in the total phenolic content in the plant extracts. The nature and the extent of these changes in the phenolic content was investigated by HPLC analysis.

Table 2 sets out the mean and standard deviation values for the total phenolic and flavonoid content (mg/g of DW) in the selected air-dried and freeze-dried aromatic plant materials. This table also summarizes the results of the LSD test for comparison of means. The findings showed that the total phenolic and flavonoid content for all the aromatic plants examined varied significantly between air-dried and freeze-dried plant materials, with the freeze-dried ones having the lowest values. However, that was not the case for *L. nobilis* and *F. vulgare* extracts, for which no significant difference between the air-dried and freeze-dried samples harvested in August and February was observed (**Table 2**). The total phenolic content for freeze-dried samples ranged from 42.40 to 63.60 (*R. officinalis*), from 50.70 to 115.10 (*M. viridis*), from 64.50 to 96.55 (*O. majorana*), from 21.50 to 41.20 (*L. nobilis*), and from 28.50 to 49.25 (*F. vulgare*) mg of GAE/g of DW. For the air-dried ones, the respective values ranged from 63.40 to 120.25 (*R. officinalis*), from 102.50 to 138.00 (*M. viridis*), from 72.40 to 125.80 (*O. majorana*), from 22.90 to 80.30 (*L. nobilis*), and from 28.70 to 69.80 (*F. vulgare*) mg of GAE/g of DW. The total flavonoid content of the selected freeze-dried samples significantly decreased in comparison to that of air-dried samples (**Table 2**). There are no data regarding the effect of air-drying and freeze-drying processes on the phenolic composition of the aromatic plants. Nevertheless, it is worth noting that in these plants significantly higher levels of total phenols and flavonoids were obtained from air-dried rather than freeze-dried samples. Freeze-drying caused a significant decrease (almost 50%) of the total phenolic and flavonoid content in all plants studied. The effect of air- and freeze-drying on the phenolic and flavonoid content of the plant extracts under investigation can be observed in more detail through HPLC analysis.

Qualitative and Quantitative Analysis. The qualitative−quantitative analysis of the five aromatic plant extracts is presented in **Tables 2** and **3**. Representative chromatograms are displayed in **Figures 2** and **3**. The HPLC analysis of the selected aromatic plant extracts, obtained from both air-dried and freeze-dried samples, showed that a large number of flavonoids and phenolic acids were present in significant amounts. The most abundant ones were quercetin (traces−6.45 mg/g of DW), luteolin (0.20−4.50 mg/g of DW), ferulic acid (0.30−7.55 mg/g of DW), gallic acid (0.15−2.50 mg/g of DW), rosmarinic acid (traces−3.00 mg/g of DW), and caffeic acid (0.10−2.00 mg/g of DW). These compounds were readily identified by comparisons to the retention time and UV spectra of authentic standards. Quercetin, caffeic acid, ferulic acid, and gallic acid were found in greater amounts in all three aromatic plant extracts of the Lamiaceae family, with the exception of gallic acid in the *R. officinalis* extracts. Among the five tested aromatic plants, these extracts exhibited the most powerful activity. In addition, although the calculated levels of rosmarinic acid were overall lower than the levels of the previously mentioned phenolic compounds, their contribution to the high radical scavenging activity was considerable. This can be attributed to the presence of four hydroxyl groups in its molecule (*39*). The antioxidant capacities of the above phenolic compounds presented in **Figure 1** further support our findings.

Other compounds with characteristic spectra of rutin and vanillic acid were also detected in variable quantities, contribut-

Table 3. Phenolic Acid Concentration for the Examined Plant Extracts[a]

sample[b]		identified phenolic acids								unidentified rosmarinic acid[d]
		gallic acid	3,4-di-HBA[c]	vanillic acid	caffeic acid	chlorogenic acid	*p*-coumaric acid	ferulic acid	rosmarinic acid	
1F	a	nd[e]	3.25 ± 0.04a	1.15 ± 0.01aA	1.40 ± 0.00a	nd	nd	4.50 ± 0.04	1.00 ± 0.06	2.80 ± 0.02a
	f	nd	4.50 ± 0.11	1.50 ± 0.03A	0.50 ± 0.01b	nd	nd	1.10 ± 0.09a	nd	0.45 ± 0.01
1M	a	nd	3.20 ± 0.01a	0.35 ± 0.01B	1.40 ± 0.04a	nd	nd	1.15 ± 0.08	0.40 ± 0.00a	2.70 ± 0.12
	f	nd	4.00 ± 0.27	0.30 ± 0.01bB	0.40 ± 0.01b	nd	nd	nd	nd	0.20 ± 0.01b
1A	a	0.20 ± 0.01	3.85 ± 0.06bA	nd	1.35 ± 0.12a	nd	nd	7.55 ± 0.10	3.00 ± 0.21	4.80 ± 0.05a
	f	nd	3.60 ± 0.04A	tr[f]	0.45 ± 0.01b	nd	nd	0.85 ± 0.01	nd	0.30 ± 0.01b
1N	a	0.90 ± 0.04	3.65 ± 0.03bB	1.20 ± 0.03a	1.40 ± 0.09a	nd	nd	3.25 ± 0.13	0.50 ± 0.08a	4.50 ± 0.07
	f	nd	3.85 ± 0.03B	0.55 ± 0.01b	0.55 ± 0.01	nd	nd	1.30 ± 0.10a	0.10 ± 0.00	1.00 ± 0.00
2F	a	nd	1.80 ± 0.00	0.80 ± 0.01	1.90 ± 0.04c	nd	nd	5.70 ± 0.10	0.80 ± 0.05	3.15 ± 0.04
	f	nd	nd	0.40 ± 0.00c	0.40 ± 0.05d	nd	nd	1.55 ± 0.00b	nd	0.70 ± 0.01
2M	a	0.15 ± 0.01a	0.50 ± 0.03	0.60 ± 0.03	0.85 ± 0.98	nd	nd	3.80 ± 0.03c	0.50 ± 0.03	8.45 ± 0.02
	f	0.60 ± 0.02	nd	1.00 ± 0.04	0.40 ± 0.01de	nd	nd	2.10 ± 0.07	nd	0.60 ± 0.01c
2A	a	0.15 ± 0.01a	2.10 ± 0.07c	5.35 ± 0.27	1.75 ± 0.06	nd	nd	3.95 ± 0.08c	1.20 ± 0.03b	5.00 ± 0.08d
	f	1.35 ± 0.01b	nd	0.60 ± 0.03c	0.50 ± 0.01e	nd	nd	1.70 ± 0.05b	nd	0.50 ± 0.01c
2N	a	0.35 ± 0.01	2.20 ± 0.04c	2.15 ± 0.04C	1.90 ± 0.04cA	nd	nd	4.30 ± 0.06	1.10 ± 0.03b	4.86 ± 0.07d
	f	1.55 ± 0.15b	nd	2.35 ± 0.03C	1.70 ± 0.11A	nd	nd	0.30 ± 0.00	nd	3.90 ± 0.15
3F	a	2.40 ± 0.12A	4.80 ± 0.09C	0.80 ± 0.01	1.10 ± 0.22	nd	nd	4.40 ± 0.04dA	0.45 ± 0.01c	5.00 ± 0.01
	f	2.20 ± 0.24A	4.95 ± 0.50C	1.65 ± 0.76	0.65 ± 0.01	nd	nd	4.35 ± 0.08A	nd	2.00 ± 0.05
3M	a	1.15 ± 0.08	1.60 ± 0.02D	nd	1.60 ± 0.01f	nd	nd	4.90 ± 0.04	1.00 ± 0.02	1.35 ± 0.06A
	f	1.50 ± 0.16	1.45 ± 0.26D	1.90 ± 0.03	0.10 ± 0.00	nd	nd	1.30 ± 0.02e	0.15 ± 0.00	1.10 ± 0.01A
3A	a	1.90 ± 0.09B	2.00 ± 0.32	2.70 ± 0.08d	1.65 ± 0.02f	nd	nd	4.25 ± 0.05d	0.60 ± 0.03c	5.45 ± 0.08
	f	1.80 ± 0.10B	3.00 ± 0.02	0.80 ± 0.05	1.00 ± 0.01 g	nd	nd	2.95 ± 0.11	nd	2.95 ± 0.03
3N	a	2.00 ± 0.11	1.10 ± 0.04	1.50 ± 0.06	2.00 ± 0.12	nd	nd	3.95 ± 0.41	1.55 ± 0.02	5.90 ± 0.22
	f	2.50 ± 0.02	2.00 ± 0.02	2.80 ± 0.03d	0.80 ± 0.00 g	nd	nd	1.20 ± 0.23e	nd	0.70 ± 0.07
4F	a	nd	2.70 ± 0.01	nd	nd	nd	nd	nd	tr.	nd
	f	nd	5.00 ± 0.40	0.55 ± 0.04e	nd	nd	nd	nd	nd	nd
4M	a	nd	1.05 ± 0.04	1.05 ± 0.04	nd	nd	nd	nd	0.15 ± 0.00dA	nd
	f	nd	nd	nd	nd	nd	nd	nd	0.10 ± 0.00A	nd
4A	a	nd	nd	2.70 ± 0.01	nd	nd	nd	nd	0.20 ± 0.00d	nd
	f	nd	0.55 ± 0.04d	5.00 ± 0.40	nd	nd	nd	nd	nd	nd
4N	a	nd	0.85 ± 0.01	0.85 ± 0.01	nd	nd	nd	nd	tr.	tr.
	f	1.40 ± 0.15	0.45 ± 0.01d	0.45 ± 0.01e	nd	nd	nd	nd	nd	nd
5F	a	nd	nd	nd	0.60 ± 0.01 h	0.45 ± 0.02a	0.80 ± 0.01a	0.75 ± 0.01f	0.30 ± 0.00e	0.40 ± 0.01e
	f	nd	3.30 ± 0.38	2.80 ± 0.80f	nd	nd	nd	nd	nd	nd
5M	a	nd	nd	nd	0.30 ± 0.01	nd	0.83 ± 0.00a	0.85 ± 0.00f	0.20 ± 0.00e	0.40 ± 0.01e
	f	nd	1.10 ± 0.00	1.10 ± 0.00	nd	nd	nd	nd	nd	nd
5N	a	nd	nd	nd	0.70 ± 0.01 h	0.60 ± 0.01a	1.50 ± 0.09	1.30 ± 0.01	0.95 ± 0.03	1.20 ± 0.00
	f	1.90 ± 0.12	4.10 ± 0.08	2.60 ± 0.26f	0.40 ± 0.01	tr	tr	0.40 ± 0.01	0.30 ± 0.00	0.90 ± 0.00

[a] Values (mg/g of DW) are expressed as mean ± standard deviation. Values with the same lower case letter within each column are not significantly ($p > 0.05$) different between the months for each plant. Values with the same upper case letter within each column are not significantly ($p > 0.05$) different between the two methods of drying for each plant. [b] (1) *Rosmarinus officinalis*, (2) *Mentha viridis*, (3) *Origanum majorana*, (4) *Laurus nobilis*, (5) *Foeniculum vulgare*; each numbered sample from 1 to 5 is followed by the upper case letter F, M, A, or N, which represents the initial of the examined period (February, May, August, and November, respectively), whereas the lower case letters a and f represent air-dried and freeze-dried samples, respectively. [c] Hydroxybenzoic acid. [d] Quantified and expressed as caffeic acid equivalents (CAE). [e] Not detected. [f] Traces.

ing to the increased antioxidant activity. The occurrence of these phenolic compounds was confirmed by other studies (*3, 6, 9, 40*). For *L. nobilis* extracts, major phenolic constituents were flavonoids (luteolin), the concentration of which was relatively high (ranging from 0.20 to 4.50 mg/g of DW), whereas rosmarinic acid was present in low concentrations. Vanillic acid and 3, 4-dihydroxybenzoic acid were also detected in small amounts in air-dried *L. nobilis*, whereas in freeze-dried samples it was found in high concentrations. Although some phenolic acids are potential antioxidants, the identified ones had a rather small contribution to the total antioxidant capacity of the air-dried *L. nobilis* extracts because they were present in small amounts. In the *F. vulgare* extracts, two flavonoids (quercetin and rutin) were identified, whereas phenolic acids, such as caffeic acid, chlorogenic acid, ferulic acid, and rosmarinic acid, were present in low levels. However, these phenolic acids made a positive contribution to the antioxidant activity of the extracts. HBA and vanillic acid were not detected in air-dried fennel, whereas in the freeze-dried ones they were found in quite high concentrations. The antioxidant activity of the aromatic plant extracts could be partially attributed to the presence of other phenolic compounds that could not be identified due to lack of reference compounds. However, their chemical classes were determined from their chromatographic behavior and UV spectra. On this basis, they were further categorized into two major groups, unknown/unconfirmed phenolic acids (group 1) (**Table 3**) and unknown/unconfirmed flavonoids and their glycosides (group 2) (**Tables 2**). The amount of these groups in the aromatic plants tested ranged from 0.40 to 8.45 mg of CAE/g of DW for group 1 and from traces to 22.00 mg of QE/g of DW for group 2. However, both groups were detected in most of the aromatic plant extracts examined, contributing thus to their high antioxidant capacity.

Overall, considerable variations were found in phenolic compounds at different harvesting periods. In the present study, the quercetin and luteolin contents of plant materials changed significantly during plant development. Generally, higher levels were reached at flowering and full flowering stages. The flavonoid content of both *R. officinalis*, *M. viridis*, and *O. majorana* extracts was significantly higher during the flowering stage and significantly lower during the later vegetative stages. In *R. officinalis* and *M. viridis* extracts, the concentration of quercetin increased significantly from 0.43 to 6.00 mg/g of DW and from 0.35 to 5.20 mg/g of DW, respectively. A considerable increase was observed in the concentration of luteolin, particularly in *R. officinalis* extracts (from 0.40 to 2.50 mg/g of DW). The same was observed in *O. majorana* extracts. The calculated levels of luteolin in *L. nobilis* extracts (the only flavonoid identified in this extract) varied significantly with seasons (from 0.20 to 3.10 mg/g of DW). The highest value was observed during May. Luteolin was not identified in the extracts of *F. vulgare*, whereas quercetin did not vary significantly between

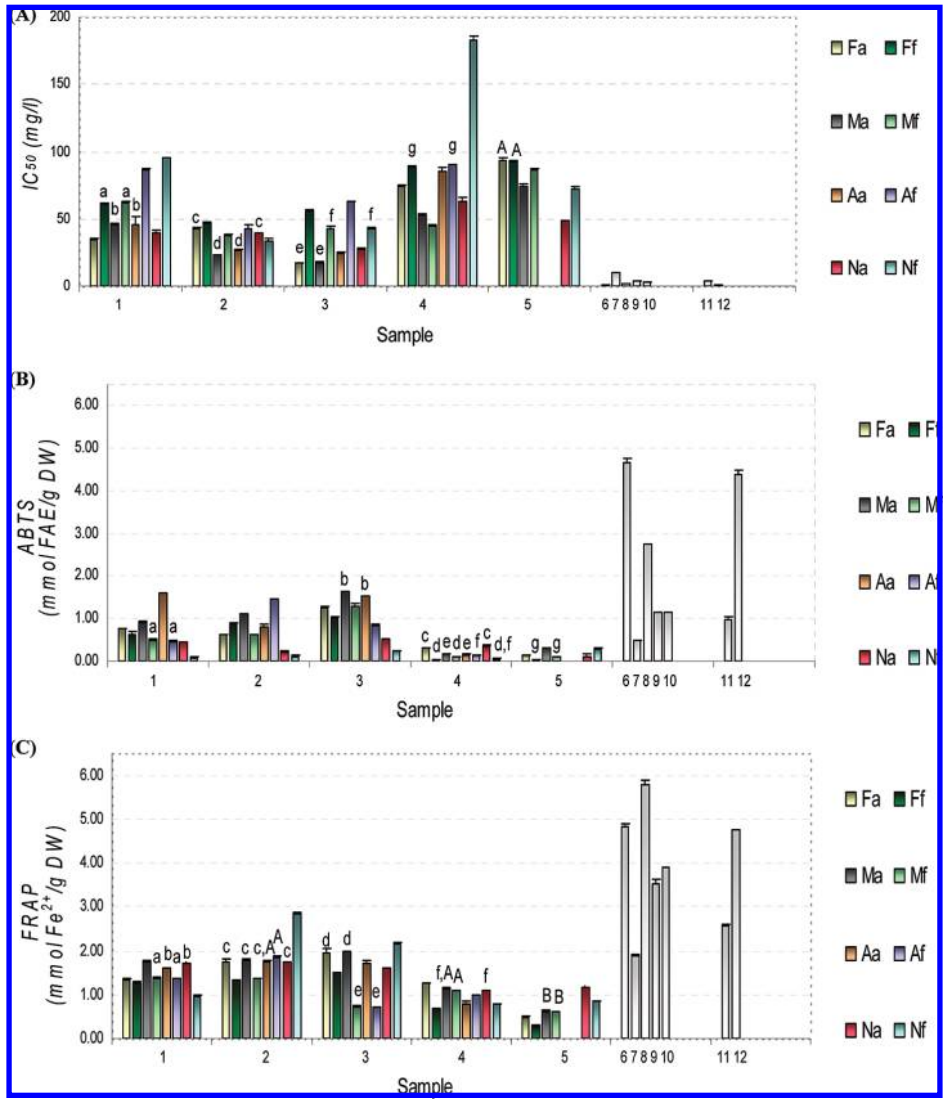

Figure 1. Total antioxidant capacity of air-dried and freeze-dried aromatic plants by (**A**) DPPH, (**B**) ABTS, and (**C**) FRAP assays. Values are expressed as mean ± standard deviation. Samples: (1) *Rosmarinus officinalis*, (2) *Mentha viridis*, (3) *Origanum majorana*, (4) *Laurus nobilis*, (5) *Foeniculum vulgare*, (6) gallic acid, (7) vanillic acid, (8) caffeic acid, (9) ferulic acid, (10) rosmarinic acid, (11) rutin, and (12) quercetin; each numbered sample from 1 to 5 is followed by the upper case letter F, M, A, or N, which represents the initial of the examined period (February, May, August, or November, respectively), whereas the lower case letters a and f represent the initials for air-dried and frieze-dried samples, respectively. Bars with the same lower case letter (a−g) are not significantly ($p > 0.05$) different between months. Bars with the same upper case letter (A, B) are not significantly ($p > 0.05$) different between air-dried and freeze-dried samples.

the late fruiting (February) and before flowering stages (May). Most of the phenolic acids seemed to be affected to a lesser extent by the time of harvest (plant development stage). This fact could be a possible explanation of the small variations in the total antioxidant capacity of the examined plant extracts during 2007. A typical example of these small differences is caffeic acid, the amount of which remained constant during the year in the *R. officinalis* extracts. In *M. viridis*, *O. majorana*, and *F. vulgare* extracts, its level ranged from 0.85 to 1.90, from 1.10 to 2.00, and from 0.30 to 0.70 mg/g of DW, respectively. Caffeic acid was not detected in all *L. nobilis* extracts. Nevertheless, fruiting or bud stages exhibited the highest concentration of most of the phenolic acids present in the examined aromatic plant extracts (**Table 3**).

The freeze-drying process, when compared with the air-drying one, resulted in decreased levels of the main flavonoids in most plant extracts (**Table 2**). Moreover, a considerable decrease of the concentration in the majority of the phenolic acids (mainly hydroxycinnamic acids) was observed in most cases (**Table 3**). This finding is in agreement with the results reported by other researchers, in which freeze-drying caused a loss of 87% of total flavonols and 95% of all simple flavonols present in *Posidonia oceanica* L. extracts (*41*). In the case of *R. officinalis*, *M. viridis*, and *O. majorana* extracts, freeze-drying caused a significant decrease (almost 50%) of the main flavonoids present in the samples. However, this decrease was not accompanied by a reduction of their hydroxybenzoic acids (gallic acid, vanillic acid, and 3,4-dihydroxybenzoic acid). A similar finding was also observed in our extracts obtained from freeze-dried *F. vulgare* and *L. nobilis*. Similar effects, that is, degradation of hydroxycinnamic acids and flavonoids and increase of gallic acid, are also reported by other workers (*42*). Although one could expect that freeze-drying may eliminate factors such as oxidation and radiation that could reduce the polyphenolic content in plant materials, the results obtained in this study do not support this claim. Another disadvantage the high cost of this treatment. The freeze-drying process seems to reduce phenolic concentration. This can be attributed to the hydrolytic degradation that might

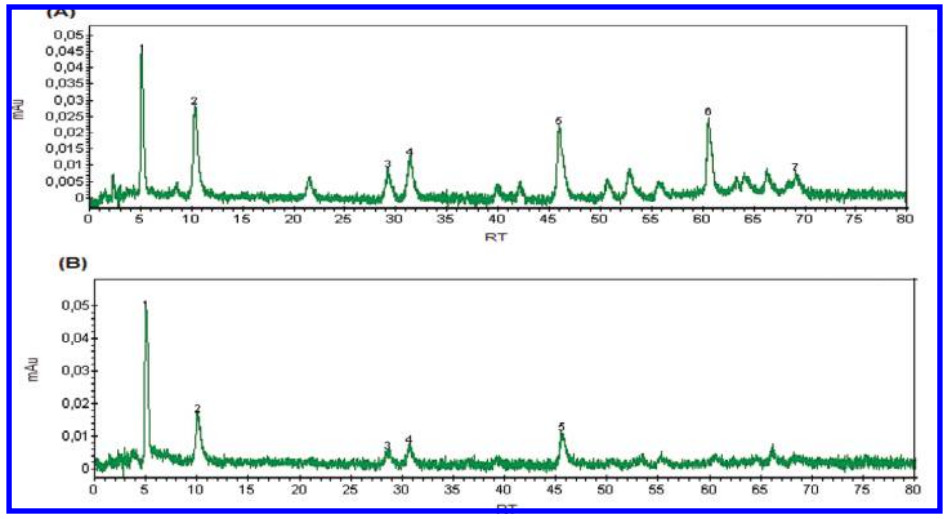

Figure 2. HPLC profiles of *Origanum majorana* obtained from (**A**) air-dried and (**B**) freeze-dried plant material during August 2007. (1) Gallic acid; (2) 3,4-dihydroxybenzoic acid; (3) caffeic acid; (4) vanillic acid; (5) ferulic acid; (6) rosmarinic acid; (7) quercetin. Detection was at 290 nm.

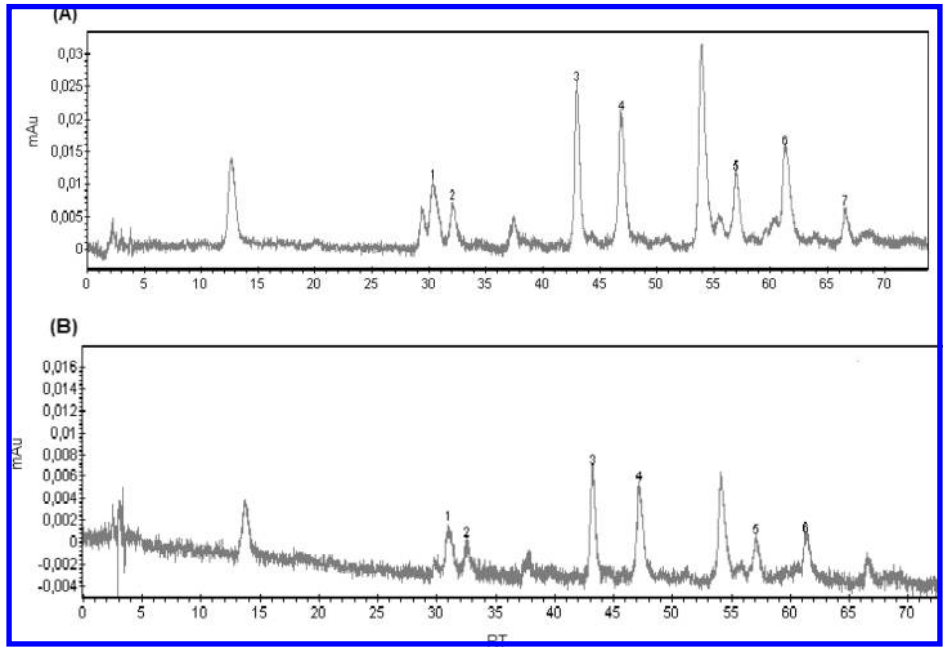

Figure 3. HPLC profiles of *Foeniculum vulgare* obtained from (**A**) air-dried and (**B**) freeze-dried plant material during November 2007. (1) Caffeic acid; (2) chlorogenic acid; (3) *p*-coumaric acid; (4) ferulic acid; (5) rutin; (6) rosmarinic acid; (7) quercetin. Detection was at 330 nm.

occur during thawing of frozen plant tissue. However, the thawing phenomenon may not be alone responsible for the observed decrease in flavonoid concentrations. Thawing in conjunction with the slow rate of the drying process may aggravate these losses and facilitate such reactions (*41, 43*). Air-drying at ambient temperature seems to be an appropriate method in preserving most of the phenolic compounds present in plant materials, especially flavonoids. However, the proper prehandling method depends upon the chemical species and the part of the plant under consideration (*43*).

Total Antioxidant Capacities. Scavenging of different types of reactive oxygen and nitrogen species, mostly free radicals (both synthetic and biologically relevant), is thought to be one of the main mechanisms of the antioxidant action exhibited by phenolic phytochemicals. In this study, two different radical scavenging models, based on two different radicals, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, were used. The synthetic nitrogen-centered $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals are not biologically relevant, but are often used as "indicator compounds" in testing hydrogen donation capacity and thus antioxidant activity. Furthermore, the FRAP assay was also employed for antioxidant activity estimation. The antioxidant properties of the methanolic extracts may correlate with their reducing capacity. This is due to the fact that the antioxidant activity is thought to be concomitant with the development of reductones, which are reported to be terminators of free radical chain reactions (*44, 45*).

Parts **A** and **B** of **Figure 1** show that the extracts had varying degrees of scavenging action against the two radicals used. In the $DPPH^{\bullet}$ assay, the three air-dried plant extracts of the Lamiaceae family showed the highest activity with IC_{50} values ranging from 34.20 to 46.00 mg/L (*R. officinalis*), from 23.30 to 42.25 mg/L (*M. viridis*), and from 17.50 to 27.35 mg/L (*O. majorana*), followed by *L. nobilis* (52.50−85.40 mg/L) and *F. vulgare* extracts (48.40−93.10 mg/L). *R. officinalis*, *M. viridis*, and *O. majorana* extracts were found to have rather similar behaviors with regard to $ABTS^{\bullet+}$. *L. nobilis* and *F. vulgare* again showed the lowest activities (0.15−0.40 and 0.10−0.30 mmol of FAE/g of DW, respectively). The FRAP assay (**Figure 1C**) revealed that the most

effective extracts were those obtained from *R. officinalis* (1.35−1.80 mmol of Fe^{2+}/g of DW), *M. viridis* (1.75−1.80 mmol of Fe^{2+}/g of DW), and *O. majorana* (1.60−2.00 mmol of Fe^{2+}/g of DW), which did not practically differ from each other in terms of activity. Less effective extracts were those obtained from *L. nobilis* (0.80−1.25 mmol of Fe^{2+}/g of DW) and *F. vulgare* (0.50−1.20 mmol of Fe^{2+}/g of DW). Our results are in agreement with most of the studies concerning the antioxidant activities of the Lamiaceae family aromatic plants (*6, 11, 46*). Possible differences in the antioxidant activity of the plants tested from those in literature can be attributed to genotype and environmental differences within species, choice of parts tested, time of taking samples, and determination methods. However, all plant extracts had antioxidant activity lower than that of the reference compounds used in this study (**Figure 1**).

Total antioxidant capacities of each of the five aromatic plant extracts indicated slight variations within the year 2007 (**Figure 1**). These variations can be attributed to different harvesting seasons of the aromatic plants under investigation (different plant development stages), the degree of which seems to be affected by the environmental changes that occurred throughout the year. All plant materials were harvested from the same location, and therefore they were subject to the same growing conditions. From the estimated IC_{50} values (**Figure 1A**), extracts from air-dried *M. viridis* and *O. majorana* demonstrated the strongest antioxidant activity during the flowering stage (May) (23.30 and 17.50 mg/L, respectively). Both values differed significantly between the periods tested but not from those observed during August (for *M. viridis*) and February (for *O. majorana*). In the case of *R. officinalis* extracts, obtained from air-dried plant material, a significantly stronger antioxidant activity was observed before the flowering stage (34.20 mg/L). Extracts from dried *L. nobilis* and *F. vulgare* were less efficient in terms of antioxidant activity than the above-mentioned plants. Nevertheless, their lowest IC_{50} values (higher antioxidant activity) were observed during the early fruiting stage (52.50 and 48.40 mg/L, respectively), differing significantly from the other stages. Both $ABTS^{\bullet}$ and FRAP assays (**Figure 1B**,**C**) showed a similar variation behavior during harvesting periods for each air-dried plant material.

Extracts of the selected air-dried plant materials showed significantly higher antioxidant activities than the extracts of the freeze-dried ones. As shown in **Figure 1A**, their IC_{50} values were reduced by almost 50% with respect to those of the freeze-dried samples. This was particularly noticeable in *R. officinalis* and *O. majorana* extracts, in which the range of the IC_{50} values decreased from 95.24−60.60 to 46.00−34.20 mg/L and from 62.80−42.40 to 27.35−17.50 mg/L, respectively. A considerable decrease (by 50−60%) of the antioxidant efficacy determined by FRAP and ABTS assays was also observed in most of the freeze-dried plant materials.

It is important to note that the degree of capability of the plant extracts examined to deactivate free radicals such as $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ and to reduce iron(III), of both air-dried and freeze-dried samples, seems to be affected by the level of phenolic content. However, the order of antioxidant activity for the *R. officinalis* and *M. viridis* extracts did not seem to depend on total phenolic content, as these plants showed similar activities. Thus, it is worth noting that the relationship between the content of particular antioxidants and antioxidant activity is difficult to explain on the basis of only a quantitative analysis, as synergistic action taking place among the phenolic constituents present in natural extracts may contribute to differences in the antioxidant ability of plant extracts. This is a point that needs further investigation (*6, 42*).

The results demonstrated the fact that the antioxidant behavior and the total phenolic content of the selected aromatic plant extracts varied considerably between air-dried and freeze-dried plant materials. Small variations were observed between harvesting periods. Extracts from air-dried *M. viridis*, *O. majorana*, and *R. officinalis* demonstrated the greatest antioxidant activity during the flowering stage, whereas extracts from dried *L. nobilis* and *F. vulgare* were less efficient in terms of antioxidant activity, with the highest values being observed during the early fruiting stage. The identified flavonoids (quercetin, luteolin, and rutin) were detected in significantly higher amounts during the flowering stages. The highest phenolic acid content was observed during the fruiting stages, although no significant differences were observed between the harvesting periods. However, in all cases, it can be pointed out that there is a tendency for flavonoids to increase with advancing development stages, whereas phenolic acids seem to follow an opposite pattern. Additionally, the antioxidant activities of all the freeze-dried samples were significantly lower than those observed in air-dried plant materials. This fact could be attributed to the significant decrease of most of the phenolic compounds present in the selected aromatic plant extracts. This finding is worthy of further investigation.

SAFETY

Ethanol is a highly flammable and slightly polluting substance; methanol is very flammable and toxic. Safety glasses should always be worn, and a fume cupboard should be used. For $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals, contact with skin and eyes should be avoided and protective clothing should be worn. Eye/face protection is also necessary. For all other reagents suitable gloves should be worn and contact with the skin and eyes should be avoided.

ABBREVIATIONS USED

DW, dry weight; GAE, gallic acid equivalents; ECE, (−)-epicatechin equivalents; CAE, caffeic acid equivalents; QE, quercetin equivalents; FAE, ferulic acid equivalents; HPLC, high-performance liquid chromatography diode array detection; DAD, diode array detector; IC_{50}, inhibitory concentration of substrate (mg/L) that causes 50% loss of the DPPH activity (color).

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