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Screening of Free Radical Scavenging Compounds in Water Extracts of *Mentha* Samples Using a Postcolumn Derivatization Method

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An on-line high-performance liquid chromatography−1,1-diphenyl-2-picrylhydrazyl (HPLC−DPPH•) method has been improved for the detection of polar and nonpolar radical scavenging compounds in complex plant extracts. Nine water extracts were prepared from different *Mentha* species, varieties, hybrids, and cultivars. After the components within each extract had been separated by reverse phase chromatography using 10−100% methanol with 2% acetic acid as a mobile phase, analytes within the eluent capable of scavenging a citric acid−sodium citrate-buffered methanol 1,1-diphenyl-2-picrylhydrazyl solution were detected by postcolumn derivatization at 517 nm. The HPLC−DPPH• on-line method was applied to the qualitative and quantitative analysis of *Mentha* extracts. There was a strong correlation between the scavenging (negative) peak area and the concentration of the radical scavenging reference substances used. The minimum detectable concentration (μg/mL) of the antioxidant compounds was determined. Caffeic acid, eriocitrin (eriodictyol-7-*O*-rutinoside), luteolin-7-*O*-glucoside, and rosmarinic acid were identified as the dominant radical scavengers in these extracts by this method.

KEYWORDS: ***Mentha*; 1,1-diphenyl-2-picrylhydrazyl (DPPH•); high-performance liquid chromatography; HPLC-DPPH•; antioxidant activity; free radicals; phenolics**

INTRODUCTION

In living organisms, various reactive oxygen species (ROS). e.g., superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and nonradical compounds, can be formed by different mechanisms. Such species are considered to be important causative factors in the development of diseases such as malaria, diabetes, stroke, arteriosclerosis, cancer, and cardiovascular diseases and the aging process. ROS can also cause a change in the organoleptic properties of foods by the oxidative degradation of their constituent lipids (*1*−*3*).

Antioxidants are added to foods, oils, and fats to retard the oxidation of lipids. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ) have been used to extend the shelf life of foods, which are susceptible to lipid oxidation. However, some toxic effects of synthetic antioxidants have been reported (*4*, *5*). Therefore, many researchers have focused on the search for natural compounds with antioxidant properties. There is an increasing interest in the radical scavenging activities of some natural compounds especially those found in edible plants, which may play a role in delaying the onset of various chronic diseases. Some fruits and berries, herbs and spices, and vegetables have been found to possess radical scavenging capacity (*6*−*8*). Phytochemical analysis of these materials revealed the presence of polyphenolic phytochemicals, viz., hydroxybenzoic and hydroxycinnamic derivatives and flavonoids among others, which play a major role in the taste and color characteristics and exhibit in vitro radical scavenging antioxidant activity (*9*, *10*). The antioxidant activity depends on the position and degree of hydroxylation, polarity, solubility, reducing potential, and stability of phenolic radicals (*11*).

According to the literature, members of the family of Lamiaceae (Labiatae) appear to be a rich source of polyphenolic compounds. Plants containing flavonoids and other phenolics are known to possess strong antioxidant properties. Some Lamiaceae plants such as *Salvia*, *Mentha*, and *Origanum* species are used as herbal teas for their (folk) medicinal properties. Some flavonoids present in tea infusions may have protective effects against coronary heart disease, cancer, or allergy (*12*−*14*).

Mentha species (Lamiaceae) are a source of essential oil and are used as a condiment in various foods such as some beverages, ice creams, candies, cakes, and meats to give taste and odor. Extracts isolated from members of the genus *Mentha*

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Table 1. Extract Yield and HPLC Qualitative–Quantitative Data for Water Soluble *Mentha* Extracts

		identified components[a]									
sample[b]	EY[c]	caffeic acid (13.4)[d]	eriocitrin (21.8)	luteolin -glu (25.8)	naringenin -glu (26.7)	isorhoifolin (28.7)	rosmarinic acid (30.1)	eriodictyol (32.9)	luteolin (40.9)	apigenin (42.2)	Σ
1	33.4	0.02 ± 0.00	6.41 ± 0.07	6.62 ± 0.13	ND[e]	0.94 ± 0.02	9.33 ± 0.13	ND	ND	ND	23.3 ± 0.19
2	34.2	0.27 ± 0.00	0.37 ± 0.02	4.36 ± 0.04	0.30 ± 0.01	0.34 ± 0.01	5.26 ± 0.01	ND	0.03 ± 0.00	0.08 ± 0.00	11.0 ± 0.04
3	28.1	0.33 ± 0.01	8.78 ± 0.08	5.57 ± 0.03	ND	0.30 ± 0.00	12.11 ± 0.12	ND	ND	ND	27.1 ± 0.15
4	33.2	0.22 ± 0.00	40.27 ± 0.18	2.70 ± 0.01	ND	0.89 ± 0.03	8.44 ± 0.15	0.32 ± 0.00	ND	ND	52.8 ± 0.06
5	36.5	0.68 ± 0.01	0.40 ± 0.01	4.26 ± 0.04	0.19 ± 0.00	0.21 ± 0.01	4.76 ± 0.12	ND	0.28 ± 0.00	ND	10.8 ± 0.13
6	28.5	0.37 ± 0.01	0.37 ± 0.00	0.28 ± 0.01	ND	0.17 ± 0.00	0.66 ± 0.02	ND	ND	ND	1.85 ± 0.02
7	31.5	0.22 ± 0.00	23.39 ± 0.09	1.90 ± 0.02	ND	0.21 ± 0.01	4.77 ± 0.07	0.17 ± 0.00	ND	ND	30.7 ± 0.12
8	23.1	0.19 ± 0.01	10.70 ± 0.09	5.57 ± 0.03	ND	1.26 ± 0.02	4.60 ± 0.08	ND	0.54 ± 0.01	0.04 ± 0.00	22.9 ± 0.13
9	36.6	ND	6.66 ± 0.15	9.15 ± 0.11	0.07 ± 0.00	1.10 ± 0.04	6.45 ± 0.16	ND	ND	ND	23.4 ± 0.25

[a] Values (mg g^{-1}) are expressed as means ± standard deviation. [b] Samples: 1, *M. aquatica*; 2, *M. arvensis* var. *japanensis*; 3, *M.* × *dalmatica*; 4, *M.* × *piperita* Frantsila; 5, *M. haplocalyx*; 6, *M.* Moroccan; 7, *M.* Native Wilmet; 8, *M. spicata* var. *crispa*; and 9, *M.* × *verticillata*. [c] EY, extract yield % (w w^{-1}). [d] Retention time (min). [e] ND, not detected; glu, glucoside.

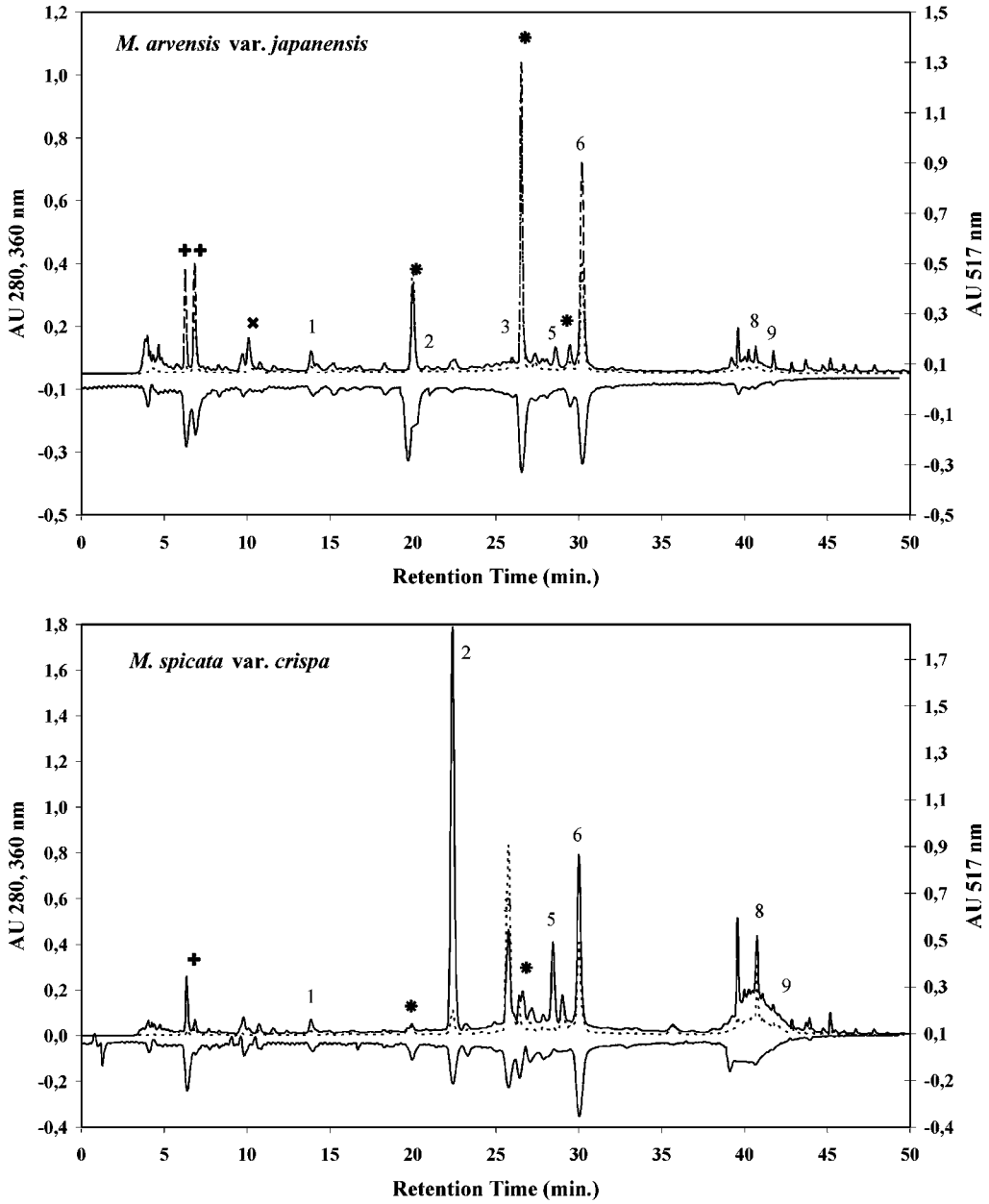

Figure 1. HPLC–PDA analysis (positive peaks) with detector responses at 280 and 360 nm overlaid and DPPH• radical quenching profile (negative peaks) for two *Mentha* extracts. Peaks: 1, caffeic acid; 2, eriocitrin; 3, luteolin-7-*O*-glucoside; 4, naringenin-7-*O*-glucoside; 5, isorhoifolin; 6, rosmarinic acid; 7, eriodictyol; 8, luteolin; 9, apigenin; +, benzoates; ×, hydroxycinnamates; and *, flavonoids.

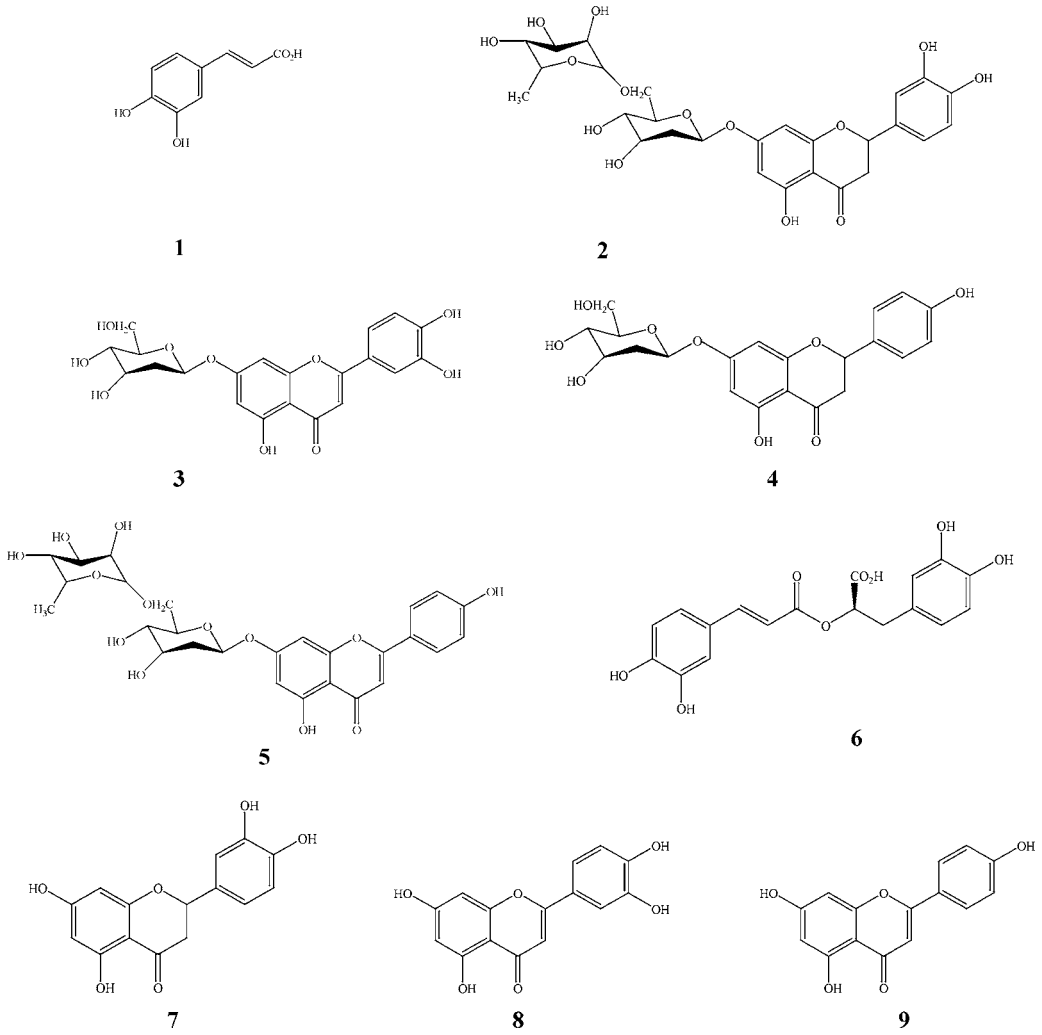

Figure 2. Structural formulas of the identified components within the extracts. Compounds: **1**, caffeic acid; **2**, eriocitrin; **3**, luteolin-7-*O*-glucoside; **4**, naringenin-7-*O*-glucoside; **5**, isorhofolin; **6**, rosmarinic acid; **7**, eriodictyol; **8**, luteolin; and **9**, apigenin.

are widely used in herbal and pharmaceutical preparations and in traditional medicine as spasmolytic and antibacterial agents. In the infusion of the mint, it has been suggested that 75% of the polyphenolic compounds present may be responsible for the claimed physiological activity (*13*).

Activity-guided fractionation of plant extracts is a time-consuming, labor intensive, and expensive process and often leads to loss of activity during the isolation and purification procedures due to dilution effects or decomposition (*15*, *16*). Furthermore, a successful fractionation may only identify previously characterized compounds and not novel ones (*15*). For this reason, the availability of a rapid and cost effective method for screening and activity evaluation of samples is essential in order to avoid many of the above-mentioned problems. A method combining separation and activity evaluation would present a major advantage for such investigations. However, reports concerning on-line separation and antioxidant activity assessment are scarce. Reported high-performance liquid chromatography (HPLC) separation coupled with activity include chemiluminescent reactions (*17*, *18*) and reactions with the relatively stable nitrogen-centered free radical species such as 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) (*16*−*20*) and 2,2′-azobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (*21*). However, the HPLC−DPPH$^{\bullet}$ on-line activity test is currently more widely used than any other technique. The main reasons for this are that (i) this free radical is the most commonly used reactive species for the determination of free radical scavenging properties of potential antioxidants and (ii) the available DPPH$^{\bullet}$-based tests are simple, convenient, and adaptable to the screening of large numbers of compounds/extracts (*22*).

Table 2. Minimum Detection Amount and Correlation Coefficients of Phenolic Standards in the On-Line HPLC–DPPH$^{\bullet}$ System

	visible detection		UV detection
standards	MDC (μg μL^{-1})	calibration coefficient (r^2)	calibration coefficient (r^2)
caffeic acid	6.65	0.9966	0.9998
eriocitrin	20.52	0.9996	0.9999
luteolin-7-*O*-glucoside	21.96	0.9927	0.9999
naringenin-7-*O*-glucoside			0.9981
isorhoifolin			0.9997
rosmarinic acid	3.67	0.9954	0.9998
eriodictyol	19.76	0.9997	0.9994
luteolin	12.74	0.9946	0.9990
apigenin			0.9991

The aim of the present article is to simultaneously determine the qualitative−quantitative composition and identify radical scavenging compounds in water extracts of selected *Mentha* samples, using a postcolumn DPPH$^{\bullet}$ radical reaction. The active phenols were analyzed quantitatively using an HPLC−UV method. In addition, the postcolumn HPLC−DPPH$^{\bullet}$ method was

Table 3. Qualitative–Quantitative Data of Water Soluble *Mentha* Extracts by the HPLC–DPPH• Assay

	UV detection				visible detection			
	280 nm			360 nm	517 nm			
sample[b]	caffeic acid	eriocitrin	rosmarinic acid	luteolin-glu	caffeic acid	eriocitrin	luteolin-glu	rosmarinic acid
1	0.02 ± 0.00 [a]	6.41 ± 0.07	9.33 ± 0.13	6.62 ± 0.13	0.015 ± 0.00	5.86 ± 0.20	5.65 ± 0.22	7.18 ± 0.75
2	0.27 ± 0.00	0.37 ± 0.02	5.26 ± 0.01	4.36 ± 0.04	0.21 ± 0.01	0.30 ± 0.06	4.07 ± 0.25	3.65 ± 0.17
3	0.33 ± 0.01	8.78 ± 0.08	12.11 ± 0.12	5.57 ± 0.03	0.27 ± 0.00	6.06 ± 0.29	5.04 ± 0.16	8.41 ± 0.63
4	0.22 ± 0.00	40.27 ± 0.18	8.44 ± 0.15	2.70 ± 0.01	0.21 ± 0.01	31.32 ± 0.56	2.49 ± 0.25	5.75 ± 0.11
5	0.68 ± 0.01	0.40 ± 0.01	4.76 ± 0.12	4.26 ± 0.04	0.65 ± 0.04	0.38 ± 0.02	3.66 ± 0.44	3.35 ± 0.18
6	0.37 ± 0.01	0.37 ± 0.00	0.66 ± 0.02	0.28 ± 0.01	0.30 ± 0.01	0.27 ± 0.03	0.25 ± 0.03	0.45 ± 0.04
7	0.22 ± 0.00	23.39 ± 0.09	4.77 ± 0.07	1.90 ± 0.02	0.20 ± 0.01	18.42 ± 0.53	1.48 ± 0.22	3.58 ± 0.42
8	0.19 ± 0.01	10.70 ± 0.09	4.60 ± 0.08	5.57 ± 0.03	0.13 ± 0.01	9.12 ± 0.45	3.86 ± 0.26	3.58 ± 0.42
9	ND	6.66 ± 0.15	6.45 ± 0.16	9.15 ± 0.11	ND	6.15 ± 0.09	7.38 ± 0.79	4.64 ± 0.59

[a] Values (mg g^{-1}) are expressed as means ± standard deviation. [b] Samples: 1, *M. aquatica*; 2, *M. arvensis* var. *japanensis*; 3, *M.* × *dalmatica*; 4, *M.* × *piperita* Frantsila; 5, *M. haplocalyx*; 6, *M.* Moroccan; 7, *M.* Native Wilmet; 8, *M. spicata* var. *crispa*; and 9, *M.* × *verticillata*. ND, not detected; glu, glucoside.

compared with previously published spectrophotometric results (*23*).

MATERIALS AND METHODS

Materials. *Mentha aquatica* was obtained from the Botanical Institute of Linz, Austria; *Mentha arvensis* var. *japanensis* and *Mentha* Native Wilmet were from the Agrifood Research North Ostrobotnia Research Station, Finland; *Mentha* × *dalmatica* was from the Agrifood Research Horticultural Institute, Finland; *Mentha* × *piperita* Frantsila was from the Frantsila Herb Garden, Finland; *Mentha haplocalyx* was from the Baoding Agricultural College, China; *Mentha* Morocco was from Joroinen, Finland; *Mentha spicata* var. *crispa* was from the Botanical Gardens, University of Helsinki, Finland; and *Mentha* × *verticillata* was from the Botanical Gardens, University of Turku, Finland. Ultrapure water (HPLC grade) was prepared using a Millipore Milli-RO 12 plus system (Millipore Corp., Bedford, MA). Standards were purchased from Extrasynthase (Genay, France). All remaining solvents and reagents were of analytical grade and were purchased from the usual sources.

Preparation of Freeze-Dried Extracts. Air-dried aerial material was suspended in ultrapure H_2O and deodorized by hydrodistillation, using a European Pharmacopoeian hydrodistillation apparatus. The resulting water extracts were then filtered, reduced in volume in vacuo (45 °C), freeze-dried, and stored at 4 °C until analyzed.

HPLC Analysis. The liquid chromatographic apparatus (Waters 600) consisted of an in-line degasser, pump, and controller coupled to a 2996 photodiode array detector equipped with a Rheodyne injector (20 μL sample loop) interfaced to a PC running Millenium^{32} chromatography manager software (Waters Corp., Milford, MA). Separations were performed on a reverse phase Hypersil BDS-C18 analytical column (250 mm × 4.6 mm i.d.; particle size, 5 μm) (Agilent Technologies, Milford, MA) operating at room temperature with a flow rate of 0.7 mL/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200 and 550 nm. Elution was effected using a ternary nonlinear gradient of the solvent mixture MeOH:H_2O:CH_3COOH (10:88:2, v/v/v) (solvent A), MeOH:H_2O:CH_3COOH (90:8:2, v/v/v) (solvent B), and MeOH (solvent C). The composition of B was increased from 15 to 30% in 15 min, increased to 40% in 3 min and held for 12 min, increased to 100% in 5 min, and then, the composition of C was increased to 15% in 2 min, increased to 30% in 11 min, and then returned to the initial condition in 2 min. The components were identified by comparison of their retention times to those of authentic standards under analysis conditions and UV spectra using our in-house photodiode array (PDA) library. A 10 min equilibrium time was allowed between injections.

Quantitative Analysis. All extracts and standards were dissolved in 70% (aqueous) methanol to a concentration of 10 and 1 mg mL^{-1}, respectively. The concentration used for the calibration of caffeic acid, naringenin-7-*O*-glucoside, eriodictyol, luteolin, isorhoifolin, and apigenin was 0.01−0.10 mg mL^{-1}, 0.008−0.255 mg mL^{-1} for rosmarinic acid, and 0.02−0.50 mg mL^{-1} for luteolin-7-*O*-glucoside and eriocitrin (eriodictyol-7-*O*-rutinoside). Quantitative data of each phenolics were obtained from both UV detection (280/360 nm) and activity detection (517 nm).

HPLC Postcolumn Derivatization. On-line postcolumn addition of the DPPH• reagent was performed using a Waters 515 HPLC pump. DPPH• radical scavenging detection was carried out at 517 nm with a sensitivity of 0.05 aufs using a 2487 Dual λ Absorbance UV−vis detector (Waters Corp.). A 13 m long reaction coil (0.25 mm i.d., 0.6 min reaction time) PEEK tubing (Waters Corp.) was interfaced between the UV detector and DPPH• reagent pump via a T-junction. The DPPH• reagent was prepared in methanol at the beginning of each day of analysis at a concentration of 70 mg mL^{-1} and kept protected from light. This solution was mixed with citrate buffer at a ratio of 3:1 (v v^{-1}). The buffer, pH 7.6, contained 0.05 M citric acid and 0.05 M sodium citrate solutions. This DPPH• reagent was filtered through a 0.45 μm membrane filter and degassed with nitrogen before use. The flow of DPPH• reagent was 0.7 mL min^{-1}.

Detection Limits and Minimum Detectable Concentrations. Detection limits (LD, arbitrary units) were calculated according to the equation described by Koleva et al. (*19*), where t is the Student's t-statistic and $\sigma_{\text{blank signal}}$ is the standard deviation of the blank signal (n = 15). The minimum detectable concentration (MDC) values in μg mL^{-1} units were calculated for each compound by using the calibration equations. These equations were used to calculate the MDC using the previously calculated LD value as y.

RESULTS AND DISCUSSION

The DPPH• radical possesses a characteristic absorption maximum between 515 and 517 nm, which is diminished in the presence of a compound capable of reducing it to it's hydrazine form by hydrogen/electron donation. The different kinetic behavior of antioxidants is an important factor in the evaluation of antiradical activity (*11*). On the basis of the results of prior experiments where different capillary lengths and i.d. were used (unpublished data), a reaction time of 0.6 min was determined as the optimum antioxidant−DPPH• reaction period. This is in agreement with the literature data, where the optimum reaction time was determined by off- and on-line experiments (*11*, *16*, *17*, *19*).

Chromatographic separations, postcolumn radical scavenging activities, and quantitative analysis of water extracts of several mint species were carried out by on-line HPLC−DPPH• and HPLC−UV methods, respectively. The plant extracts were produced and deodorized (removal of the terpene fraction) by hydrodistillation. The yields of extracts are given in **Table 1**. All of these extracts were separated on the reverse phase HPLC column using an acidic aqueous methanol solvent system, and analyte detection was carried out with PDA detection. After UV detection, the analytes were derivatized with buffered

methanolic DPPH• solution, and the radical scavengers in the extracts were detected at 517 nm. UV and DPPH• quenching chromatograms simultaneously obtained under gradient conditions are presented in **Figure 1**. Reference phenolic compounds in the extracts were also analyzed by on-line HPLC−DPPH• methods both qualitatively and quantitatively. Caffeic acid, rosmarinic acid, eriocitrin (eriodictyol-7-*O*-rutinoside), luteolin-7-*O*-glucoside, naringenin-7-*O*-glucoside, isorhoifolin, eriodictyol, luteolin, and apigenin (**Figure 2**) in the extracts were analyzed quantitatively, and the amounts of phenols in the extracts are given in **Table 1**. The identification of the known compounds in the extracts was carried out by comparing their t_R values and UV spectra with those of standards. Eriodictyol and luteolin derivatives, rosmarinic acid, hesperetin-7-*O*-rutinoside, and apigenin-7-*O*-rutinoside have been reported in different *M.* × *piperita* genotypes by Aerias et al. (*13*). Eriocitrin (eriodictyol-7-*O*-rutinoside) and rosmarinic acid were found as the major compounds (59−67% of total phenolics, respectively) whereas luteolin-7-*O*-rutinoside and hesperidin (10% of total phenolics) were reported to play an important role in the same study. According to the UV spectra and chromatographic behavior, eriocitrin, luteolin-7-*O*-glycoside, and rosmarinic acid were identified as major compounds in the extracts. *M.* × *piperita* Frantsila and *M.* Native Wilmet contained the highest amount of eriocitrin (40.27 and 23.39 mg g^{-1}, respectively). Triantaphyllou et al. (*12*) reported that the *Mentha* extracts contained bound phenolic acids and flavonoids. Therefore, flavone aglycones such as luteolin, apigenin, and eriodictyol were found in lesser amounts in all of the extracts.

For the determination of detection limits of the tested compounds ($LD_{compound}$, μg mL^{-1}), the Student's *t*-statistic $t_{0.05} = 2.07$ for $n = 15$ measurements of the blank signal with a confidence interval of 95% was used. The negative peak resulting from the bleaching of DPPH• by a radical scavenging compound is considered detectable if its height exceeds the calculated $LD_{compound}$. MDCs of compounds were calculated using calibration curves and are shown in **Table 2**.

The calibration curves of negative peaks of each reference phenolics were obtained from the same calibration solutions to validate the on-line HPLC−DPPH• method. As can be seen in **Table 2**, a linear correlation ($r^2 = 0.9927-0.9997$; $n = 4$) was found between the concentration and the negative peak areas. According to these results, this method can be used for quantitating the antiradical activity after HPLC separation. Caffeic acid, eriocitrin (eriodictyol-7-*O*-glucoside), luteolin-7-*O*-glycoside, and rosmarinic acid in the extracts were also calculated using their calibration equations obtained from negative peaks, and the results are given in **Table 3**. As seen in **Figure 3**, linear correlation were found between the UV and the DPPH• methods. The correlation factors ($r^2 = 0.9715-0.9971$; $n = 4$) obtained for the relations of all tested radical scavengers were very high (**Figure 2**). The activity responses were not obtained from naringenin-7-*O*-glycoside, isorhoifolin, or apigenin by using this method. Unknown compounds were identified to the level of phytochemical classes (e.g., benzoate, hydroxycinnamate, and flavonoid) from their recorded spectral data and are shown in **Figure 1**.

Antioxidant and free radical scavenging activities of the samples were tested in our previous study using different methods (*23*). Free radical scavenging activities of the samples were also tested using an in vitro spectrophotometric DPPH• assay. All of the extracts exhibited variable activities, but the extract of *M.* × *piperita* Frantsila was found as the most active

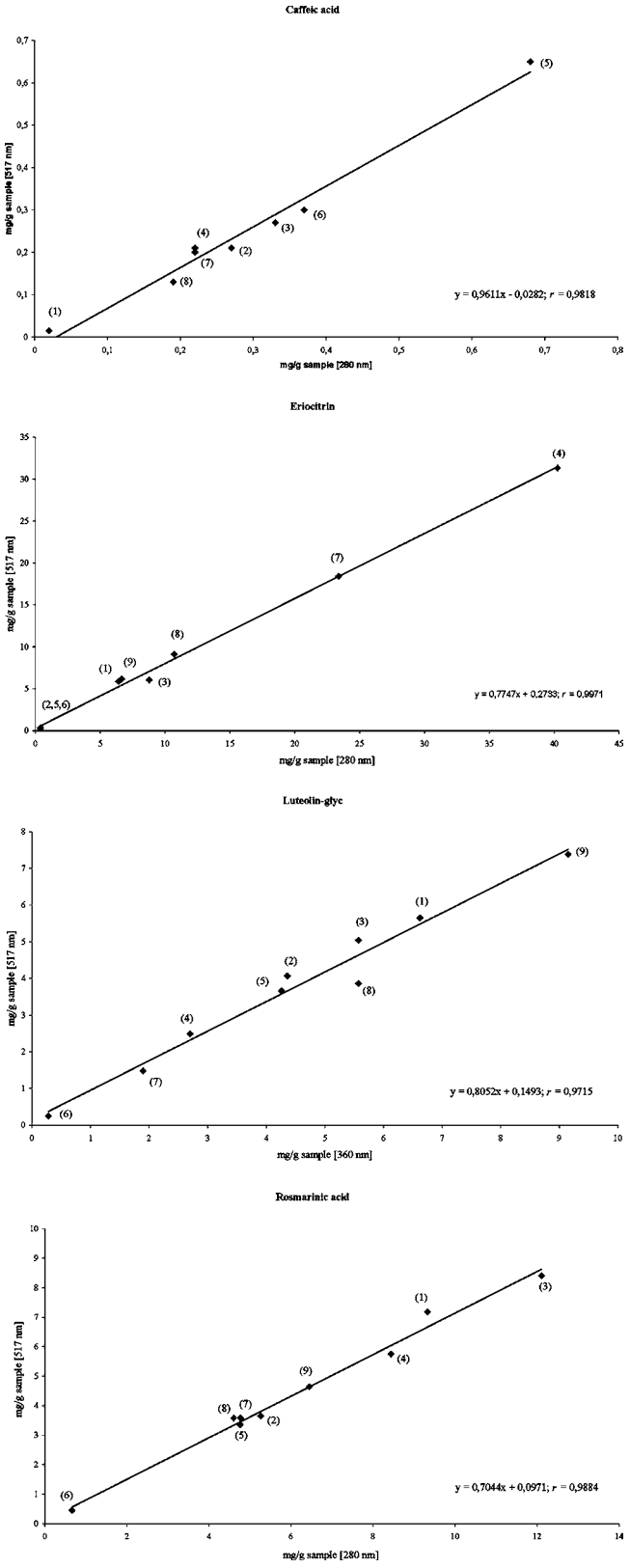

Figure 3. Correlation of amounts of phenolics by the HPLC–DPPH• assay at 280/360 and 517 nm. Samples: 1, *M. aquatica*; 2, *M. arvensis* var. *japanensis*; 3, *M.* × *dalmatica*; 4, *M.* × *piperita* Frantsila; 5, *M. haplocalyx*; 6, *M.* Moroccan; 7, *M.* Native Wilmet; 8, *M. spicata* var. *crispa*; and 9, *M.* × *verticillata*.

in each test system. The maximum total negative peaks were also obtained from *M.* × *piperita* Frantsila using the postcolumn derivatization method. Three unknown radical scavengers, which

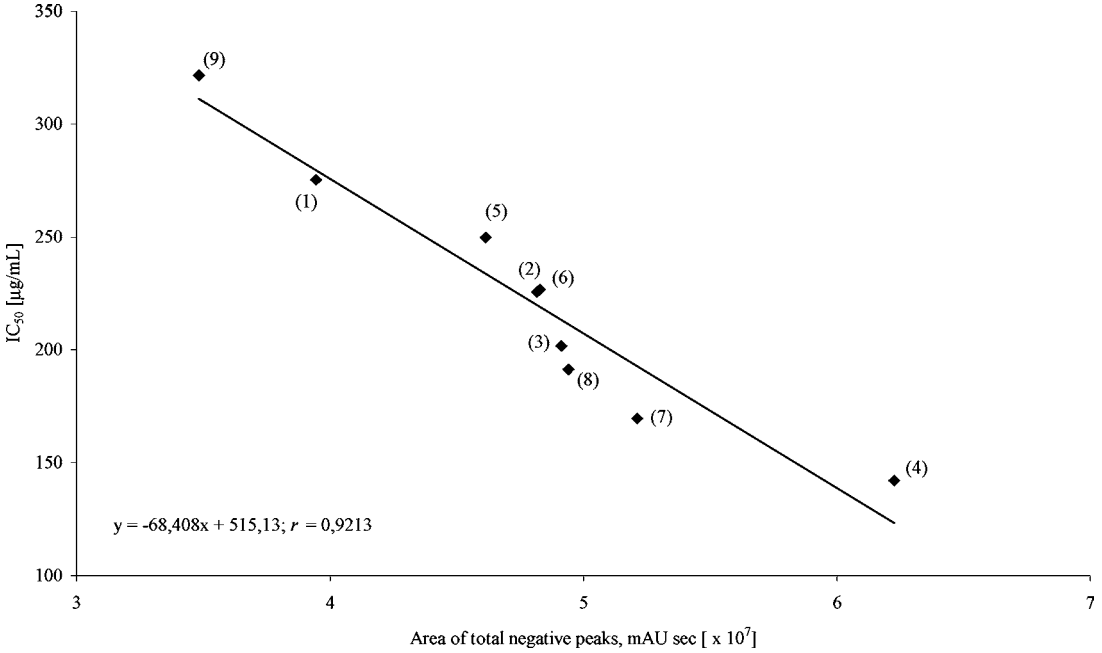

Figure 4. Association between off-line and on-line DPPH• radical scavenging. Samples: 1, *M. aquatica*; 2, *M. arvensis* var. *japanensis*; 3, *M.* × *dalmatica*; 4, *M.* × *piperita* Frantsila; 5, *M. haplocalyx*; 6, *M.* Moroccan; 7, *M.* Native Wilmet; 8, *M. spicata* var. *crispa*; and 9, *M.* × *verticillata*.

were identified as flavonoid glycosides using PDA spectral data, were detected in the extract of *M.* × *piperita* Frantsila. The same active compounds were also found in the extract of *M.* Native Wilmet. This extract was also found as the second active sample in our previous study (*23*), and it has a second maximum negative peak area. The IC_{50} (μg mL^{-1}) values of the extracts obtained from spectrophotometric analysis in our previous study (*23*) were compared with the total negative peak areas of each extract from the HPLC−DPPH• assay, and a high correlation was found ($r^2 = 0.9158$; $n = 4$) (**Figure 4**). The calibration equation calculated between total negative peak areas and IC_{50} values ($y = -7^{-0.6}x + 516.64$) can be used for calculation of IC_{50} values of different *Mentha* extracts.

Gradient elution and acidic water/organic solvent mixtures are commonly used in reverse phase chromatography for the separation of phenolics from complex plant extracts. Changes in the pH of the mobile phase during an analysis can cause baseline instability and a higher S/N ratio in the on-line HPLC−DPPH• scavenging detection. This baseline shifting and increased baseline noise are the main problems associated with on-line HPLC−DPPH• analyses. Therefore, it is very important to ensure that the eluant is buffered to between 5.0 and 6.5 pH units, the optimum pH range for DPPH•−hydrogen donation reactions. An HPLC technique coupled to an on-line (postcolumn) DPPH• radical method is presented, which is an improvement on previously published systems due to a stable, nondrifting baseline with minimal baseline noise throughout a chromatographic gradient elution of widely differing polarity and pH. This allows for a greater sensitivity in the detection of polar and nonpolar antioxidative (free radical scavenging) components in complex crude plant extracts. Furthermore, standard analytical laboratory equipment can be used, e.g., a standard chromatographic pump can be used for delivery of the DPPH• reagent rather than a syringe pump (*17*, *19*), and no special treatments are required, e.g., helium sparging of the DPPH• reagent (*17*).

Plant extracts/fractions and products have complex structures, and isolation and identification of active compounds is a difficult, long, and expensive process. Postcolumn derivatization techniques can be used as a cheap, fast, and efficient alternative. The postcolumn HPLC−DPPH• method given in this study can be successfully used for the qualitative and quantitative analysis of free radical scavengers in complex mixtures. From the chromatogram and combined activity profile figures, sufficient data are available to identify known compounds or the chemical class of unknown components, which possess radical scavenging activity. The observed chromatographic behavior provides important information about conditions required for the isolation of new analytes with such important activity.

LITERATURE CITED

(1) Prior, R. L.; Cao, G. Analysis of botanicals and dietary supplements for antioxidant capacity: A review. *J. AOAC Int.* **2000**, *83*, 950−956.

(2) Yamaguchi, F.; Saito, M.; Ariga, T.; Yoshimura, Y.; Nakazawa, H. Free radical scavenging activity of garcinol from *Garcinia indica* fruit rind. *J. Agric. Food Chem.* **2000**, *48*, 2320−2325.

(3) Alma, M. H.; Mavi, A.; Yildirim, A.; Digrak, M.; Hirata, T. Screening chemical composition and antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. growing in Turkey. *Biol. Pharm. Bull.* **2003**, *26*, 1725−1729.

(4) Choi, H.-S.; Song, H. S.; Ukeda, H.; Sawamura, M. Radical-scavenging activities of citrus essential oils and their components: Detection using 1,1-diphenyl-2-picrylhydrazyl. *J. Agric. Food Chem.* **2000**, *48*, 4156−4161.

(5) Dapkevicius, A.; Venskutonis, R.; van Beek, T. A.; Linssen, J. P. H. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J. Sci. Food Agric.* **1998**, *77*, 140−146.

(6) Shahidi, F.; Naczk, M. *Food Phenolics: Sources, Chemistry, Effects, Applications*; Technomic Publishing: Lancaster, PA, 1995; pp 75−108.

(7) Kähkönen, M. P.; Hopia, A. I.; Heinonen, M. Berry phenolics and their antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 4076−4082.

(8) Shahidi, F.; Janitha, P. K.; Wanasundara, P. D. Phenolic antioxidants. *Crit. Rev. Food Sci.* **1992**, *32*, 67−103.

(9) Escarpa, A.; Gonzáles, M. C. Total extractable phenolic chromatographic index: An overview of the phenolic class contents from different sources of foods. *Eur. Food Res. Technol*. **2001**, *212*, 439−444.
(10) Jiménez, M.; García-Carmona, F. Myricetin, an antioxidant flavonol, is a substrate of polyphenol oxidase. *J. Sci. Food Agric*. **1999**, *79*, 1993−2000.
(11) Bandonienè, D.; Murkovic, M. On-line HPLC-DPPH• screening method for evaluation of radical scavenging phenols extracted from Apples (*Malus domestica* L.). *J. Agric. Food Chem*. **2002**, *50*, 2482−2487.
(12) Triantaphyllou, K.; Blekas, G.; Boskou, D. Antioxidative properties of water extracts obtained from herbs of the species Lamiaceae. *Int. J. Food Sci. Nutr*. **2001**, *52*, 313−317.
(13) Aeias, F. M.; Valentão, P.; Andrade, P. B.; Ferreres, F.; Seabra, R. M. Phenolic fingerprint of peppermint leaves. *Food Chem*. **2001**, *73*, 307−311.
(14) Zgórka, G.; Glowniak, K. Variation of free phenolic acids in medicinal plants belonging to the Lamiaceae family. *J. Pharm. Biol. Anal*. **2001**, *26*, 79−87.
(15) Hostettmann, K.; Wolfender, J.-L.; Terreaux, C. Modern screening techniques for plant extracts. *Pharm. Biol*. **2001**, *39*, 18−32.
(16) Bandonienè, D.; Murkovic, M.; Pfannhauser, W.; Venskutonis, P. R.; Gruzdienè, D. Detection and activity evaluation of radical scavenging compounds by using DPPH• free radical and on-line HPLC-DPPH• methods. *Eur. Food Res. Technol.* **2002**, *214*, 143−147.
(17) Dapkevicius, A.; van Beek, T. A.; Niederländer, H. A. G. Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates. *J. Chromatogr*. **2001**, *912*, 73−82.
(18) Dapkevicius, A.; van Beek, T. A.; Niederländer, H. A. G.; De Groot, A. On-line detection of antioxidative activity in high-performance liquid chromatography eluates by Chemiluminescence. *Anal. Chem*. **1999**, *71*, 736−740.
(19) Koleva, I. I.; Niederländer, H. A. G.; van Beek, T. A. An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. *Anal. Chem.* **2000**, *72*, 2323−2328.
(20) Bandonienè, D.; Murkovic, M. The detection of radical scavenging compounds in crude extract of borage (*Borago officinalis* L.) by using an on-line HPLC-DPPH• method. *J. Biochem. Biophys*. *Methods* **2002**, *53*, 45−49.
(21) Koleva, I. I.; Niederländer, H. A. G.; van Beek, T. A. Application of ABTS•+ radical cation for selective on-line detection of radical scavengers in HPLC eluates. *Anal. Chem.* **2001**, *73*, 3373−3381.
(22) Bouchet, N.; Barrier, L.; Fauconneau, B. Radical scavenging activity and antioxidant properties of tannins from *Guiera senegalensis* (Combretaceae). *Phytother. Res.* **1998**, *12*, 159−162.
(23) Dorman, H. J. D.; Koşar, M.; Kahlos, K.; Holm, Y.; Hiltunen, R. Antioxidant properties and composition of aqueous extracts from *Mentha* species, hybrids, varieties, and cultivars. *J. Agric. Food Chem.* **2003**, *51*, 4563−4569.

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