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QUANTITATIVE DETERMINATION OF PHENOLIC COMPOUNDS IN *Mentha piperita* LEAVES

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A spectrophotometric method using a dual-wavelength Firordt method that enabled simultaneous determination of the flavonoid and phenolic-acid contents was developed for quantitative analysis of phenolic compounds in Mentha piperita *L. (Lamiaceae) leaves. Hesperidin and rosmaric acid were used as standards. The optimal extraction parameters of the phenolic compounds were determined. Metrological analysis of the developed method was performed. It was found that the determination error of the analyzed classes of compounds was less than 3%. The studied batches of* M. piperita *raw material contained flavonoids 3.02–6.32%; phenolic acids, 2.70–5.52%; total phenolic compound content, 5.72–11.51%.*

Keywords: *Mentha piperita* L., Lamiaceae, flavonoids, phenolic acids, quantitative analysis, Firordt method, dual-wavelength spectrophotometer.

Peppermint (*Mentha piperita* L., Lamiaceae) is a medicinal plant that is widely used in practice as a cholegogic, carminative, and aromatic and spasmolytic agent [1]. The biological activity of *M. piperita* is associated primarily with the essential oil, the principal components of which are monoterpenes (menthol, menthylacetate, etc.) [2]. Nevertheless, recent research showed that the phenolic compounds of *M. piperita* (flavonoids, phenolic acids) exhibit antioxidant, cholegogic, anti-allergic, and other types of pharmacological activity [3–5]. Several researchers isolated from *M. piperita* leaves about 40 phenolic compounds including isorhoifolin, menthoside [6], piperitoside [7], nevadensin, hymenoxin, menthocubanone [8], dimethoxy- and dimethylsudachitin, 5-hydroxy-6,7,3′,4′-tetramethoxyflavone [9], eriocitrin, luteolin-7-rutinoside [10], xanthomicrol, gardenin B and D, 5-*O*-demethylnobiletin, 5,3′,4′-trihydroxy-6,7,8-tetramethoxyflavone [11], eupatorin, salvigenin [12], luteolin, sorbifolin, thymusin, thymonin, apigenin, 5,6-dihydroxy-7,3′,4′-trimethoxyflavone, sideritoflavone, 5,6-dihydroxy-7,8-3′,4′-tetramethoxyflavone, ladanein, pebrellin, acacetin [13], luteolin-7-glucoside, eriodictyol-7-glucoside, narirutin, hesperetin, diosmin, rosmaric acid [14], rhoifolin [15], 5,7-dihydroxychromon-7-rutinoside [3], luteolin-7-glucuronide, and caffeic and lithospermic acids [16]. According to HPLC, the dominant components are eriocitrin, hesperidin, and luteolin-7-rutinoside in addition to rosmaric acid. The flavonoid and phenolic acid contents can make up 2.81–17.79 and 0.71–3.86%, respectively [14–16]. Phenolic compounds are reported to have pronounced biological activity and have a significant content in *M. piperita*. They in addition to essential oil components should be considered responsible for the pharmacological effect of this species and must be determined quantitatively for certification of raw material. Quantitative determination of phenolic compounds is not presently required for standardization of *M. piperita*. Therefore, our goal was to develop a quantitative determination method for flavonoids and phenolic acids in *M. piperita* leaves.

The absorption spectrum of the alcohol extract of *M. piperita* shows two maxima at 285 ± 3 and 326 ± 2 nm (Fig. 1). Considering data on the chemical composition of this species, it can be assumed that the first maximum is due mainly to flavonoids (eriocitrin, hesperidin, etc.); the second, to phenolic acids (rosmaric, caffeic, etc.).

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TABLE 1. Regression Analysis, Specific Absorption Coefficients ($E,^{1\%}_{1cm}$), and Working Concentrations for Hesperidin and Rosmaric Acid at 285 and 326 nm

λ, nm	Calibration curve equation[a]	r^2	S_{YX}[b]	$E,^{1\%}_{1\ cm}$	Range[c], μg/mL
		Hesperidin			
285	A = 0.0302·c + 0.0060	0.9935	$2.62\cdot10^{-2}$	312	6.4–26.3
326	A = 0.0063·c + 0.0039	0.9967	$3.84\cdot10^{-3}$	62	31.1–126.4
		Rosmaric acid			
285	A = 0.0300·c + 0.0163	0.9901	$1.89\cdot10^{-2}$	314	6.1–26.1
326	A = 0.0455·c + 0.0112	0.9957	$1.87\cdot10^{-2}$	495	4.2–17.3

[a]A, optical density; *c*, concentration (mg/mL); [b]S_{YX}, standard deviation; [c]region of working concentrations in optical density range 0.2–0.8.

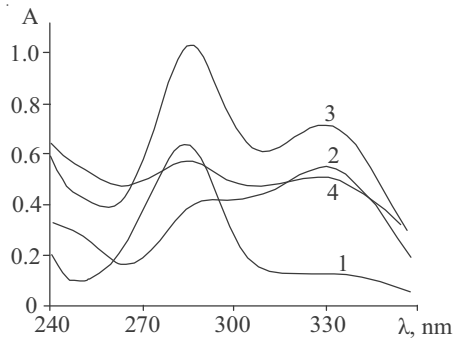

Fig. 1. Absorption spectra of solutions of hesperidin (1, $5.55\cdot10^{-4}$ M), rosmaric acid (2, $5.55\cdot10^{-4}$ M), equimolar mixture of hesperidin and rosmaric acid (3, $5.55\cdot10^{-4}$:$5.55\cdot10^{-4}$ M), and *M. piperita* alcohol extract (4).

Absorption spectra of standards, hesperidin, rosmaric acid, and their mixure, were determined in order to confirm this assumption. It was found that hesperidin typically shows in the studied wavelength range maxima at 283 ± 1 and 326 ± 1 nm; rosmaric acid, 290 ± 2 and 327 ± 1 nm (Fig. 1). The spectrum of an equimolar mixture of the two compounds contains bands at 285 ± 2 and 326 ± 2 nm and is similar to the spectrum of the alcohol extract of *M. piperita*. Therefore, both maxima can be used for analytical purposes. The presence of menthol, the dominant component of *M. piperita* essential oil, has no effect on the absorption spectrum because it does not absorb in the studied wavelength range.

We selected dual-wavelength spectrophotometry (Firordt method) for the analysis. It can take into account the effect from the presence of compounds with spectra that partially overlap each other [17]. Calibration curve equations and specific absorption coefficients of hesperidin and rosmaric acid at 285 and 326 nm were determined in order to perform the calculations (Table 1).

Light absorption was linear for solutions of the tested compounds for optical density values 0.2–0.8 opt. units. The range of working concentrations was 6.4–126.4 μg/mL for hesperidin; 4.1–26.1 μg/mL, rosmaric acid.

A series of hesperidin and rosmaric acid mixtures was analyzed in order to check the accuracy of the dual-wavelength method (Table 2). The error of the concentration determination was less than 5% and could be negative or positive.

Results for hesperidin with dominant rosmaric acid and for rosmaric acid with dominant hesperidin were significantly elevated by using direct spectrophotometry to determine flavonoids at 285 nm and phenolic acids at 326 nm in these two-component solutions (Table 2).

The study of the extraction of phenolic compounds from *M. piperita* leaves established the following optimal parameters: raw material particle size 0.5–2.0 mm, ethanol (70%) extractant, double extraction on a boiling water bath for 60 min each with a 1:50 ratio to extract 98% of the determined groups of compounds.

TABLE 2. Accuracy of Dual-wavelength and Single-wavelength Determination Methods

Mixture No.	Starting wt., μg		Found, μg		Relative determination error, %	
	hesperidin	rosmaric acid	hesperidin	rosmaric acid	hesperidin	rosmaric acid
			Dual-wavelength method			
1	189.00	20.00	179.86	19.72	–4.84	–1.40
2	135.20	40.00	129.74	41.18	–4.04	+2.95
3	101.40	60.00	105.32	57.45	+3.87	–4.25
4	67.60	80.00	66.90	76.33	–1.04	–4.59
5	33.80	100.00	32.55	98.97	–3.70	–1.03
			Single-wavelength method			
1	189.00	20.00	173.07	36.81	+2.41	+84.05
2	135.20	40.00	166.02	56.60	+22.80	+41.50
3	101.40	60.00	163.14	70.51	+60.89	+17.52
4	67.60	80.00	141.19	82.10	+108.86	+2.63
5	33.80	100.00	129.97	94.60	+284.53	–5.40

TABLE 3. Quantitative Determination of Phenolic Compounds in *M. piperita* using Spectrophotometric Methods

Raw matl. No.	Method 1[a]			Method 2[b]; flavonoids, %	Method 3[c]; phenolic compounds, %
	flavonoids, %	phenolic acids, %	total phenolic compounds, %		
1	3.02±0.06	2.70±0.05	5.72	1.18±0.02	3.04±0.08
2	3.81±0.07	5.52±0.12	9.33	0.115±0.002	5.51±0.14
3	6.32±0.12	5.19±0.10	11.51	2.56±0.06	5.34±0.14
4	4.59±0.09	3.74±0.07	8.33	1.72±0.04	3.99±0.10
5	3.74±0.07	4.52±0.09	8.26	0.223±0.004	4.94±0.12

[a]Dual-wavelength spectrophotometry; [b]differential spectrophotometry; [c]Folin method.

Five batches of raw material were analyzed quantitatively for content of phenolic acids using the developed method (Table 3). Raw material was analyzed for comparison using the previously proposed differential spectrophotometry method for determining flavonoids [18] and the Folin method [19] for *M. piperita*.

The flavonoid content in *M. piperita* was 3.02–6.32%; phenolic acids, 2.70–5.52%; total content of phenolic compounds, 5.72–11.51%. The differential spectrophotometry method gave significantly lower results, 0.12-2.56%. Moreover, this analytical method did not consider the content of phenolic acids. Determination by the Folin method also gave low (3.24–5.34%) values although the results agreed satisfactorily with the content of phenolic acids obtained by dual-wavelength spectrophotometry (regression equation: $y = 0.902 \cdot x + 0.655$, where y is the content of phenolic acids determined by the developed method, x, the total content of phenolic compounds by the Folin method; $r^2 = 0.9854$). The reason for this is the fact that the dominant flavanones in *M. piperita* (hesperidin and eriocitrin) reacted much more poorly with Folin reagent than the phenolic acids (rosmaric and caffeic). For example, the optical densities of solutions of the products from reaction of hesperidin and rosmaric acid with Folin reagent were 0.089 and 0.409 opt. units, respectively. This clearly confirms the situation.

A test of the method by the added–found method using hesperidin, rosmaric acid, and their mixture found that the relative determination errors of flavonoids and phenolic acids were less than 3% (Table 4). Table 5 gives the metrological parameters of the method.

The developed method was used to analyze the commercial *M. piperita* preparation "Tincture of peppermint." It was found that the contents of flavonoids and phenolic acids were 0.110–0.119% and 0.054–0.092%, respectively.

TABLE 4. Determination of Phenolic Compounds in *M. piperita* by Added–Found Method

Content, mg[a]		Added, mg		Theoretical content, mg		Found, mg		Determination error			
								1[b]		2[c]	
1[b]	2[c]	3	4	1[b]	2[c]	1[b]	2[c]	abs. mg	rel., %	abs. mg	rel., %
28.457	25.312	7.100	0.000	35.557	25.312	36.127	25.344	+0.570	1.60	+0.032	0.13
28.457	25.312	21.300	0.000	49.757	25.312	50.852	25.590	+1.095	2.20	+0.278	1.10
28.457	25.312	0.000	12.100	28.457	37.412	28.144	37.973	–0.313	1.10	+0.561	1.50
28.457	25.312	0.000	24.200	28.457	49.512	28.998	50.502	+0.541	1.90	+0.990	2.00
28.457	25.312	14.200	12.100	42.657	37.412	43.396	38.043	+0.739	1.73	+0.631	1.69
28.457	25.312	21.300	18.150	49.757	43.462	49.315	44.210	–0.442	0.89	+0.748	1.72

[a]Per 1 g raw matl.; [b]calculated as hesperidin; [c]calculated as rosmaric acid; 1, flavonoids; 2, phenolic acids; 3, hesperidin; 4, rosmaric acid.

TABLE 5. Metrological Parameters of Developed Method (n = 11, P = 95%, $t(p,f)$ = 2.23)[a]

Sample	x_{av}, %	S^2	S_x	±Δx, %	±E, %
		Flavonoids			
M. piperita leaves[b]	6.32	$3.19\cdot10^{-2}$	$5.38\cdot10^{-2}$	0.12	1.90
Tincture of peppermint	0.112	$8.87\cdot10^{-6}$	$8.97\cdot10^{-4}$	0.002	1.79
		Phenolic acids			
M. piperita leaves[b]	5.19	$2.21\cdot10^{-2}$	$4.48\cdot10^{-2}$	0.10	1.93
Tincture of peppermint	0.084	$2.20\cdot10^{-6}$	$4.47\cdot10^{-4}$	0.001	1.19

[a]x_{av}, average value; S^2, dispersion; S_x, mean square deviation; ±Δx, absolute error of the arithmetic mean; E, relative error; [b]raw matl. No. 3.

EXPERIMENTAL

Raw material (*M. piperita* L. leaves) was purchased at drugstores. The manufacturers (raw material batch and number) were ZAO Ivan-Chai (010108, No. 1), OOO Lek C+ (021108, No. 2), ZAO APF Fito-EM (200209, No. 3; 080300, No. 4), ZAO Travy Bashkirii (140807, No. 5), Tincture of peppermint, ZAO EKOlab (series 031106). All studied batches of raw material met requirements of pharmacopoeial monograph FS No. 18 of the State Pharmacopoeia, XIth Ed. [20].

The standards were rosmaric acid (Fluka), hesperidin, and menthol (Acros Organics). Spectrophotometric studies were carried out on a UV-Vis-mini spectrophotometer (Shimadzu) in 10-mm quartz cuvettes.

Preparation of Hesperidin Starting Solution. Hesperidin (25 mg) was dried beforehand, transferred to a 25-mL volumetric flask, dissolved in DMSO (2 mL), and adjusted to the mark with ethanol (70%) (Solution A, $1.64\cdot10^{-3}$ M). Solution A (1.69 mL) was transferred to a 5-mL volumetric flask and adjusted to the mark with ethanol (70%) (Solution B, $5.55\cdot10^{-4}$ M).

Preparation of Rosmaric Acid Starting Solution. Rosmaric acid was dried beforehand (5 mg), transferred to a 25-mL volumetric flask, dissolved in ethanol (70%), and adjusted to the mark with ethanol (70%) ($5.55\cdot10^{-4}$ M).

Preparation of an Equimolar Mixture of Hesperidin and Rosmaric Acid. Starting solutions of hesperidin (Solution B) and rosmaric acid (300 μL each) were transferred to a 5-mL volumetric flask and adjusted to the mark with ethanol (70%).

Construction of Hesperidin and Rosmaric Acid Calibration Curves. Serial dilutions of the standard solutions were prepared in order to construct the calibration curves. Starting hesperidin (Solution B) and rosmaric acid solutions (100–1000 μL) were placed into volumetric flasks and adjusted to the mark with ethanol (70%). Optical densities of the solutions

were determined at 285 and 326 nm. Curves in coordinates of optical density vs. concentration (μg/mL) were constructed using the resulting values. Regression analysis of the resulting plots was performed using the Advanced Grapher 2.11 program set (Alenthum Software Inc.). Specific absorption coefficients were calculated (E, $^{1\%}_{1\ cm}$) by the standard method [21].

Accuracy of Dual-wavelength Spectrophotometry Method. A series of standard solutions was prepared for this. Aliquots of starting solutions of hesperidin (Solution B) and rosmaric acid (Solution No.) were placed into 5-mL volumetric flasks [500 and 100 μL (No. 1), 400 and 200 μL (No. 2), 300 and 300 μL (No. 3), 200 and 400 μL (No. 4), 100 and 500 μL (No. 5)] and adjusted to the mark with ethanol (70%). Optical densities of the solutions were determined at 285 and 326 nm.

Quantitative Determination of Phenolic Compounds in *M. piperita* Leaves. An analytical sample of raw material was ground to particle size that passed through a sieve with 2-mm openings. About 0.5 g (accurate weight) of ground raw material was placed into a 150-mL flask with a ground-glass joint, treated with ethanol (70%, 50 mL), connected to a reflux condenser, and heated on a boiling water bath for 60 min. The extract was cooled and filtered into a 100-mL volumetric flask. The extraction was repeated under the same conditions. The volume of the combined filtrate was adjusted to the mark with ethanol (70%) (Solution A).

Solution A (1 mL) was transferred to a 25-mL volumetric flask and adjusted to the mark with ethanol (70%, Solution B). Optical density of Solution B was determined at 285 and 326 nm. The reference solution was ethanol (70%).

The content of flavonoids was calculated as hesperidin (X_1); of phenolic acids, as rosmaric acid (X_2) per absolute dry weight of raw material in percent using the formulas:

$$X_1 = \frac{(E_2^{326} \cdot A^{285} - E_2^{285} \cdot A^{326}) \cdot k^V}{(E_2^{326} \cdot E_1^{285} - E_1^{326} \cdot E_2^{285}) \cdot m} \cdot \frac{100}{100 - W} \qquad X_2 = \frac{(E_1^{285} \cdot A^{326} - E_1^{326} \cdot A^{285}) \cdot k^V}{(E_1^{285} \cdot E_2^{326} - E_2^{285} \cdot E_1^{326}) \cdot m} \cdot \frac{100}{100 - W}$$

where A^{285} and A^{326} are optical densities of the studied solution at 285 and 326 nm; k^V, dilution coefficient of the studied solution (2500); E_1^{285} and E_1^{326}, specific absorption coefficients of hesperidin at 285 and 326 nm (312 and 62); E_2^{285} and E_2^{326}, specific absorption coefficients of rosmaric acid at 285 and 326 nm (314 and 495); m, mass of raw material (g); W, mass loss of raw material on drying (%).

The content of flavonoids in *M. piperita* leaves was also determined by differential spectrophotometry and calculated as hesperidin at 306 nm [18] and by the Folin method and calculated as rosmaric acid [19].

Reaction of Hesperidin and Rosmaric Acid with Folin Reagent. A solution of the studied compound (1 mL) in ethanol (70%, c = 400 μg/mL) was transferred to a 100-mL volumetric flask, treated with Folin reagent (Sigma) (1 mL) and Na_2CO_3 solution (20%, 10 mL), and adjusted to the mark with water. Optical density of the solutions was determined after 60 min at 763 nm.

Tincture of peppermint preparation (0.5 mL) was analyzed by placing it into a 25-mL volumetric flask, adjusting to the mark with ethanol (70%), and proceeding as above.

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