EKSPERIMENTINIAI TYRIMAI

Investigation of contribution of individual constituents to antioxidant activity in herbal drugs using postcolumn HPLC method

Raimondas Raudonis, Valdas Jakštas, Deividas Burdulis, Raimondas Benetis[1], Valdimaras Janulis

Department of Pharmacognosy,

[1]Department of Pharmaceutical Chemistry, Kaunas University of Medicine, Lithuania

Key words: *antioxidants; postcolumn high-performance liquid chromatography; DPPH radical; herbal drug.*

Summary. *The most important attention is paid to the search of natural antioxidants and their evaluation in medicinal and food raw materials of plant origin. A number of plants, their extracts, food products, and medicinal preparations appear to be the objects of scientific research. Effectiveness and informative character of research, undoubtedly, depend on relevance, sensitivity, and efficiency of the methods chosen.*

The aim of this work was to develop and validate the postcolumn high-performance liquid chromatography (HPLC)-DPPH method as well as its application in the evaluation of antioxidant activity of known and unknown compounds scavenging free radicals and existing in medicinal plant raw materials.

HPLC-separated compounds were identified at the wavelength of 275 nm, and then the mobile phase with analytes flowed through a mixing tee to the reaction coil, where DPPH reagent solution was supplied. The solution flow rate was 0.4 mL/min. The reaction coil was connected with UV/VIS type detector, which measured absorption of flowing solution at the wavelength of 520 nm.

It was determined that vitexin rhamnoside, the dominant compound in the leaves of Crataegus monogyna, was not a significant radical scavenger. The most active antioxidant in the leaves and flowers of Crataegus monogyna was chlorogenic acid. The most active antioxidant in Origanum vulgare raw material was rosmarinic acid. Identified analytes in the extracts of Achillea millefolium that possessed radical-scavenging properties were chlorogenic acid, luteolin-7-O-glucoside, rutin, and luteolin.

Introduction

Numerous scientific studies have investigated the role of intermediate derivatives of oxidation process, free radicals, in various (patho)physiological processes, such as development of diseases and aging, in particular (1, 2). Free radicals have been established to determine the beginning of various diseases, including heart diseases, stroke, arteriosclerosis, diabetes, and cancer (3). All above-mentioned is the reason to look for effective compounds – antioxidants – that would reduce a harmful impact of free radicals.

Lately, increasing attention has been paid to the antioxidants of natural origin as the problem of safety of synthetic antioxidants is still open (4). The most important attention is paid to the search for antioxidants and their evaluation in medicinal and food raw materials of plant origin (5). A number of plants, their extracts, food products, and medicinal preparations appear to be the objects of research. Effectiveness and informative character of research, undoubtedly, depend on relevance, sensitivity, and efficiency of the methods chosen.

The following standard stable free radicals are often used to evaluate the antioxidant activity: DPPH, or 1,1-diphenyl-2-picrylhydrazyl radical, and ABTS, or 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) cation (6). Photocolorimetric method is used to determine the decrease in the activity of the above-mentioned radicals. Photocolorimetric method is not selective enough; therefore, to compare activity of single

Correspondence to V. Jakštas, Department of Pharmacognosy, Kaunas University of Medicine, A. Mickevičiaus 9, 44307 Kaunas, Lithuania. E-mail: valdas.jakstas@med.kmu.lt

active compounds of the aliquot, purification of compounds or some other method is necessary. Purification of active compounds or their groups from the aliquots of plant origin is time- and labor-consuming and expensive solution. Besides, when extraction and depuration procedures are applied, some of the initial antioxidant activity is lost due to dissociation (or structural deformation) of compounds with antioxidant activity and partial yield (7). To avoid the abovementioned problems, new research technologies are required that would enable to determine the activity of different plant compounds directly in the tested aliquot and, at the same time, to maintain proportions, characteristic of that raw material or preparation.

Lately, composite methods of on-line determination have been developed. These methods are qualified as selective, short in analysis duration, and very sensitive for exact determination of activity and comparison. Separation of the investigated compounds by high-performance liquid chromatography (HPLC) together with postcolumn reaction is used for such methods. DPPH and ABTS radicals are used in postcolumn reaction, and the principle of common photocolorimetric method is followed (6). On-line postcolumn methods are employed in the rapid identification of antioxidants, determination of activity, and comparison. They are particularly informative and possess important advantages if compared with colorimetric methods (7).

The aim of this study was to develop and validate the postcolumn HPLC-DPPH method as well as its application in the evaluation of antioxidant activity of known and unknown compounds scavenging free radicals and existing in medicinal plant raw materials.

Material and methods

This paper describes optimization, validation, and application of HPLC-DPPH method on-line. Reference antioxidant trolox ((R)-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used for standardization of antioxidant activity. Trolox activity equivalent was calculated for individual compound and for the analyzed plant raw material.

Eluents of analytical purity were used. Acetonitrile (ACN) of gradient purity was received from Sigma-Aldrich GmbH (Buchs, Switzerland); 99.8% trifluoroacetic acid (TFA) – from Riedel-de-Haen (Germany). Refined deionized water (18.2 $m\Omega cm^{-1}$) was prepared with Millipore (USA) water cleaning system. DPPH radical (95%) was received from Sigma-Aldrich Chemie (Germany); chlorogenic acid, hyperoside, and quercetin were received from Roth (Germany); trolox (97%), from Acros Organics (New Jersey, USA); ethanol (rectified spirit 96.3%), from Stumbras (Kaunas, Lithuania); sodium citrate and citric acid, from Fluka Chemie (Buchs, Switzerland).

Scheme of HPLC-DPPH system for on-line identification of free radical scavengers is presented in Fig. 1. The binary pump system Beckman solvent module 126 (Fullerton, USA) was used for the elution gradient formation. Chromatographic separation was performed at mobile phase flow rate of 0.4 mL/min. Elution gradient component A – 0.1% TFA water solution, component B – 0.1% TFA solution ACN. The following linear variation of gradient was used: 0 min, 95% A and 5% B; 45 min, 55% A and 45% B; 50 min, 55% A and 45% B; 55 min, 95% A and 5% B; 60 min, 95% A and 5% B. Rheodyne injector with a 20-μL injection loop was used for injection of aliquots. Analytes were separated in X-Terra RP_{18} (Waters) analytical column (3.5 μm, 3.0×150 mm), where X-Terra RP_{18} (3.5 μm, 3.0×20 mm, Waters) was used as precolumn. The separated compounds were identified with UV absorption detector Beckman System Gold 166 (Fullerton, USA) at the wavelength of 275 nm. From the detector, the mobile phase with analytes flowed through a mixing tee to the reaction coil, where DPPH reagent solution was supplied. HPLC pump Gilson pump 305 (Middleton, USA) was used to supply DPPH solution. The solution flow rate was 0.4 mL/min. The reaction coil was 3 m in length, made of PEEK (polyetheretherketone) tube with an inner diameter of 0.25 mm and outer diameter of 0.36 mm. The reaction coil was connected with UV/VIS type detector Gilson UV/VIS detector 118 (Middleton, USA), which measures absorption of flowing solution at the wavelength of 520 nm. The obtained data and equipment were managed with two computers, and original software System Gold (Beckman) and Unipoint (Gilson) was used.

Results and discussion

Development and optimization of DPPH solution. Medium pH influences kinetics of plant antioxidant reaction with DPPH radical. Koleva et al. (9) state the importance of maintaining the medium pH between 5.0 and 6.5, as this is an optimal condition for the reaction of hydrogen electron release. In the course of gradient elution of analytes, the medium pH may fluctuate. The fluctuations negatively influence the stability of the chromatogram baseline and, at the same time, S/N (ratio of analyte peak height to baseline noise) ratio. The instability of the baseline is named as the main problem in HPLC-DPPH analysis (4).

Eluents of the gradient described in the *Material and methods* have pH of <2.3. At low values of the

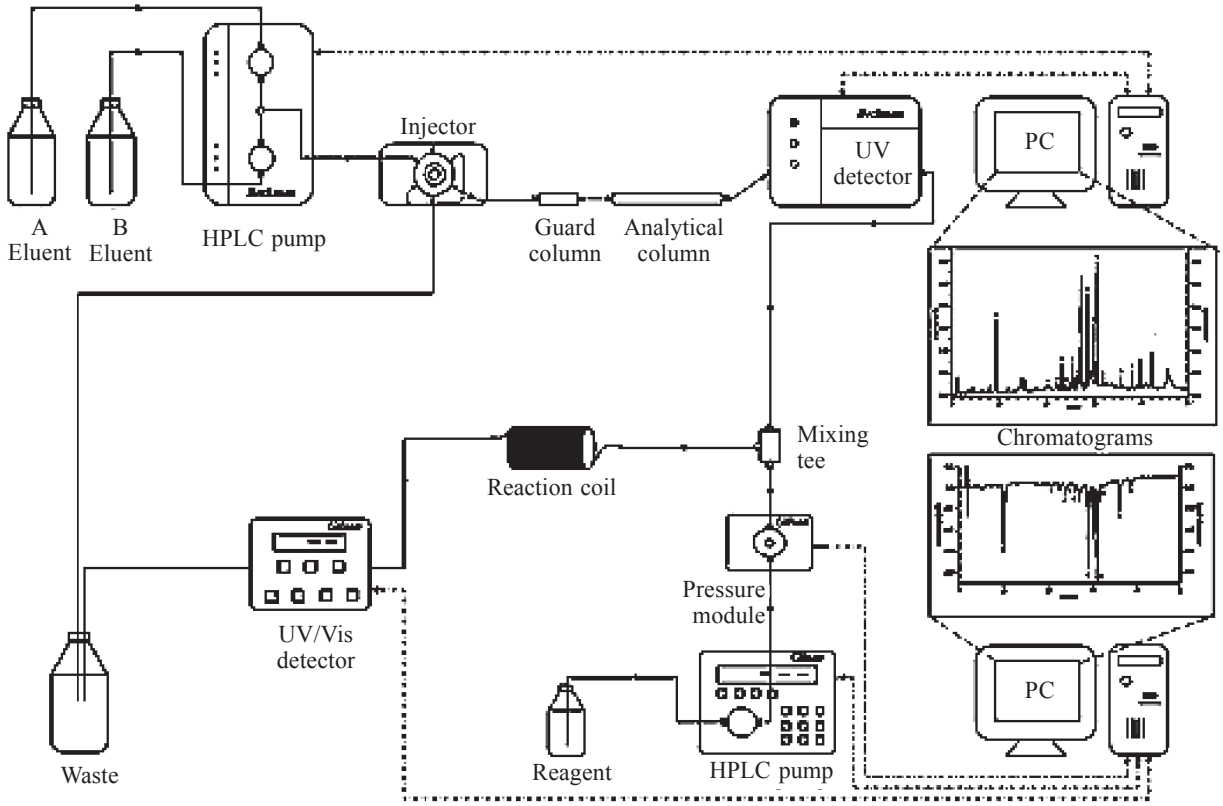

Fig. 1. **HPLC-DPPH equipment system for analysis of individual antioxidants**

medium pH, absorption of DPPH solution also decreases (9). The pH increase in the reaction solution in the reaction coil is possible only by means of adding some buffer solution to DPPH reagent solution.

The phosphatic buffer (Na_2HPO_4) system, suggested by Dapkevičius et al. (5) was employed. DPPH crystals were dissolved in CAN, and 0.03 M of sodium hydrophosphate buffer solution was added in a 1:2 ratio, pH 7.6. As water content decreased during gradient analysis, sodium hydrophosphate crystals started to form in the reaction coil, which increased the instability of the baseline. The accumulation of hydrophosphate crystals might obstruct the reaction coil. The phosphatic buffer solution was refused. Pukalskas et al. (10) described the application of ammonium acetate buffer solution to DPPH solution. DPPH solution in ACN was prepared for the experiment, and 0.005 M of ammonium acetate (CH_3COONH_4) buffer solution was added in a 1:2 ratio, pH 7.4. In the case of application of ammonium acetate buffer solution, no crystals were formed in the reaction coil; however, stabilization of the chromatogram baseline was unsuccessful. Therefore, ammonium acetate buffer solution was also refused.

Application of sodium citrate/citric acid buffer solution, described in publications by Kosar et al. (4, 7), resulted in no crystals formed in the reaction coil. The baseline of chromatogram was stable. The decision was made to apply this buffer solution for the maintenance of optimal medium pH. To increase buffering capacity of solution, double concentration of sodium citrate as that suggested by Kosar was used. For the final method, 0.1 M of sodium citrate buffer solution was prepared, and its pH was corrected with 0.05 M of citric acid solution (up to pH 7.6). The buffer solution was mixed with DPPH reagent dissolved in ACN solution, ACN being one of mobile phase components. Both solutions (buffer and reagent) were mixed in a 1:1 ratio. This proportion was chosen with the purpose to maintain sufficient solubility of DPPH in the mobile phase during gradient elution.

Choice of optimal concentration of DPPH solution is very important for qualitative evaluation of antioxidant activity. Increasing concentration of DPPH solution was shown to increase the peak area of active compound in the chromatogram. This dependence is shown in Fig. 2. Chlorogenic acid, hyperoside, and quercetin showed curves of similar character. The highest peak area was reached at the DPPH solution

concentration of 0.02–0.03 mg/mL, and further increase in the concentration had a slight if any impact on an increase in the peak area (quercetin curve). Trolox curve reached a plateau at the DPPH concentration close to 0.04 mg/mL.

At the increasing concentration of DPPH solution, baseline noise also increased (Fig. 3). Instability of chromatogram baseline might negatively influence the accuracy of quantitative evaluation and determination of minimum concentration of active compound.

DPPH solution at the concentration of 0.02–0.03 mg/mL was decided to be the most suitable for analysis of aliquots of plant origin. At this concentration, chlorogenic acid, hyperoside, and quercetin (plant antioxidants) had the highest peak areas. At the concentration of 0.02 mg/mL, baseline noise was lower than that in case of 0.03 mg/mL; therefore, DPPH solution concentration of 0.02 mg/mL was chosen for further experiments.

Kinetics of plant antioxidant reaction with the

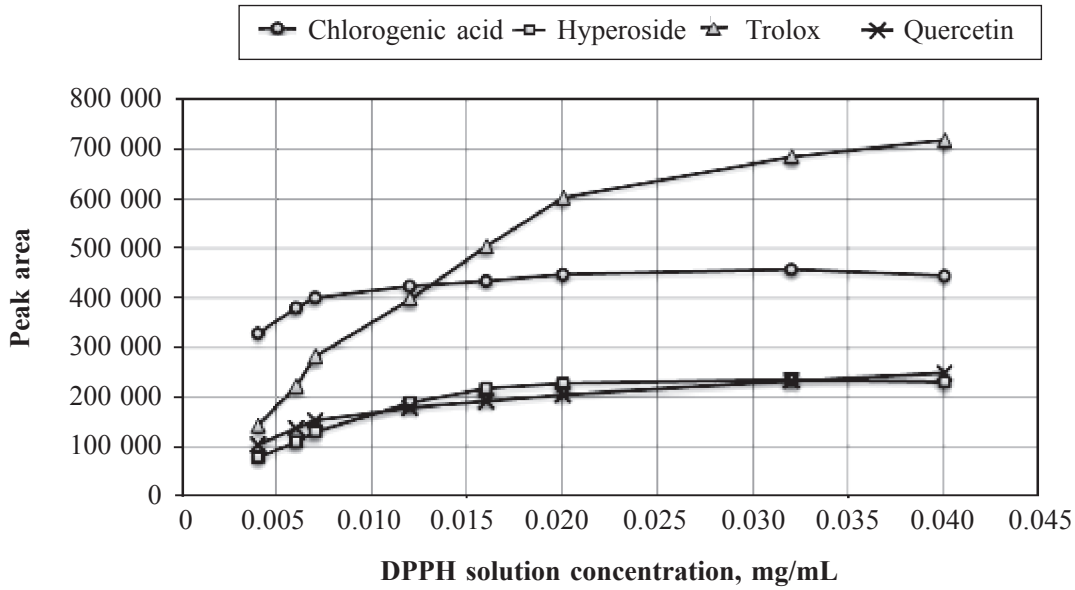

Fig. 2. **Dependence of standard peak area on DPPH solution concentration**

The following standards were used: chlorogenic acid, 0.04 mg/mL; hyperoside, 0.02675 mg/mL; trolox, 0.04 mg/mL; quercetin, 0.025 mg/mL (injected volume, 20 μL; λ=520 nm).

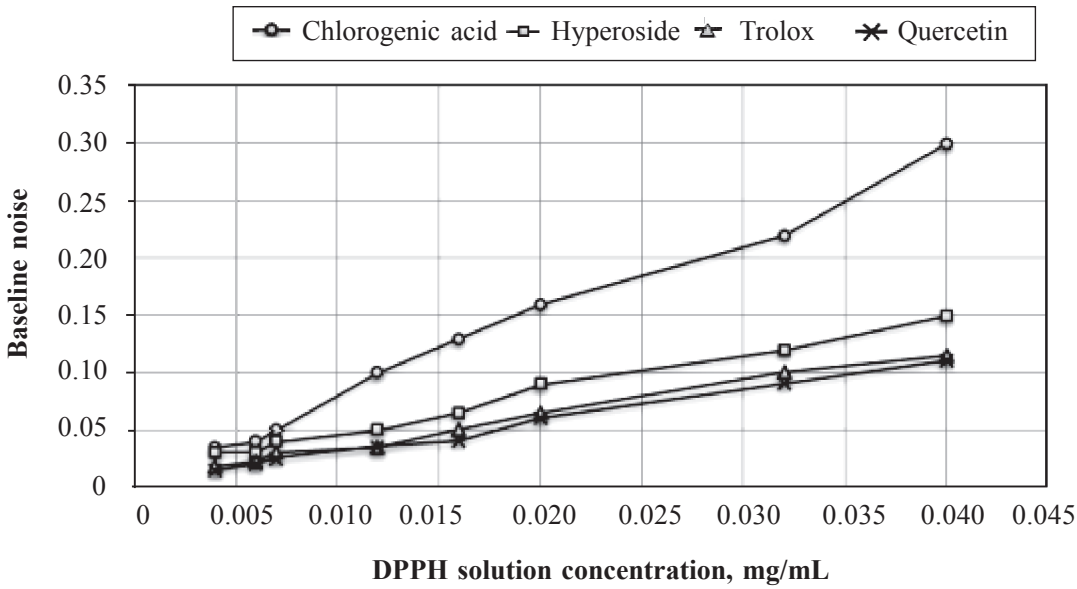

Fig. 3. **Dependence of baseline noise on DPPH solution concentration**

The following standards were used: chlorogenic acid, 0.04 mg/mL; hyperoside, 0.02675 mg/mL; trolox, 0.04 mg/mL; quercetin, 0.025 mg/mL (injected volume, 20 μL; λ=520 nm).

DPPH radical is different (11). Some antioxidants show rapid kinetics of reaction, others – significantly lower one. Bandonienė et al. (11) established spectrophotometrically 30–40 s to be the optimal reaction time with DPPH radical for most plant antioxidants. In the chosen method, the reaction coil of fixed length (3 m) was used. This length was sufficient to keep the reaction mixture in the coil for the indicated period.

Rate of DPPH solution supply to postcolumn directly determines the duration of presence of antioxidant and DPPH reagent in the reaction coil, which, in turn, influences baseline noise as well as peak height and area of analyte in a chromatogram. Therefore, choice of optimal rate of DPPH solution supply to postcolumn is necessary in order to have quantitative evaluation of antioxidant activity.

Low concentrations of known compounds were chosen for this experiment, which enables to have the most exact evaluation of the influence of rate of DPPH solution supply to postcolumn on baseline noise and peak height of analyte in the chromatogram (Fig. 4). At the increasing flow rate of DPPH solution, baseline noise and peak height of analyte decreased in the chromatogram, and on the contrary, at the decreasing flow rate – noise and peak height increased.

The flow rate of DPPH solution of 0.4–0.6 mL/min is the best for quantitative evaluation of antioxidants. For saving expensive DPPH reagent, flow rate of DPPH solution of 0.4 mL/min was chosen for further experiments.

Very little amounts of DDPH crystals were weighed for the preparation of DPPH solution; they were dissolved in a certain volume of ACN, and a particular amount of buffer solution was added. Some errors, which can distort quantitative evaluation of antioxidant activity, are possible each time when fresh reagent solution is produced. To avoid this, standardization of final DPPH solution should be performed before each run of the analysis. Standardization was carried out off-line; spectrophotometer DU-70 (Beckman) and 10-mm quartz cell were used. Wavelength was λ=520 nm; optical density of DPPH solution at 0.02 mg/mL concentration was corrected up to 0.500±0.005 AU.

HPLC-DPPH method validation. For the purpose of direct validation of HPLC-DPPH method, several standard assessments of HPLC method have been done (Table 1). Limit of determination, as minimum detectable concentration, was determined for standards by signal-to-noise ratio of 7:1. The precision of present method was evaluated using repeatability and intermediate precision (day-to-day) of variation coefficients (RSD%), calculated from 5 replicated analyses.

To perform quantitative evaluation of antioxidant compounds, trolox calibration curve was made. Trolox solution (70% ethanol in water) of four different concentrations was injected: 1.0 μg/mL, 12.5 μg/mL, 40.0 μg/mL, and 70 μg/mL (injection volume, 20 μL). A direct correlation between the amount of trolox (μg) and negative peak area, formed by it, was indicated by high correlation factor (R^2=0.997).

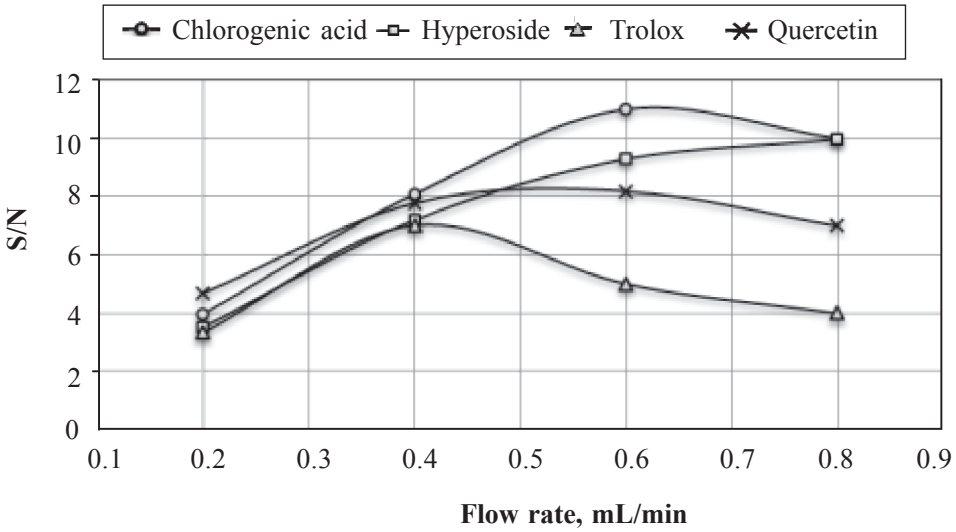

Fig. 4. **Dependence of peak height-to-baseline noise ratio (S/N) on DPPH solution flow rate**
The following standards were used: chlorogenic acid, 0.01 mg/mL; hyperoside, 0.004 mg/mL; trolox, 0.001 mg/mL; quercetin, 0.002 mg/mL (injected volume, 20 μL; λ=520 nm).

Table 1. **Precision characteristics and limits of determination**

Standard	Retention time, min	MDC, μg/mL	Repeatability (RSD, %)	
			Run-to-run	Day-to-day
Chlorogenic acid	15.5	10	0.76	1.33
Hyperoside	27.0	4.3	0.65	1.55
Trolox	35.5	1.0	0.61	2.22
Quercetin	38.4	2.1	0.74	1.92

MDC – minimum detectable concentration; RSD – coefficient of variation (relative standard deviation).

Trolox content equivalent was used for quantitative evaluation of activity of antioxidant compounds present in extractions and pharmaceutical preparations from raw materials of medicinal plants. The equivalent (E_T) is expressed by the amount of trolox (μg), which under the same experimental conditions shows an appropriate free DPPH radical-scavenging activity, evaluated by trolox calibration curve (y=686344x+18680).

Antiradical activity equivalent (A) was used to evaluate the activity of antioxidantically active compounds in plant raw material. Antiradical activity equivalent of taken compound present in one amount of plant raw material – gram – is expressed by the amount of trolox (μg), which under the same conditions shows an appropriate free DPPH radical-scavenging activity, evaluated by the same trolox calibration curve as the taken compound:

$$A_{comp} = \frac{E_{T(comp)}(\mu g)}{V_{inj}(mL)} \times \frac{V_{extr}(mL)}{m\ (g)}\ , (\mu g/g)$$

Where: $E_{T(comp)}$ – trolox content equivalent (μg) of the taken antioxidantically active compound; V_{inj} – injected volume of the aliquot (mL); V_{extr} – extraction amount of the analyzed plant raw material (mL); m – exact amount of the analyzed plant raw material.

Application of HPLC-DPPH method on-line. Potential application of optimized HPLC-DPPH method was perfectly illustrated by the investigations aimed at quantitative evaluation of antioxidants, present in *Crataegus* (hawthorn), *Origanum* (oregano), and yarrow (*Achillea*) extracts.

Preparations of *Crataegus* are prescribed in functional heart disorders, after serious illness, in case of increased blood pressure. Commonly plant raw material is collected from *Crataegus monogyna* Jacq., *C. rhiphidophylla,* and *C. laevigata*. Various flavonoids and their glycosides (vitexin, vitexin-2"-*O*-rhamnoside, izovitexin, orientin, hyperoside, rutin, epicatechin and oligomeric procyanidins) have been determined in *C. monogyna* (12). UV/DPPH chromatogram of ethanolic extract from flowers, obtained by performing evaluation of bioactive compounds of *Crataegus* by on-line HPLC-DPPH method, is presented in Fig. 5. The peak of hyperoside was dominant in the UV chromatogram; however, antioxidant activity of hyperoside, estimated by calculated trolox equivalent, was lower than chlorogenic acid (Table 2).

Using on-line HPLC-DPPH method, it was determined that vitexin rhamnoside, the dominant compound in leaves, was not a significant radical scavenger. The most active antioxidant in leaves of *Crataegus*, as well as flowers, was chlorogenic acid (Fig. 6, Table 3).

Preparations of *Origanum* are known for their antimicrobial, anti-inflammatory, demulcent, and pain-relieving actions. A significant antioxidant determined in the herb of *Origanum vulgare* is phenolic acid. Rosmarinic acid is the dominant phenolic acid in *Herba Origani* as described by Ivanauskas et al. (13). Two main compounds influencing antioxidant activity the most were determined by on-line HPLC-DPPH method. This was rosmarinic acid and unknown compound, which peak was marked in the UV/DPPH chromatogram by number 2 (Fig. 7). UV/DPPH analysis of ethanolic extract from *Origanum* leaves revealed that the most active antioxidant was rosmarinic acid (Fig. 8). It was determined that the general antioxidant activity in the herb of *Origanum* was attributed to compounds, present in the leaves of *Origanum*.

Effectiveness of the designed instrumental and experimental setup was also confirmed by applying key principles of the coupled approach to the other method of chromatographic separation performed on HPLC column with different particle size, internal diameter, and chemical properties of sorbent. Separations were carried out using 0.1% TFA solution in water (solvent A) and 0.1% TFA solution in acetonitrile (solvent B) as mobile phase with the previously published (14) gradient elution program on a 5-μm AscentisTM RP-Amide analytical column (150×4.6 mm) guarded with

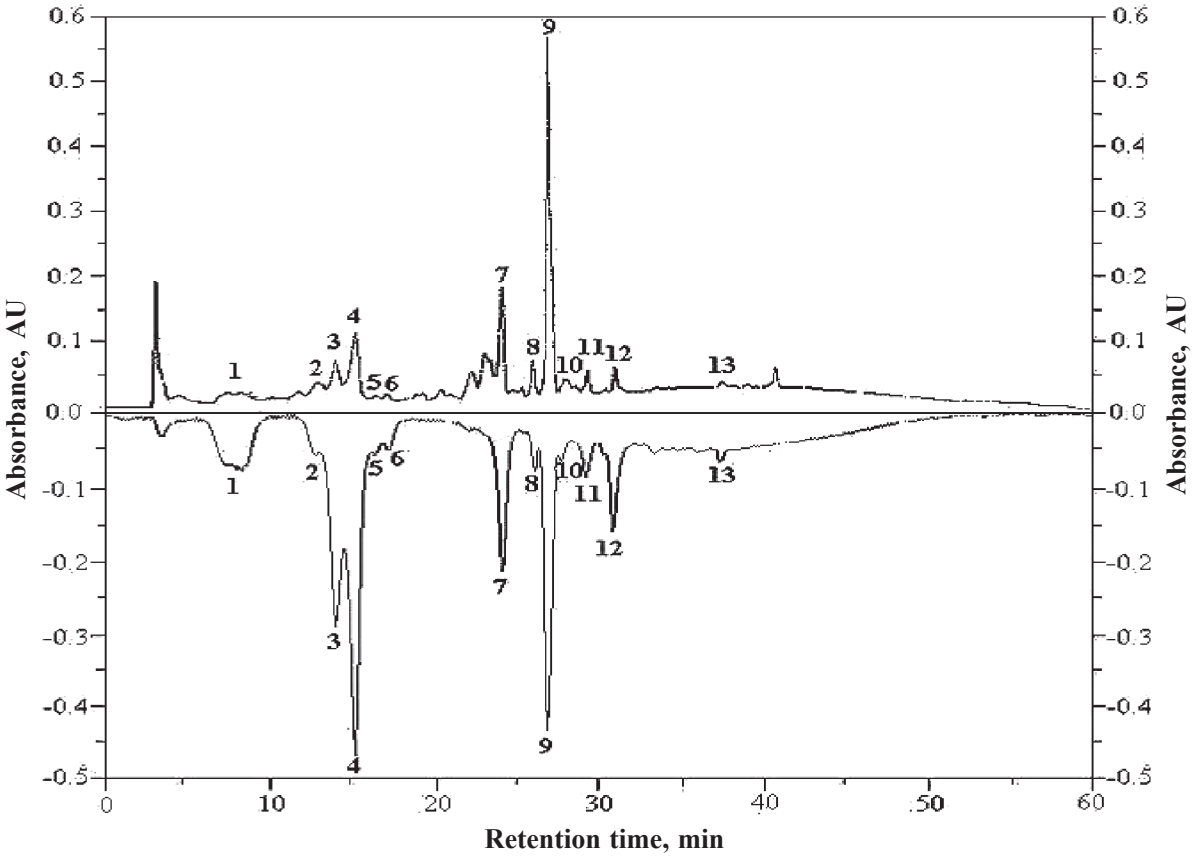

Fig. 5. **HPLC-UV-DPPH coupled chromatograms of the flowers of *Crataegus monogyna***
For exact compound refer to Table 2.

Table 2. **Antioxidant activity of individual compounds in the extract of the flowers of *Crataegus monogyna***

Peak No. in Fig. 5	Compound	Retention time, min	Peak area	E, µg	A, µg/g
1	Unknown	11.08	543 130.50	0.7641	1857.55
2	Unknown	12.65	152 763.69	0.1954	474.91
3	Unknown	13.85	929 618.31	1.3272	3226.45
4	**Chlorogenic acid**	15.08	1 572 074.00	2.2633	5501.96
5	Unknown	16.40	93 651.83	0.1092	265.54
6	**Epicatechin**	17.14	94 529.11	0.1105	268.65
7	Unknown	23.95	490 308.38	0.6872	1670.46
8	**Rutin**	25.87	101 498.73	0.1207	293.34
9	**Hyperoside**	26.83	909 067.69	1.2973	3153.66
10	Unknown	28.50	61 341.23	0.0622	151.10
11	Unknown	29.24	92 753.63	0.1079	262.36
12	Unknown	30.84	275 392.62	0.3740	909.25
13	Unknown	37.34	26 283.78	0.0111	26.93
			Total:	7.4301	18 062.17

E – trolox content equivalent; A – antiradical activity equivalent.

a 5-µm guard column SupelguardTM AscentisTM RP-Amide (20×4.00 mm) (SUPELCO, Bellefonte, PA, USA). Since the separation conditions were optimized for a flow rate of 1.5 mL/min to ensure sufficient time for reaction kinetics, it was necessary to increase the length of reaction coil from 3 m to 15 m. Furthermore, preliminary experiments were performed to optimize the reaction time by setting different

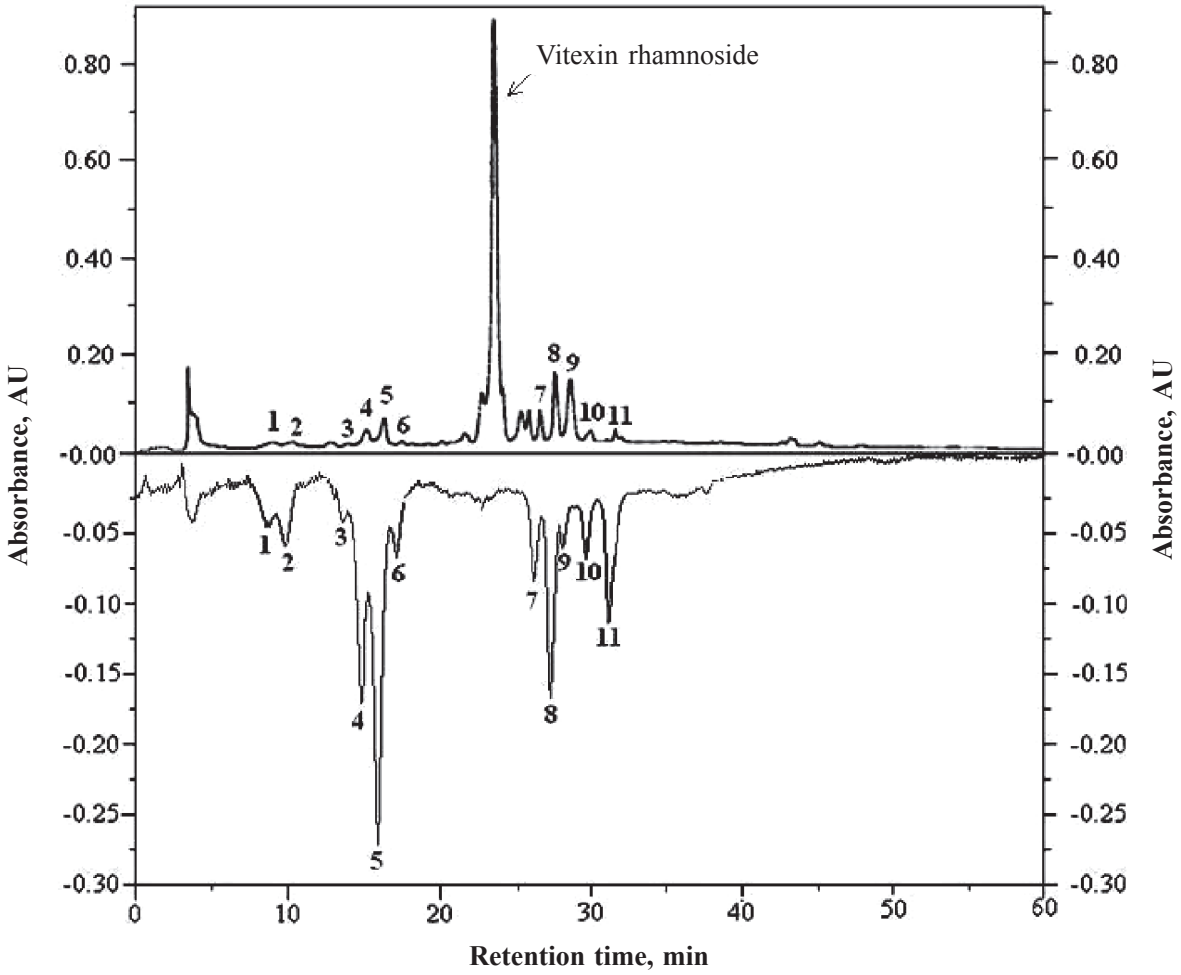

Fig. 6. **HPLC-UV-DPPH coupled chromatograms of the leaves of *Crataegus monogyna***
For exact compound refer to Table 3.

Table 3. **Antioxidant activity of individual compounds in the extract of the leaves of *Crataegus monogyna***

Peak No. in Fig. 6	Compound	Retention time, min	Peak area	E, µg	A, µg/g
1	Unknown	8.65	114 653.45	0.1398	302.33
2	Unknown	9.85	154 735.78	0.1982	428.59
3	Unknown	13.55	59 734.83	0.0598	129.33
4	Unknown	14.74	458 564.53	0.6409	1385.69
5	**Chlorogenic acid**	15.88	666 903.94	0.9445	2041.99
6	**Epicatechin**	17.07	98 875.24	0.1168	252.62
7	**Rutin**	26.21	127 444.84	0.1585	342.62
8	**Hyperoside**	27.24	311 920.00	0.4272	923.74
9	Unknown	28.10	61 873.73	0.0629	136.07
10	Unknown	29.62	80 408.05	0.0899	194.45
11	Unknown	31.18	219 184.66	0.2921	631.61
			Total:	3.1308	6769.05

E – trolox content equivalent; A – antiradical activity equivalent.

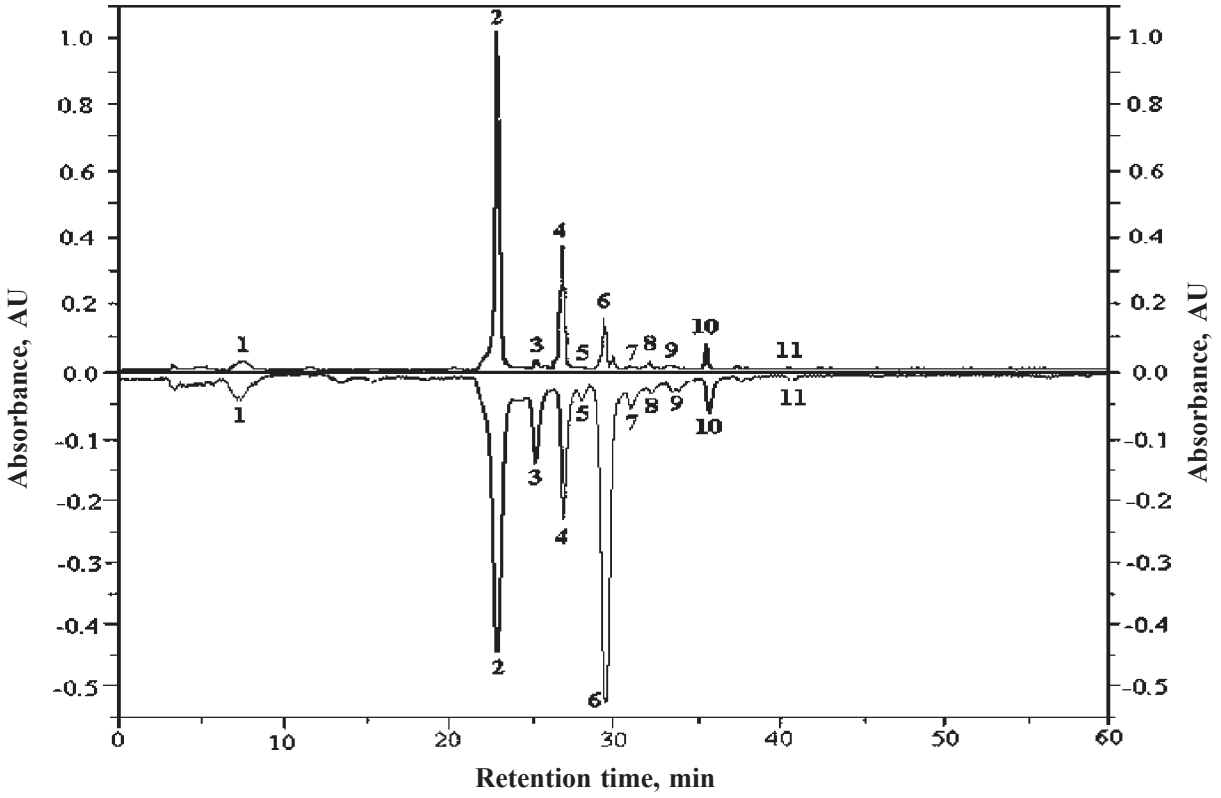

Fig. 7. **HPLC-UV-DPPH coupled chromatograms of the *Origanum vulgare* herb**
For exact compound refer to Table 4.

Table 4. **Antioxidant activity of individual compounds in the extract of *Origanum vulgare* herb**

Peak No. in Fig. 7	Compound	Retention time, min	Peak area	E, µg	A, µg/g
1	Unknown	10.07	185 927.14	0.2437	1213.05
2	Unknown	22.91	1 349 594.75	1.9391	9653.21
3	**Vitexin-2**	25.25	321 897.62	0.4418	2199.26
4	**Rutin**	27.06	571 311.49	0.8052	4008.27
5	**Hyperoside**	28.04	75 511.98	0.0828	412.21
6	**Rosmarinic acid**	29.57	1 402 083.88	2.0156	10 033.92
7	Unknown	30.96	124 611.75	0.1543	768.33
8	Unknown	32.17	50 798.95	0.0468	232.96
9	Unknown	33.62	67 791.07	0.0716	356.21
10	Unknown	35.71	99 061.89	0.1171	583.01
11	Unknown	40.81	19 474.79	0.0012	5.76
			Total:	5.9192	29 466.19

E – trolox content equivalent; A – antiradical activity equivalent.

flow rates (1, 1.5, 2 mL/min) of the working DPPH solution. The flow rate of 1.5 mL/min was chosen, because it gave relatively low baseline disturbances and an acceptable peak height of analytes. R^2 value of the trolox was 0.998, thus confirming the linearity as well as suitability of the optimized HPLC-DPPH method for quantitative determinations. The presented system was applied for the screening of 70% (v/v) ethanolic extract of yarrow (*Achillea millefolium* L. s.l.) herb for radical-trapping components (Fig. 9). The results of DPPH quenching showed the presence of radical-scavenging compounds in yarrow extracts,

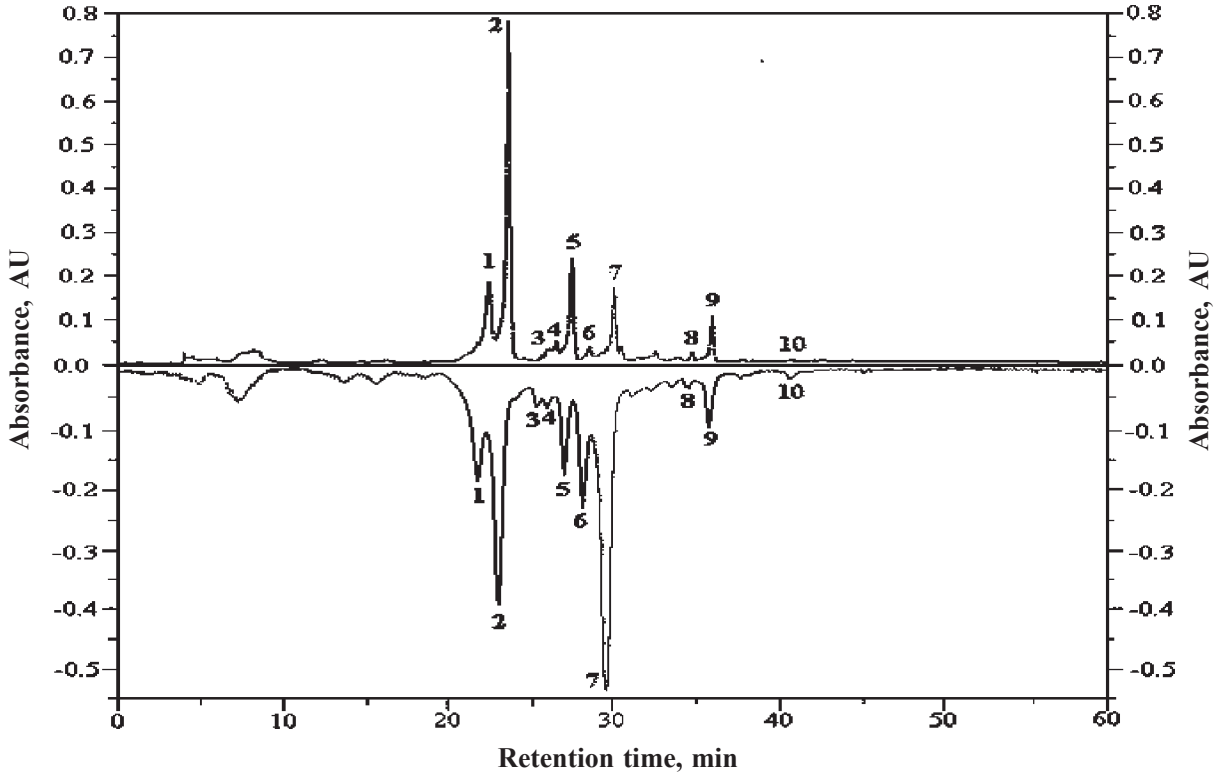

Fig. 8. **HPLC-UV-DPPH coupled chromatograms of the *Origanum vulgare* leaves**
For exact compound refer to Table 5.

Table 5. **Antioxidant activity of individual compounds in the extract of leaves of *Origanum vulgare***

Peak No. in Fig. 8	Compound	Retention time, min	Peak area	E, μg	A, μg/g
1	Unknown	21.80	625 604.44	0.8843	2204.54
2	Unknown	23.03	1 078 805.25	1.5446	3850.71
3	**Vitexin-2**	25.36	69 468.54	0.0740	184.48
4	Unknown	25.96	94 892.65	0.1110	276.83
5	**Rutin**	27.01	273 483.53	0.3712	925.53
6	**Hyperoside**	28.13	439 825.94	0.6136	1529.74
7	**Rosmarinic acid**	29.64	1 531 188.88	2.2037	5493.91
8	Unknown	34.43	18 733.46	0.0501	90.19
9	Unknown	35.74	135 209.02	0.1698	423.27
10	Unknown	40.71	22 416.32	0.0054	13.57
			Total:	5.9778	14 992.78

E – trolox content equivalent; A – antiradical activity equivalent.

namely flavonoids and phenolcarbonic acids. These bioactive compounds represent one of the most important groups of pharmacologically active substances in yarrow (15–20). It has been reported (21, 22) that antioxidant activity of extracts from several *Achillea* L. species might be correlated with their total phenolic and flavonoid contents. In our previous study (23), a considerable variation was observed in accumulation of phenolic compounds among the yarrow flowers, which, due to anatomical plant characteristics, constitute the most important part of the raw material of yarrow. Thereby, the investigation of contribution of individual constituents to antioxidant activity of yarrow extracts is of crucial importance for the comprehensive evaluation of pharmaceutical quality of the crude drug.

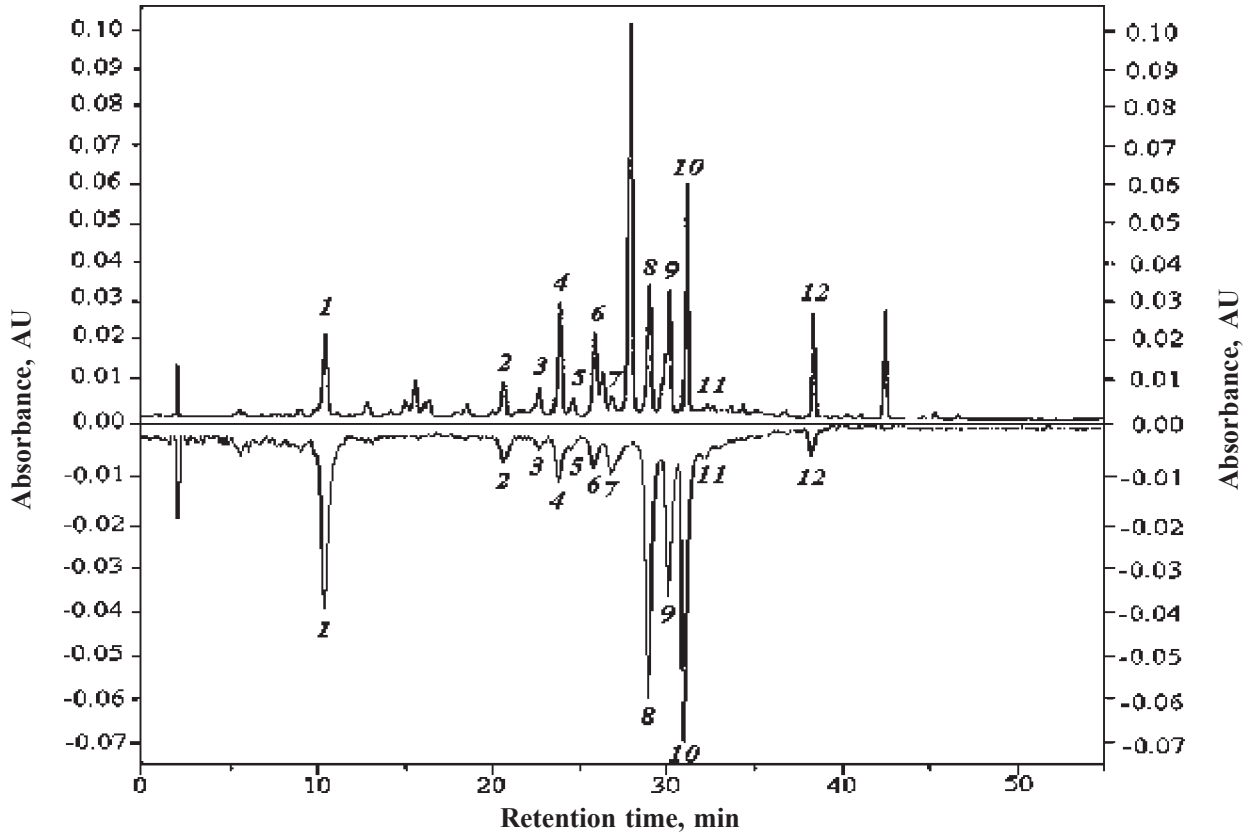

Fig. 9. **HPLC-UV-DPPH coupled chromatograms of the *Achillea millefolium* herb**
For exact compound refer to Table 6.

Table 6. **Antioxidant activity of individual compounds in the extract of *Achillea millefolium* herb**

Peak No. in Fig. 9	Compound	Retention time, min	Peak area	E, µg	A, µg/g
1	**Chlorogenic acid**	10.40	192 635.91	1.0550	5275.16
2	Unknown	20.65	23 358.72	0.1630	814.89
3	Unknown	22.67	9718.56	0.0911	455.48
4	**Luteolin-7-glucoside**	23.72	50 005.82	0.3034	1517.01
5	**Rutin**	24.46	10 135.76	0.0933	466.48
6	Unknown	25.75	24 806.33	0.1706	853.03
7	Unknown	26.76	46 631.44	0.2856	1428.10
8	**DCQA**	28.88	245 227.12	1.3322	6660.88
9	Unknown	30.07	135 881.36	0.7559	3779.74
10	**DCQA**	30.92	241 661.62	1.3134	6566.93
11	Unknown	32.08	9988.49	0.0925	462.59
12	**Luteolin**	38.20	24 619.66	0.1696	848.11
			Total:	5.8257	29 128.40

E – trolox content equivalent; A – antiradical activity equivalent;
DCQA – dicaffeoylquinic acid (correct form not identified).

Conclusions

This study describes an application of a simple, quick, selective, and sensitive method. This method does not require any exceptional equipment, preparation of complex aliquot and is available to many laboratories and scientific institutions. The optimized DPPH solution is suitable for both gradients used for analysis of different raw material samples. Using

postcolumn HPLC-DPPH method, it was determined that vitexin rhamnoside, the dominant compound in the leaves of *Crataegus monogyna*, was not a significant radical scavenger. The most active antioxidant in the leaves and flowers of *Crataegus* was chlorogenic acid. It was determined that the most active antioxidant in *Origanum vulgare* raw material was rosmarinic acid. This study revealed that the main components among the identified analytes in the extracts of *Achillea millefolium* that possessed radical-scavenging properties were chlorogenic acid, luteolin-7-*O*-glucoside, rutin, and luteolin.

Acknowledgments

Authors thank the Foundation of Science of Kaunas University of Medicine for support of this study.

Individualių junginių, lemiančių augalinių žaliavų antioksidantinį aktyvumą, tyrimas pokolonėlinės ESC metodu

Raimondas Raudonis, Valdas Jakštas, Deividas Burdulis, Raimondas Benetis[1], Valdimaras Janulis

Kauno medicinos universiteto Farmakognozijos katedra, [1]Farmacinės chemijos katedra

Raktažodžiai: *antioksidantai, pokolonėlinė ESC, DFPH radikalas, vaistinė žaliava.*

Santrauka. Didelis dėmesys skiriamas natūralių antioksidantų augalinės kimės vaistinėse ir maistinėse žaliavose paieškai ir įvertinimui. Mokslinių tyrimų objektas – didelis skaičius augalų rūšių, ekstraktų, maisto papildų ir vaistinių preparatų. Tiriamųjų darbų našumas ir informatyvumas, be abejonės, priklauso nuo pasirinktų metodų tinkamumo, jautrumo bei efektyvumo.

Darbo tikslas. Patobulinti bei įteisinti pokolonėlinę ESC-DFPH metodiką, pritaikyti ją laisvuosius radikalus surišančių žinomų ir nežinomų junginių, esančių vaistinėje augalinėje žaliavoje, antioksidantiniam aktyvumui įvertinti.

ESC išskirstyti junginiai nustatomi UV detektoriumi 275 nm ilgio bangoje. Mobilioji fazė su analitėmis per maišymo trišakį tiekiama į reakcijos kilpą, kur 0,4 ml/min. tėkmės greičiu paduodamas DFPH tirpalas. Reakcijos kilpa sujungta su UV/VIS tipo detektoriumi, matuojančiu pratekančio tirpalo absorbciją, esant 520 nm bangos ilgiui.

Nustatyta, jog *Crataegus monogyna* lapuose vyraujantis flavonoidas viteksino ramnozidas nepasižymi antioksidaciniu aktyvumu. Reikšmingiausias antioksidantas *C. monogyna* lapuose ir žieduose yra chlorogeno rūgštis. Didžiausiu antioksidaciniu aktyvumu *Origanum vulgare* augalinėje žolėje pasižymi rozmarino rūgštis. *Achillea millefolium* ekstraktuose identifikuotos šios analitės, turinčios radikalus surišantį aktyvumą: chlorogeno rūgštis, luteolin-7-O-gliukozidas, rutinas ir luteolinas.

Adresas susirašinėti: V. Jakštas, KMU Farmakognozijos katedra, A. Mickevičiaus 9, 44307 Kaunas
El. paštas: valdas.jakstas@med.kmu.lt

References

1. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford Univ. Press; 1999.
2. Davies KJA. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp 1995;61:1-31.
3. Lugasi A, Hóvári J, Sági KV, Bíró L. The role of antioxidant phytonutrients in the prevention of diseases. Acta Biologica Szegediensis 2003;47(1-4):119-25.
4. Kosar M, Dorman D, Baser K, Hiltunen R. An improved HPLC post-column methodology for the identification of free radical scavenging phytochemicals in complex mixtures. Chromatographia 2004;60:635-8.
5. Dapkevičius A, van Beek TA, Niederlander HAG. Evaluation and comparison of two improved techniques for the on-line detection of antioxidants in liquid chromatography eluates. J Chromatogr A 2001;912:73-82.
6. Koleva II, Niederlander HAG, van Bekk TA. Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates. Analytical Chemistry 2001;73: 3373-81.
7. Kosar M, Dorman HJD, Bachmayer O, Baser KHC, Hiltunen R. An improved on-line HPLC-DPPH method for the screening of free radical scavenging compounds in water extracts of *Lamiaceae* plants. Chem Nat Comp 2003;39(2):161-6.
8. Bartašiūtė A, Westerink BHC, Verpoorte E, Niederlander HAG. Improving the in vivo predictability of an on-line HPLC stable free radical decoloration assay for antioxidant activity in methanol-buffer medium. Free Radic Biol Med 2007;42:413-23.
9. Koleva II, Niederlander HAG, van Bekk TA. An on-line HPLC method for detection of radical scavenging compounds in complex mixtures. Anal Chem 2000;72:2323-8.
10. Pukalskas A, van Beek TA, de Waard P. Development of a triple hyphenated HPLC-radical scavenging detection-DAD-SPE-NMR system for the rapid identification of antioxidants

in complex plant extracts. J Chromatogr A 2005;1074 (1-2):81-8.
11. Bandonienė D, Murkovic M. On-line HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from apples (*Malus domestica* L.). J Agric Food Chemistry 2002;50:2482-7.
12. Urbonavičiūtė A, Jakštas V, Kornyšova O, Janulis V, Maruška A. Capillary electrophoretic analysis of flavonoids in single-styled hawthorn (*Crataegus monogyna* Jacq.) ethanolic extracts. J Chromatogr A 2006;1112(1-2):339-44.
13. Ivanauskas L, Jakštas V, Radušienė J, Lukošius A, Baranauskas A. Evaluation of phenolic acids and phenylpropanoids in the crude drugs. Medicina (Kaunas) 2008;44(1):48-55.
14. Benetis R, Radušienė J, Jakštas V, Janulis V, Malinauskas F. Development of an RP-HPLC method for the analysis of phenolic compounds in *Achillea millefolium* L. J Liq Chromatogr Rel Technol 2008;31(4):596-610.
15. Blumenthal M, Goldberg A, Brinckmann J. Herbal medicine. Expanded Commission E Monographs. Newton: Integrative Medicine Communications; 2000. p. 419-23.
16. Lemmens-Gruber R, Marchart E, Rawnduzi P, Engel N, Benedek B, Kopp B. Investigation of the spasmolytic activity of the flavonoid fraction of *Achillea millefolium* s.l. on isolated guinea-pig ilea. Arzneimittelforschung 2006;56(8):582-8.
17. Aljancic I, Vajs V, Menkovic N, Karadzic I, Juranic N, Milosavljevic S, et al. Flavones and sesquiterpene lactones from *Achillea atrata* subsp. *multifida*: antimicrobial activity. J Nat Prod 1999;62(6):909-11.
18. Garcia MD, Puerta R, Martinez S, Saenz NT. Analgesic, antipyretic and antiinflammatory effects of *Achillea ageratum*. Phytother Res 1997;11(5):376-9.
19. Benedek B, Geisz N, Jager W, Thalhammer T, Kopp B. Choleretic effects of yarrow (*Achillea millefolium* s.l.) in the isolated perfused rat liver. Phytomedicine 2006;13(9-10): 702-6.
20. Trifunovic S, Vajs V, Juranic Z, Zizac Z, Tesevic V, Macura S, et al. Cytotoxic constituents of *Achillea clavennae* from Montenegro. Phytochemistry 2006;67(9):887-93.
21. Konyalioglu S, Karamenderes C. The protective effects of *Achillea* L. species native in Turkey against H_2O_2-induced oxidative damage in human erythrocytes and leucocytes. J Ethnopharmacol 2005;102(2):221-7.
22. Ardestani A, Yazdanparast R. Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. Food Chemistry 2007;104(1):21-9.
23. Benetis R, Radušienė J, Janulis V. Variability of phenolic compounds in flowers of *Achillea millefolium* wild populations in Lithuania. Medicina (Kaunas) 2008; 44(10):775-81.

Received 8 May 2008, accepted 4 May 2009
Straipsnis gautas 2008 05 08, priimtas 2009 05 04