Journal of Chromatography A, 1217 (2010) 7690–7698

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Optimization and validation of post-column assay for screening of radical scavengers in herbal raw materials and herbal preparations

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ARTICLE INFO

Article history:
Received 28 April 2010
Received in revised form
28 September 2010
Accepted 4 October 2010
Available online 12 October 2010

Keywords:
Post-column
On-line HPLC-ABTS
Antioxidant capacity
Perilla

ABSTRACT

On-line method, which combines HPLC distribution and post-column reaction, was designed for the search of individual antioxidants. Optimization of the assay was performed evaluating optimal $ABTS^{\bullet+}$ radical cation concentration in the reactor, reaction time, impact of flow rate, reaction coil length. HPLC-ABTS assay validation in this work was performed by assessing reference antioxidant negative peak areas in radical scavenging chromatogram. Sample free radical scavenging activity is expressed as trolox equivalent antioxidant capacity (TEAC). Optimized and validated method was applied in detection of compounds possessing free radical scavenging ability in complex mixtures. Antioxidant compounds were studied in perilla (*Perilla frutescens* (L.) Britton var. *crispa* f. *viridis*) herbal raw material and its preparations. The HPLC-separated antioxidant compounds were identified using HPLC-photodiode array coupled to mass spectrometer, using a reference mass for determining accurate masses. Radical scavenging characteristics of rosmarinic acid, which is the dominant phenolic compound in medicinal herbal raw material of perilla and its preparations, were confirmed by the calculated TEAC values. Compounds responsible for antioxidant effect in herbal raw materials and herbal preparations were identified, evaluated and compared.

1. Introduction

Large quantities of free radicals are produced by exogenous sources, such as ionizing radiation, tobacco smoke, pesticides, air pollutants, pharmaceuticals; also quite a big part of free radicals is continuously produced as by-products in cells of numerous intracellular systems: cytoplasm molecules, membranes ferment systems, peroxisomes, mitochondria electron transmission systems, and microsome electron transmission systems [1–3]. When the protective antioxidant systems of the organism are insufficient, or if there is a lack of intrinsic anti-oxidants, free radicals may cause oxidative stress [4]. Oxidative stress in a human body causes initiation and development of most of the neuro-degenerative, cancerous diseases.

Research of raw herbal materials is the first step in the search of natural antioxidants [5]. Biologically active compounds of the herbal raw materials are distinct for different strength of the effect and different activity mechanisms [6–8]. Phenols of herbal origin are known for their radical scavenging activity [9–13].

Perilla frutescens (L.) Britton var. *crispa* f. *viridis* is an annual medicinal herbal plant of deadnettle (*Lamiaceae* Lindl.) family, originated from East Asia [14]. Rosmarinic acid, caffeic acid, luteolin, apigenin, scutellarein glycosides, triterpenic acids, which are main biologically active compounds accumulated by perilla herb, determine the most important pharmacological affects of the perilla raw material [8]. Many scientific studies have been carried out, and antimicrobial, immunostimulating, antiallergic, antitumor effects of *Perilla frutescens* L. preparations have been determined [15].

The initial selection of herbal compounds possessing the antioxidant effects is carried out by *in vitro* methods. Spectrophotometric *in vitro* methods are widely applied [6,12,16–22]. They determine total amount of antioxidants and evaluate the total antioxidant activity in the researched complex samples. Most frequently these methods use stable free radicals, thus, 1,1′-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) are the most common ones. After the reaction with an antioxidant these stable free radicals convert to colorless compounds and the

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doi:10.1016/j.chroma.2010.10.017

decrease of absorption is recorded in the visible light wave spectrum [23,24]. Radical scavenging ability is evaluated according to alteration in absorption and evaluated by standard estimates [24].

The main problem of natural antioxidants is that they are not pure substances, and there is a lack of data on the safety use [25]. The original nature of active compounds does not guarantee safe effect on organism's systems [26]. It is necessary to perform efficacy and safety researches of natural antioxidants [27]. Here arises the need for the systems that would help to distinguish, evaluate and, if possible, to identify individual compounds possessing antioxidant activity in complex samples. Once the antioxidant active substance is determined and the scavenging power of radicals is assessed according to standard estimates, the performance of efficacy and safety researches inside *in vivo* systems becomes possible.

Currently the on-line methods, which combine HPLC distribution and post-column reaction, are designed for the search of individual antioxidants. The greatest advantages of on-line methods are their selectivity, informatory capability and high sensitivity for precise determination of antioxidant-active compound and evaluation of its activity in complex compounds. These systems could also be applied to assess the quality of herbal raw materials, food supplements and herbal preparations. Manufacturers straightforwardly present numerous food supplements and herbal preparations as a source of antioxidants; however, there is no reliable methodology for assessing the antioxidant activity, safety and stability control during the release.

The objective of this work is (1) to develop and validate the on-line HPLC-ABTS assay; (2) to apply the latter in detection of compounds possessing the radical scavenging ability in complex mixtures; (3) to evaluate and compare the antioxidant activity of separate compounds; (4) to identify the compounds responsible for antioxidant effect in herbal raw materials and herbal preparations.

2. Materials and methods

2.1. Materials

All the used solvents are of HPLC grade. Methanol (99.9%) was purchased from Sigma–Aldrich GmbH (Buchs, Switzerland), acetic acid (99.8%) from Fluka Chemie (Buchs, Switzerland). Ultrapure water (18.2 $m\Omega\,cm^{-1}$) was prepared by Millipore (USA) water purification system.

The following reagents were used: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 99%), potassium persulfate (99%) from Fluka (Buchs, Switzerland); potassium permanganate (99%), potassium chloride (99.5%), potassium phosphate (99.5%), chlorogenic acid (98%), rosmarinic acid (96%) and caffeic acid (98%) which were delivered from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); (R)-6-methoxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®, 97%) from Acros Organics (New Jersey, USA); ethanol (96.3%) from Stumbras (Kaunas, Lithuania); sodium citrate (p.a.) and citric acid (p.a.) from Roth (Karlsruhe, Germany).

2.2. Sample materials

Perilla frutescens (L.) Britton var. *crispa* f. *viridis* medicinal herbal raw material was prepared in the collection of medicinal plants trial area in Kaunas Botanical Garden of Vytautas Magnus University in August. The raw material was air dried at room temperature (20–25 °C) in well ventilated room, protected from direct sun rays. Loss on drying was determined by the method indicated by European pharmacopoeia. Data of the study was recalculated for absolute dry herbal raw material by assessing the received data obtained from loss on drying samples.

The dry extract (10:1) of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* was produced by extracting the perilla medicinal raw material with water according to percolation method. One centimeter thickness layer of extract frozen on the surfaces of the sublimation vessels in the rotating freezing module of the liophilisator Freeze Dryer FD8512S (ilShin® Europe, Ede, The Netherlands), and then dried in this liophilisator at 5 milithorium pressure (condenser temperature −85 °C).

The following herbal preparations were obtained commercially: *Perilla frutescens* (L.) Britton concentrated leaf extract (5:1) (CLE) and capsulated herbal preparation (CHP). One capsule of capsulated extract contains 150 mg standardized *Perilla frutescens* (L.) Britton leaf extract, which is equivalent to 8 g of fresh perilla leaves.

2.3. Preparation of sample solutions

Reference compounds (trolox 80 μM, rosmarinic acid 110 μM, caffeic acid 60 μM, chlorogenic acid 150 μM) were dissolved in 96.3% ethanol.

Air-dried *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* medicinal raw material was crushed to particles passing through the 355 μm sieve. About 0.5 g (precise weight) of crushed perilla medicinal raw material was weighed and extracted with 50 ml 60% (V/V) ethanol.

The samples of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* dry extract (10:1) and of concentrated *Perilla frutescens* (L.) Britton leaf extract (CLE) (5:1) were prepared by dissolving 0.1 g (precise weight) of dry extract powder in 25 ml of water. Herbal preparation capsules were taken apart, 3 g of powder was weighted and dissolved in 25 ml of water. The concentrations of herbal preparations samples were selected according to minimum detectible concentrations (MDC) and minimum detectible amounts (MDA) of standard compounds. All samples were filtered through 0.22 μm pore size membrane filters (Carl Roth Gmbh, Germany) and 20 μl of the sample solution was injected for HPLC-ABTS analysis.

2.4. HPLC-ABTS radical scavenging system

The principle diagram of on-line HPLC-ABTS assay is shown in Fig. 1. Linear binary gradient is formed by chromatograph Beckman Programmable Solvent Module 126 (Fullerton, USA) at constant flow rate of 1 ml/min. Solvent A is 0.5% acetic acid, solvent B is methanol. Changes in gradient: 0 min, 90% A and 10% B; 40 min, 20% A and 80% B; 50 min, 10% A and 90% B; 52 min, 0% A and 100% B; 53 min, 90% A and 10% B; 60 min, 90% A and 10% B. Samples are injected by Rheodyne 7125 manual injector (Rheodyne, RohnertPark, CA) with 20 μl injection loop. Analytes are distributed by ACE C18 analytic column (5 μm, 150 mm × 4.6 mm) fitted with guard column ACE 5 μm C18 (Aberdeen, Scotland). The distributed compounds are detected by UV absorption detector Beckman System Gold 166 Programmable Detector Module (Fullerton, USA) at 290 nm wavelength. After the detection the eluent is directly mixed with $ABTS^{\bullet+}$ reagent solution for the assessing the scavenging of free radicals. 110 μM (A_{650} = 0.90 AU) of $ABTS^{\bullet+}$ working solution is supplied to the post-column at constant 0.5 ml/min flow rate by continuously working pump Waters Reagent Manager (Milford, USA). Reaction between $ABTS^{\bullet+}$ radical cation and analyte happens in the reaction coil. The following reaction coils were used: 3 and 15 m (0.3 mm ID and 1.58 mm OD) made of TFE (Teflon®) tube, 15 and 30 m (0.25 mm ID and 1.58 mm OD) made of PEEK (polyetheretherketone) tube. The decrease of reaction mixture absorption after reaction with antioxidant is directly recorded at 650 nm wavelength by UV/Vis type detector Gilson 118 (Middleton, USA). The compounds possessing antioxidant activity are recorded as negative peaks in radical scavenging (RS) chromatogram. Data received from experimental research is processed

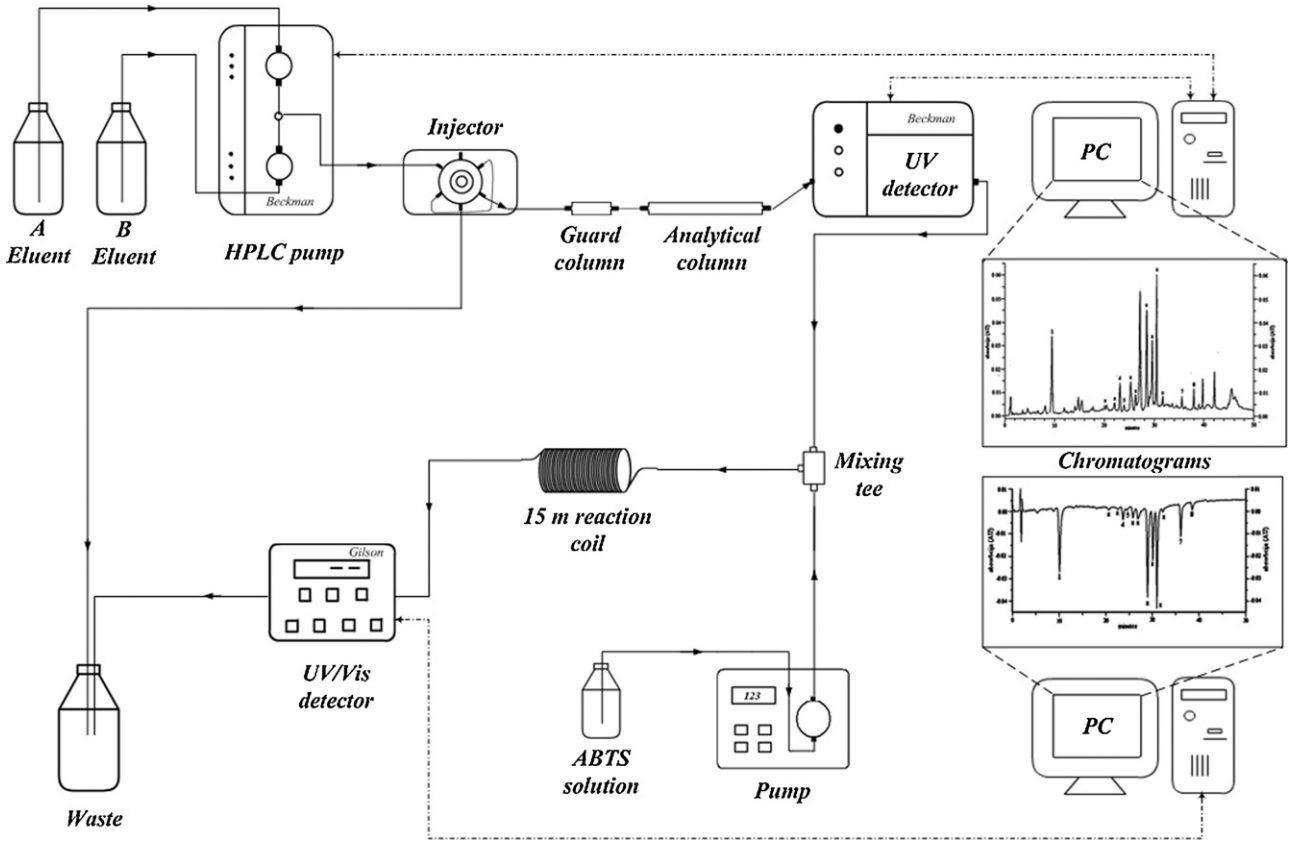

Fig. 1. HPLC-ABTS equipment system for radical scavenging analysis of individual antioxidants.

by Beckam (System Gold) and Gilson (UniPoint) software. Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, USA) and Microsoft Excel. All determinations were done in triplicate, and results were calculated as mean ± standard error (SE). Differences between means were compared by using nonparametric Mann–Whitney *U*-test. Linear relationship regression R^2 model was used for evaluating calibration curves. Level of significance $\alpha = 0.05$.

2.5. HPLC–MS system

HPLC–MS analysis of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* extracts was performed on a LC–MS system equipped with Waters 1525 binary pump (Waters, Milford, MA) and Rheodyne manual injector (Rheodyne, RohnertPark, CA). Compounds were separated on an ACE C18 LC-MS column (5 μm, 150 mm × 4.6 mm), (Aberdeen, Scotland), fitted with a ACE 5 μm C18 guard column (Aberdeen, Scotland), at a flow rate of 1.0 ml/min using a gradient starting from 90% of solvent A and 10% B and in 40 min increasing the content of B to 80%, then in 10 min—to 90%, then in following 2 min—to 100%. Afterwards in 1 min the eluents were returned to initial composition and the column was equilibrated for 7 min. Eluent A was 0.5% acetic acid in water and B—methanol. Compounds eluting from the column were detected with Waters DAD 996 detector (Waters, Milford, MA) operated in a range 210–400 nm, and Waters-Micromass ZQ 2000 single quadrupole mass detector, operated in negative ion electrospray ionization mode. The total ion recording in a range from 100 to 950 amu (Da), was used for monitoring compounds eluting from the column. Desolvation and cone gas (nitrogen) was set to 300 and 80 l/h, respectively. The ion source was operated at 120 °C, the desolvation temperature was 300 °C, the capillary voltage was maintained at 3 kV, and cone voltage—at 30 V.

2.6. Antioxidant activity assessment

According to the formula presented by Koleva et al. [28], reference compound relative $TEAC_{rel}$ values are calculated using the slopes (a) of the calibration curves. Trolox equivalent antioxidant capacity ($TEAC_{rel}$) shows how many times the researched known standard antioxidant is more active than reference antioxidant trolox. TEAC is calculated according to the formula:

$$TEAC_{rel} = \frac{a_{sample}}{a_{trolox}}$$

Trolox equivalent antioxidant capacity (TEAC) is calculated in order to perform quantitative assay of antioxidant active compounds present in extract of medicinal herbal raw materials and in their medicinal preparations. TEAC calculations were adapted from spectrophotometric assays [10,21,29,30]. TEAC of an antioxidant active compound corresponds to trolox quantity (μmol), which corresponds to the same antioxidant activity as 1 g of antioxidant active compound present in medicinal herbal raw material or pharmaceutical preparation. TEAC is calculated according to formula:

$$TEAC = \frac{TE_{comp.}(\mu M)}{1000} \times \frac{V_{sampl.}(ml)}{m_{sampl.}(g)}, \quad (\mu mol/g)$$

here $TE_{comp.}$ is the quantity equivalent of antioxidant active compound trolox (μM); TE is calculated according to formula:

$$TE = \frac{S_{comp.} - b}{a}, \quad (\mu M)$$

$S_{comp.}$ is the negative peak area of radical scavenging active compound in radical scavenging chromatogram; a is the slope, and b is the y-intercept from trolox calibration curve regressive equation; $V_{sampl.}$ is the volume of herbal raw material extract or pharmaceutical preparation (ml); $m_{sampl.}$ is the weighed (precise) quantity of herbal raw material or pharmaceutical preparation (g).

Attention shall be paid to the fact that the calculated TEAC value is equivalent to trolox quantity in μmol g of the researched sample. It does not indicate which of the compounds is more active; it exceptionally expresses the compound quantity equivalent to trolox quantity (mol). $TEAC_{rel}$ assesses antioxidant activity of a known compound in relation with trolox, as the calibration curve slopes are used as quantitative calculation estimates.

3. Results and discussion

3.1. Preparation and stability of ABTS$^{\bullet+}$ solutions

Literature suggests various methodologies for ABTS$^{\bullet+}$ radical cation generation [31–38].

ABTS$^{\bullet+}$ radical cation generation in our study was performed with the following strong oxidizers: potassium persulfate and potassium permanganate. When activating ABTS$^{\bullet+}$ with potassium permanganate, problems with the MnO_2 sediments arise; additional procedures are necessary to remove the sediments. The ABTS$^{\bullet+}$ activated this way is less stable, because the divalent manganese present in the ABTS solution increases ABTS$^{\bullet+}$ radical cations decomposition [39].

When increasing ABTS/persulfate molar ratio in the solution, stability of the produced ABTS$^{\bullet+}$ increases. Optimal ABTS/persulfate molar ratio equals to 2 or 3 [39].

During the research the stock ABTS$^{\bullet+}$ solution is prepared by oxidizing with potassium persulfate before addition of the antioxidant. 2 mM ABTS are dissolved in purified water and 0.7 mM of potassium persulfate are added. The mixture is kept for 16–17 h in the dark at room temperatures till complete reaction occurs, and the stable amount of ABTS$^{\bullet+}$ radical cations is obtained. Stock ABTS$^{\bullet+}$ solution, when protected from light, is stable for more than 2 days when working at room temperatures [38,39].

Before every serial analysis stock ABTS$^{\bullet+}$ solution is diluted till working concentration. The long-term stability of working ABTS$^{\bullet+}$ solution is an important parameter. In serial analyses the decrease of working ABTS$^{\bullet+}$ solution concentration conditions the decrease of ABTS$^{\bullet+}$ radical cations in the reactor, which influences the sensitivity of the assay and precision of antioxidant or its equivalent quantitative assessment. When reactor detection limits are reached, the quantitative assay is not possible. The diluter's impact on the stability of long-term working ABTS$^{\bullet+}$ solution is assessed by diluting with 8 mM phosphate-buffered saline (PBS) buffer (pH 7.4), 0.1 M citric acid–sodium citrate buffer (pH 7.6) and purified water. ABTS$^{\bullet+}$ solution of working concentration was kept for 8 h in room temperatures and protected from light. Dilution of the stock ABTS$^{\bullet+}$ solution with PBS buffer till the working concentration induces ABTS$^{\bullet+}$ radical cations degradation, and in 5 h the concentration of ABTS$^{\bullet+}$ working solution reduces to 50% (~0.2 μM/min). Similar tendency was determined when diluting stock ABTS$^{\bullet+}$ solution by citrate buffer (~0.17 μM/min). Long-term stability of ABTS$^{\bullet+}$ working solution was determined when the purified water was used as a diluter. In 8 h the concentration of ABTS$^{\bullet+}$ working solution reduces only by ~5%. In the further researches before every serial analysis stock ABTS$^{\bullet+}$ solution is diluted by purified water till the working concentration.

The influence of mobile organic phase in the reactor does not have great impact on ABTS$^{\bullet+}$ radical cation absorption. Possibility to apply post-column reaction with stable ABTS$^{\bullet+}$ radical cations on gradient analysis emerges [28].

3.2. Optimization of the online experimental setup

When validating on-line HPLC-ABTS assay, it is critically important to select the optimal ABTS$^{\bullet+}$ radical cation concentration in the reactor. ABTS$^{\bullet+}$ radical cation concentration in the reactor and reaction time impacts the negative peak area in RS chromatogram and S/N (the ratio between antioxidant peak height and baseline noise). When ABTS$^{\bullet+}$ radical cation concentration in the reactor increases, the reactor's detection range is increased and the sensitivity of the assay is impacted. The assay's sensitivity can be adjusted by changing the reaction time and the ABTS$^{\bullet+}$ radical cation concentration in the reaction coil, respectively, to the kinetics and antioxidant power of the researched samples.

HPLC-ABTS assay was designed by taking into account the common concentrations of analytes in HPLC analysis. ABTS$^{\bullet+}$ radical cation concentration in the reactor was optimized by assessing standards' peak areas and by calculating S/N ratios after injection of a fixed amount of standards (20 μl) (measurements in triplicate). The following mixture of standards was used: trolox 80 μM, rosmarinic acid 110 μM, caffeic acid 60 μM, chlorogenic acid 150 μM. ABTS$^{\bullet+}$ radical cation concentration in the reactor can be adjusted by adjusting concentration of the working solution (10–120 μM and its flow rate to the reactor (0.25–1.5 ml/min)). Having selected the fixed flow rate to the reactor and 15 m (0.3 mm ID and 1.58 mm OD) TFE (Teflon®) reaction coil (volume ~1 ml), the ABTS$^{\bullet+}$ concentration in the reactor was adjusted by adjusting the concentration of the working solution. It was determined that upon increased ABTS$^{\bullet+}$ radical cation concentration in the reactor, the standard antioxidant negative peak area in RS chromatogram increases. This dependence is shown in Fig. 2 The largest fixed areas are reached at different ABTS$^{\bullet+}$ concentrations due to different reaction kinetics. At low ABTS$^{\bullet+}$ concentration in the reactor (10 μM), the antioxidants may scavenge all the radicals, and thus the maximum detection area of the reactor is reached. At ABTS$^{\bullet+}$ radical cation concentrations above 35 μM, no statistically reliable difference between standard antioxidant negative peak areas was determined. At increased working ABTS$^{\bullet+}$ solution flow rate to the reactor the antioxidant peak area in RS chromatogram decreases, as the reaction time in the reactor decreases. The longer the reaction time in the reactor, the greater peak areas are.

Working pressure in the post-column system is an important parameter, as it increases when the flow rate of ABTS$^{\bullet+}$ radical cation solution increases. Attention shall be paid to the pressure limitations of the detector's flow cell and the reaction coil; the pressure shall not exceed 1000 si.

It was found that when ABTS$^{\bullet+}$ radical cation concentration in the reactor increases, the standard antioxidant peak area increases; however, at the same time there is an increase in baseline noise,

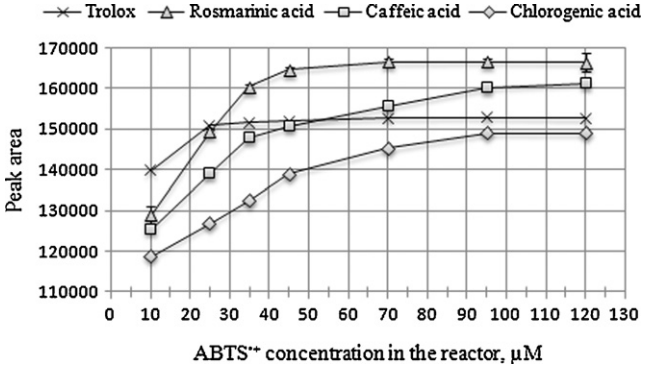

Fig. 2. Dependence of standard negative peak area on ABTS$^{\bullet+}$ radical cation concentration in the reactor. Used 15 m (0.3 mm ID and 1.58 mm OD) TFE (Teflon®) reaction coil (volume ~1 ml). HPLC flow rate 1 ml/min. Reagent flow rate 0.5 ml/min.

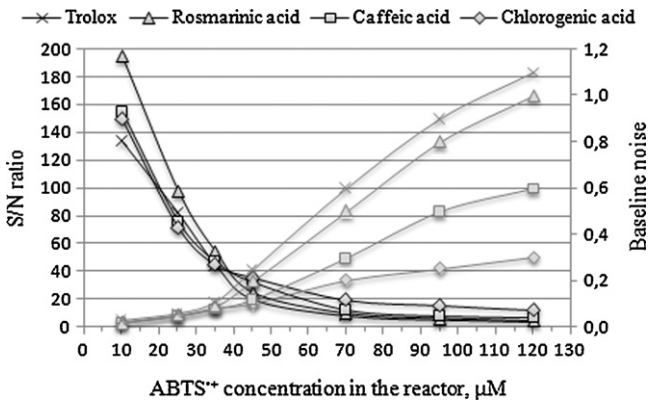

Fig. 3. Dependence of peak height-to-baseline noise ratio (S/N) and baseline noise on ABTS$^{\bullet+}$ radical cation concentration in the reactor. Used 15 m (0.3 mm ID and 1.58 mm OD) TFE (Teflon®) reaction coil (volume ~1 ml). HPLC flow rate 1 ml/min. Reagent flow rate 0.5 ml/min. ABTS$^{\bullet+}$ radical cation concentration in the reactor is 35 μM.

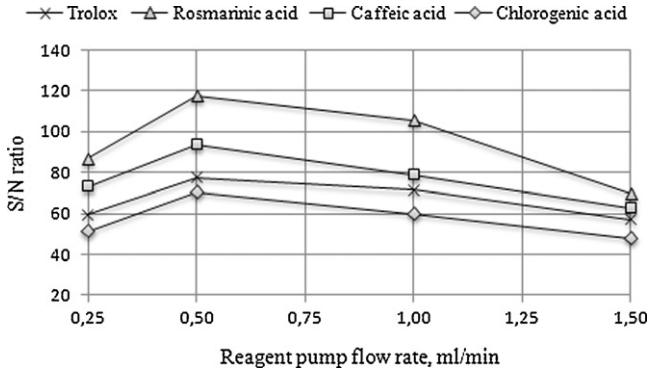

Fig. 4. Dependence of S/N ratio on reagent pump flow rate. Used 15 m (0.3 mm ID and 1.58 mm OD) TFE (Teflon®) reaction coil (volume ~1 ml). HPLC flow rate 1 ml/min. ABTS$^{\bullet+}$ radical cation concentration in the reactor is 35 μM. Reagent flow rate relate to reaction time: 0.25 ml/min, 48 s; 0.5 ml/min, 40 s; 1 ml/min, 30 s; 1.5 ml/min, 24 s.

which reduces S/N ratio (Fig. 3.). When ABTS$^{\bullet+}$ concentration in the reactor increases, the S/N ratio decreases. The instability of RS chromatogram baseline makes negative impact on the sensitivity of the assay, on the precision of quantitative evaluation of antioxidant or its equivalent, on minimum detectable concentrations (MDC) in an active compound, and on minimum detectable amounts (MDA) in an active compound. The instability of baseline is named to be the main problem of HPLC-ABTS analysis [40].

Statistically significant baseline noise change ($p < 0.05$) was determined when ABTS$^{\bullet+}$ concentration in the reactor was 40 μM. If the ABTS$^{\bullet+}$ concentration in the reactor is increased further, the instability of baseline increases.

Optimized ABTS$^{\bullet+}$ radical cation concentration in the reactor is 35 μM. At this concentration the optimal S/N ratio is reached, and the optimal negative peak area of standard oxidants is determined. Concentration of 35 μM ABTS$^{\bullet+}$ in the reactor has been selected for the further research.

Reaction kinetics of different free radical scavengers with ABTS$^{\bullet+}$ radical cation varies [41]. Literature states that optimal reaction time between most of herbal antioxidants and ABTS$^{\bullet+}$ is 30–40 s [41,42]. Reaction time can be alternated in two ways: by reducing the flow rate and by extending the reaction coil (increasing its volume). A fixed-length (15 m) reaction coil was selected to assess the flow rate impact on S/N ratio. ABTS$^{\bullet+}$ working solution of certain concentration is supplied to the reactor in order to reach 35 μM ABTS$^{\bullet+}$ concentration at different flow rates (0.25–1.5 ml/min) in the reactor. The ABTS$^{\bullet+}$ solution supply flow rate to the reactor directly determines the reaction time of antioxidant and ABTS$^{\bullet+}$ radical cation in the reactor; the duration impacts the chromatogram baseline noise, analyte peek height and area, respectively. Selection of optimal ABTS$^{\bullet+}$ solution supply flow rate to the reactor is essential when performing quantitative evaluation of antioxidant activity.

Mixture of standards was used for this experiment. Dependence between ABTS$^{\bullet+}$ solution supply flow rate to reactor, analyte peak height and chromatogram baseline noise (S/N) was determined (Fig. 4). S/N ratio decreases at increased ABTS$^{\bullet+}$ solution flow rate in the system, and vice versa, at decreased flow rate the S/N ratio increases. At 0.25 ml/min flow rate a small enlargement of peaks in RS chromatogram was noticed, therefore S/N ratio decreased.

At 35 μM ABTS$^{\bullet+}$ radical cation concentration in the reactor, as shown in Fig. 4, the best S/N ratio is reached at 0.5 ml/min flow rate. ABTS$^{\bullet+}$ solution of 110 μM (0.90 AU) concentration was selected for quantitative assessment of antioxidants or their equivalents. Optimal 35 μM ABTS$^{\bullet+}$ radical cation concentration in the reactor was reached at 0.5 ml/min flow rate. These experimental conditions were applied in further experiments.

Optimization of reaction time is performed not only by assessing the impact of flow rate, but also the influence of reaction coil length on S/N ratio is also evaluated. Four different reaction coils were used for this experiment. At total flow rate of 1.5 ml/min in 3 m (0.3 mm ID and 1.58 mm OD) tube the mixture of reaction lingers ~8 s, in 5 m (0.25 mm ID and 1.58 mm OD)—~30 s, in 15 m (0.3 mm ID and 1.58 mm OD)—~40 s and in 30 m (0.25 mm ID and 1.58 mm OD) reaction coil—~60 s.

It was determined that short (3 m) reaction coil does not provide enough time for the reaction between ABTS$^{\bullet+}$ radical cations and the antioxidant to occur, therefore relatively low S/N ratio is reached. In 30 m 0.25 mm ID reaction coil the reaction lasts for 60 s; however, at the same time the undesirably huge reverse counter pressure to the post-column system is caused. Peaks enlargement appears, and this conditions decrease of S/N ratio. No statistically significant differences between 30 and 40 s reaction coils were noticed; therefore 15 m (0.3 mm ID and 1.58 mm OD) long reaction coil was selected for further experiments, thus guaranteeing long reaction time for compounds of low reaction kinetics.

3.3. Online HPLC-ABTS system validation

The literature data regarding validation of post-column reaction assay is scarce, there are no unified standard estimates for validation of such assays. Post-column reaction is an extension of HPLC method. At the time of post-column reaction no distribution is occurring; previously distributed analytes pass through the reactor, therefore the main HPLC separation parameters do not apply for post-column assay validation. HPLC separation method is validated separately. HPLC-ABTS assay validation in this work was performed by assessing reference antioxidant negative peak areas in RS chromatogram. Several standard assay validation parameters were chosen: assay precision, minimum detectable concentration, minimum detectable amount, linearity and specificity.

Assay validation is carried out under optimized conditions: ABTS$^{\bullet+}$ working solution of 110 μM (0.90 AU) concentration, 0.5 ml/min flow rate to reactor, 35 μM ABTS$^{\bullet+}$ radical cation concentration in the reactor, and 15 m (0.3 mm ID and 1.58 mm OD) reaction coil.

Specificity was assessed by identification test discriminating of active compounds (the reaction of ABTS$^{\bullet+}$ radical cation and particular active ingredient present in the sample, conforming its radical scavenging identity).

Table 1
Minimum detectable concentrations (MDC), minimum detectable amounts (MDA) and precision characteristics of some antioxidants.

Compounds	MDC, μM	MDA, ng	Repeatability	Intermediate precision
Trolox	1.60	8	1.53	2.99
Rosmarinic acid	1.67	12	1.6	3.13
Caffeic acid	2.22	8	1.28	2.16
Chlorogenic acid	2.26	16	3.21	5.84

*Obtained under the optimized experimental conditions. RSD, coefficient of variation (relative standard deviation). Determined with a 20-μl loop; 40 s reaction time.

Precision of the assay was determined by analyzing ethanolic solution of standard compounds (trolox 80 μM, rosmarinic acid 110 μM, caffeic acid 60 μM, and chlorogenic acid 150 μM). The precision of assay was determined by repeatability and intermediate precision, and these values were expressed as the RSD of a series of measurements.

Repeatability maximum RSD value equals to 3.21%, and intermediate precision maximum RSD value equals to 5.84% (Table 1). Such precision of the results is acceptable; therefore, it is possible to perform quantitative evaluation of antioxidant active compounds or their equivalents.

Reference antioxidant minimum detectable concentrations (MDC) and minimum detectable amounts (MDA) were determined by dilution method; there is also a possibility to quantify them (S/N∼10) [43] (Table 1.). The lowest MDC is of trolox (1.60 μM), the highest one is of chlorgenic acid (2.26 μM). The lowest MDA is of trolox and caffeic acid (8 ng); double amount (16 ng) of chlorgenic acid is necessary for quantitative evaluation.

The evaluation of assay applicability for quantitative researches was performed by injecting four different caffeic acid concentrations under isocratic conditions. After recalculation of received proportional negative peak areas, it was determined that the assay is linear in this interval (560, 280, 140, and 70 μM) of caffeic acid concentrations.

Reference compound linearity evaluations were also performed. The obtained data are presented in Table 2. Different concentrations (560, 280, 140 and 70 μM) (triplicate injections) of a known standard antioxidant were used to construct calibration curve $h = a \times c + b$, where h stands for negative peak area in RS chromatogram; c is the compound concentration in μM; a is the slope and b is the y-intercept of the obtained curve. The evaluated determination coefficients (R^2) of analytes' calibration curves are higher than 0.99; $p < 0.0001$, which proves the linearity of quantitative determination method. The received values of high-performance liquid chromatography-ABTS post-column reaction method vali-

Table 2
Calibration curve ($h = a \times c + b$; R^2) and $TEAC_{rel}$ data for several known antioxidants.

Compounds	Slope, a	Intercept, b	Linear range, μM	Data points in linear range, n	R^2	$TEAC_{rel}$
Trolox	2812.7	−9576.6	20–320	6	0.9997	1.00
Rosmarinic acid	4896.6	−1760.9	18–180	5	0.9986	1.74 ± 0.09
Caffeic acid	2166.3	−6228.6	70–555	5	0.9958	0.77 ± 0.08
Chlorogenic acid	1203.8	−17352.0	35–283	5	0.9910	0.43 ± 0.06

*Obtained under the optimized experimental conditions. $TEAC_{rel}$ are means ± SD.

Table 3
HPLC–MS-DAD data.

Peak No.	RT (min)	λ_{max} (nm)	ESI/MS m/z		Compounds	References
			$[M-H]^-$	Fragments		
1	12.99	324.5	179		Caffeic acid	[44,46]
2	15.62	329.0	593	179	Apigenin-7-O-caffeoylglucoside	[46,47]
3	16.37	348.0	637	285, 351	Luteolin-7-O-diglucuronide	[46–48]
4	18.52	338.8	621	351, 269	Apigenin-7-O-diglucuronide	[46–48]
5	20.30	334.0	461	285	Scutellarein-7-O-glucuronide	[46,49,50,51]
6	22.50	329.0	359	197, 161	Rosmarinic acid	[44–46]

Table 4
Antiradical activity equivalents of individual compounds present in the researched samples.

Peak No.	RT, min	Compounds	Perilla herb		Perilla extract powder (10:1)		CLE (5:1)		CHP	
			TE	TEAC	TE	TEAC	TE	TEAC	TE	TEAC
–	6.93	Unknown	n.d.	n.d.	n.d.	n.d.	71.38	17.84	16.40	0.14
–	7.90	Unknown	n.d.	n.d.	n.d.	n.d.	10.33	2.58	4.18	0.03
–	10.07	Unknown	n.d.	n.d.	n.d.	n.d.	13.37	3.34	4.66	0.04
–	11.99	Unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.44	0.09
1	12.99	Caffeic acid	13.40	1.34	24.95	6.24	37.55	9.39	6.75	0.06
–	14.87	Unknown	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.91	0.04
2	15.62	Apigenin-7-O-caffeoylglucoside	10.84	1.08	14.96	3.74	10.61	2.65	4.91	0.04
3	16.37	Luteolin-7-O-diglucuronide	11.93	1.19	24.58	6.14	13.87	3.47	6.46	0.05
4	18.52	Apigenin-7-O-diglucuronide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
–	19.12	Unknown	n.d.	n.d.	n.d.	n.d.	20.21	5.05	5.07	0.04
5	20.30	Scutellarein-7-O-glucuronide	89.26	8.93	89.59	22.40	20.01	5.00	4.79	0.04
–	21.36	Unknown	15.09	1.51	26.67	6.67	n.d.	n.d.	n.d.	n.d.
–	22.01	Unknown	56.02	5.60	26.00	6.50	n.d.	n.d.	n.d.	n.d.
6	22.50	Rosmarinic acid	302.66	30.27	350.28	87.57	73.47	18.37	15.60	0.13
–	27.07	Unknown	5.32	0.53	9.48	2.37	11.83	2.96	n.d.	n.d.
–	28.66	Unknown	8.15	0.82	4.87	1.22	n.d.	n.d.	n.d.	n.d.
		Total:	512.68	51.27	571.37	142.84	282.63	70.66	84.16	0.70

dation criteria confirm its reliability, accuracy and applicability for quantitative evaluation of known and unknown antioxidant active compounds according to TEAC (μmol/g).

3.4. Online HPLC-ABTS assay application

Combined detection system (HPLC–MS-DAD) was applied for extracts analysis in order to identify the components possessing antioxidant effect. Components were identified, and data of both systems was assessed according to retention time, diode matrix spectrum, mass fragmentation spectrum, and *m*/*z* proportion. Retention times, compound molecular mass, mass fragmentation, and λ_{max} absorption were compared to literary data [44–51]. Four flavonoids and two phenolic acids were identified in the raw material of Britton var. *crispa* f. *viridis* (Table 3). Apigenin-7-O-caffeoylglucoside *m*/*z* 593 [M−H]−, 179 [caffeic acid−H]−. Luteolin-7-O-diglucuronide *m*/*z* 637 [M−H]−, 285 [M−H−diglucuronide]− = [aglycone−H]−, 351 [diglucuronide−H−H_2O]−. Fragment 351 is specific for diglucuronide group after dehidration [49]. Apigenin-7-O-caffeoylglucoside *m*/*z* 621 [M−H]−, 351 [diglucuronide−H−H_2O]−, 269 [M−H−diglucuronide]− = [aglycone−H]−. Scutellarein-7-O-glucuronide *m*/*z* 461 [M−H]−, 285 [M−H-176]− due to the loss of glucuronic acid in the deprotonized molecule (176 u). Caffeic acid *m*/*z* 179 [M−H]−. Rosmarinic acid *m*/*z* 359 [M−H]−, 197 [M−H−caffeic acid]−, 161 [M−H-197]− due to the loss of caffeoyl substitute (162 u) [44,51].

Developed, optimized and validated on-line HPLC-ABTS assay was applied on medicinal herbal raw material (*Perilla herba*) and on *Perilla extract powder* (10:1) of *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis*, also on *Perilla frutescens* (L.) Britton capsulated herbal preparation and on concentrated extract (5:1) in order to determine the presence of antioxidant active compounds. Reference antioxidant trolox was used to assess the free radical scavenging capacity. Table 4 presents the calculated TEAC values of individual compounds that determine the antioxidant activity of the above stated samples.

Koleva et al. point out that hydrogen-donating groups determine the antioxidant activity of the compounds [28]. The ability of flavonoids to scavenge free radicals is the extent and nature of the hydroxylation pattern of the aromatic rings. If compared to apigenin, the stronger characteristics of luteolin and scutellarein are determined by a higher number of hydroxyl groups in the molecule. The antioxidant activity of luteolin is increased by free hydroxyl group in B ring *ortho* position, and of scutellarein—in C_6 position of A ring. Apigenin structure does not contain these free hydroxyl groups, which explains its low antioxidant activity [52]. Antiradical activity of flavonoid glucosides can be increased by their acylated sugar moieties [10]. It was determined that phenolic compounds of *Lamiaceae* family plants containing caffeoyl group possess strong antioxidant activity [10,53].

HPLC-UV-ABTS chromatograms (according to the formed negative inactivated $ABTS^{\bullet+}$ radical cation peaks) of the researched samples revealed that rosmarinic acid possesses the greatest radical scavenging capacity (Figs. 5 and 6, Peak no 6). Antioxidant characteristics of rosmarinic acid, which is the dominant phenolic compound in medicinal herbal raw material of *Perilla frutescens* (L.) Britton and its preparations were confirmed by the calculated TEAC values (Table 4). The greatest TEAC value of 87.57 μmol/g was determined in *Perilla extract powder* (10:1) sample; this determines ~60% of total compound antioxidant activity (142.84 μmol/g). The lowest TEAC value of 0.13 μmol/g (~20% total antioxidant activity) was determined in capsulated herbal preparation. Rosmarinic acid has two *ortho*-dihydroxy functional groups, which determine high antioxidant activity of this compound. *Lamiaceae* family plants, in which the higher rosmarinic acid amounts have been

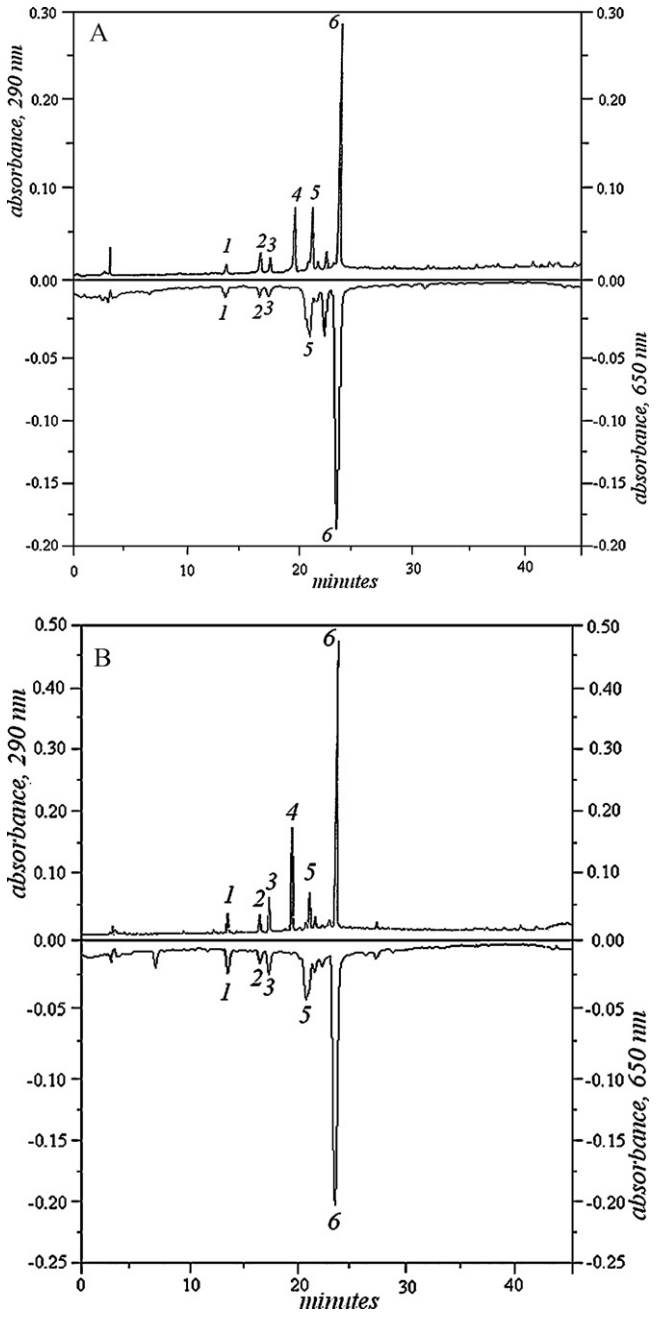

Fig. 5. HPLC-UV-ABTS coupled chromatograms of the *Perilla frutescens* (L.) Britton var. *crispa* f. *viridis* herb (A) and concentrated extract powder (10:1) (B). For exact compound refer to Table 4.

detected, are significant for greater free radical scavenging activity [54].

The literature states that high quantities of apigenin formations are specific for the green perilla form [44]. Their quantities vary from 27 till 78% of total flavone amount [44]. On-line HPLC-ABTS assay determined that apigenin-7-O-diglucuronide is not significant for antioxidant characteristics; however, the acylated derivative apigenin-7-O-caffeoylglucoside was able to scavenge free radicals in all the researched samples (Figs. 5 and 6, Peak no 4 and 2, respectively). Caffeoyl group provides apigenin-7-O-caffeoylglucoside with antioxidant effect.

Caffeic acid, luteolin-7-O-diglucuronide and scutellarein-7-O-glucuronide were detected in all the researched samples, and the antioxidant activity of those compounds was assessed (Table 4,

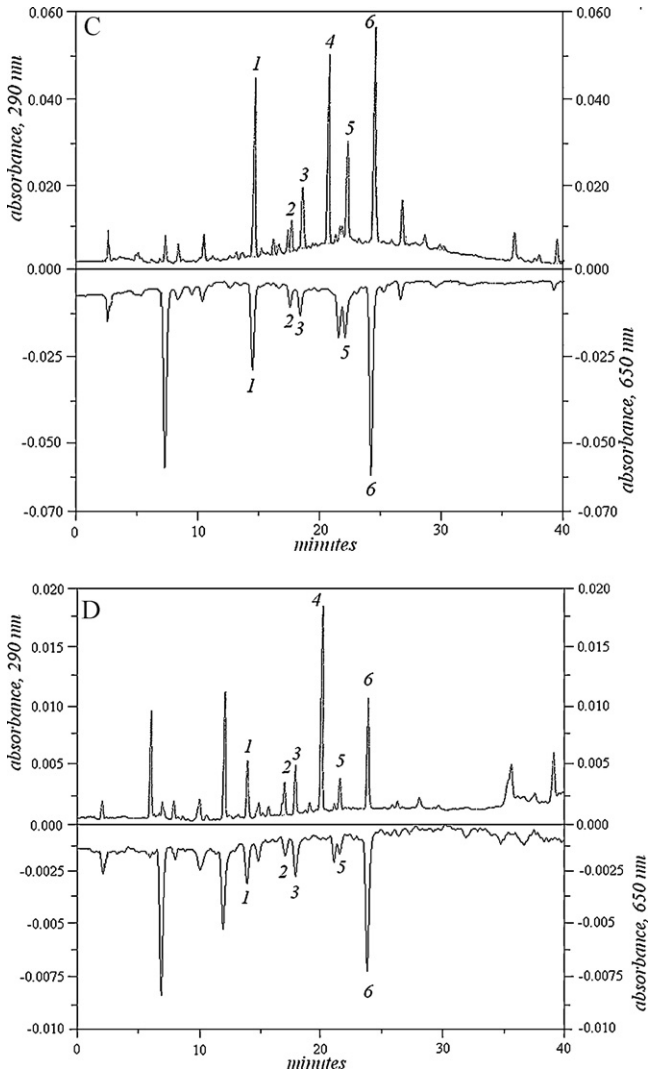

Fig. 6. HPLC-UV-ABTS coupled chromatograms of the *Perilla frutescens* (L.) Britton leaf concentrated extract powder (5:1) (C) and medicinal herbal preparation (standardized leaf extract (D)). For exact compound refer to Table 4.

Figs. 5 and 6, Peak no 1, 3, 5, respectively). Unidentified components also impact the total antioxidant activity of the researched samples. Unknown compounds determine only ~15% of total antiradical activity of Britton var. *crispa* f. *viridis* medicinal herb raw material (*Perilla herb*) and its dry extract (*Perilla extract powder* (10:1)) (TEAC 51.27 and 142.84 μmol/g, respectively), whereas antioxidant axtive unidentified compounds in *Perilla frutescens* (L.) Britton concentrated extract (5:1) and in capsulated herbal preparation determine even ~50% of total activity (TEAC 70.66 and 0.70 μmol/g, respectively). This can be attributed to the additional materials with antioxidant characteristics that were used in the manufacturing process.

4. Conclusion

Sensitive and selective on-line HPLC assay for detection of free radical scavenging compounds in complex samples by employing $ABTS^{\bullet+}$ radical cation was optimized and validated. On-line HPLC-ABTS assay enables scientists to evaluate and standardize the quality of raw materials, and to select the species that are rich with specific antioxidants. The assay also provides the possibility to determine antiradical properties of unknown antioxidants, to make precise assessment of antioxidant activity of known compounds, their amount and contribution to total antioxidant activity of a medicinal herbal raw material or medicinal preparation. The assay that we designed is significant for its sufficient repeatability. This assay is suffessfully used in scientific researches, as well as it can be applied for evaluation of quality parameters of compex medicinal herbal raw materials and medicinal preparations.

Acknowledgement

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

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