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Comparative evaluation of post-column free radical scavenging and ferric reducing antioxidant power assays for screening of antioxidants in strawberries

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ABSTRACT

ABTS and FRAP post-column techniques evaluate the antioxidant characteristics of HPLC separated compounds with specific reagents. ABTS characterize their ability to scavenge free radicals by electron-donating antioxidants, resulting in the absorbance decrease of the chromophoric radical. FRAP – is based on the reduction of Fe(III)–tripyridyltriazine complex to Fe(II)–tripyridyltriazine at low pH by electron-donating antioxidants, resulting in an absorbance increase. Both post-column assays were evaluated and compared according to the following validation parameters: specificity, precision, limit of detection (LoD), limit of quantitation (LoQ) and linearity. ABTS and FRAP post-column assays were specific, repeatable and sensitive and thus can be used for the evaluation of antioxidant active compounds. Antioxidant active compounds were quantified according to TEAC for each assay and ABTS/FRAP ratio was derived. No previous records of antioxidative activity of leaves and fruits of strawberries (*Fragaria viridis*, *Fragaria moschata*) research have been found. The research results confirm the reliability of ABTS and FRAP post-column assays for screening of antioxidants in complex mixtures and the determination of radical scavenging and ferric reducing ability by their TEAC values.

1. Introduction

Oxidative stress plays a pivotal role in pathogenesis of cardiovascular, neurodegenerative diseases, cancer and aging [1]. Antioxidants reduce oxidative stress by various mechanisms [2]. Research of natural antioxidants has increased in area of functional foods, agriculture and disease prevention [3–5]. Numerous assays with different mode of action have been established for the assessment of antioxidant activity [3,6,7]. Spectrophotometric studies evaluating the total antioxidant capacity are convenient and easy adaptable. These assays depend on single electron transfer or hydrogen atom transfer [8]. Most popular assays, based on single electron transfer, evaluate radical scavenging abilities (ABTS – 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), DPPH – 2,2-diphenyl-1-picrylhydrazyl) and potential of ferric or cupric reducing (FRAP – ferric reducing antioxidant power, CUPRAC – cupric reducing antioxidant capacity) capacity expressed as Trolox equivalents [3,9–12]. Yet, they evaluate the additive and synergistic interrelational impact of all compounds present in the sample as herbal extracts contain a body of polyphenolic compounds with different structure and activity [13]. To elucidate the activity of separate compounds in complex extracts is not possible. Structure–activity relationships well established in numerous studies confirmed different modes of action of separate polyphenolic compounds [14,15]. It is purposeful to evaluate individual polyphenolic compounds in herbal extracts with antioxidant activity.

Recently a body of researches has established on-line post-column methods for screening of antioxidants in complex mixtures [16–21]. HPLC separation is coupled with rapid identification of antioxidative active compounds. The pivotal advantages of these post-column reaction methods are that the antioxidant activity of an individual compound can be measured and its contribution to the total activity of a complex mixture can be estimated, and also the activity of an individual compound can be compared to other constituents in the mixture and their structure–activity relationships can be determined [21,22]. These methods exclude the interactional effects of compounds as the detection occurs with separated analytes. Most of post-column assays use DPPH and ABTS radicals [16,21,23], while FRAP post-column assay, to the best of our knowledge, has not been installed previously. The latter assays can be performed in aqueous medium and low pH. As the assays are installed in the same conditions, it becomes possible to compare the radical scavenging and reducing abilities of antioxidant active compounds.

Leaves and fruits of strawberries (*Fragaria* L. species) have long been used for medicinal and nutritional purposes [24]. The herbal

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material contains a complex mixture of various polyphenolic compounds (flavonoids, phenolic acids, tannins, anthocyanins). The potential health benefits have been associated with antioxidant effects [25]. Therefore it is important to study polyphenolic-rich extracts and to identify the antioxidant active components.

Thus, the aim of this study was to compare ABTS and FRAP post-column assays through the validation parameters and to apply the assays for the determination and evaluation of radical scavenging and ferric reducing abilities of antioxidants in strawberries.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and HPLC grade methanol were purchased from Sigma–Aldrich (Buchs, Switzerland). Formic acid (98–100%), acetic acid (99.8%) and hydrochloric acid were obtained from Fluka Chemie (Buchs, Switzerland). Ethanol (96.3%) was provided by Stumbras (Kaunas, Lithuania). Ultrapure water was prepared using a Millipore water purification system (Bedford, MA). The following reagents were used: 2,2-azinobis (ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulfate from Fluka (Buchs, Switzerland); iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), 2,4,6-tripyridyl-s-triazine (TPTZ) and sodium acetate trihydrate from Sigma–Aldrich Chemie (Steinheim, Germany). The following standards were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), chlorogenic acid, (−)-epicatechin, caffeic acid, (+)-catechin, rutin, ellagic acid, quercetin, isoquercitrin, quercitrin, hyperoside, epigallocatechin gallate were purchased from Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), Sigma–Aldrich (Buchs, Switzerland). Individual stocks of standard solutions were prepared in ethanol. For HPLC post-column analysis the stock solutions were respectively diluted with ethanol to the required working concentrations. Ellagic acid was prepared and diluted with acetonitrile and purified water mixture (1:1).

2.2. Sample materials and preparation

Fragaria viridis, *Fragaria vesca* and *Fragaria moschata* leaves and fruits, which were obtained from Vilnius University Botanical Garden, Lithuania, were the plant material used for this research. Strawberry leaves were collected in July and air-dried at room temperature (20–25 °C), in a ventilated chamber. Fully ripened fruits were frozen and stored in deep freezer at −30 °C.

The air-dried parts of *F. viridis*, *F. vesca*, *F. moschata* leaves were milled. The fruits were homogenized using a rotating blade mixer. The milled strawberry leaves were extracted with 70% ethanol (1:100, v/v) in ultrasonic bath (BioSonic UC100 Mawai, USA) for 30 min. The homogenized fruits were extracted with 100% methanol (1:10, v/v) in ultrasonic bath for 10 min. The extracts were centrifuged (13,000 rpm, 15 min) and obtained supernatants were kept in the refrigerator at 8 °C. All the samples were filtered through 0.22 μm pore size membrane filters (Roth, Karlsruhe, Germany).

2.3. HPLC post-column antioxidant detection system conditions

The HPLC post-column equipment system for screening of individual antioxidants has been previously published by our group [12]. The HPLC system applied consisted of Waters 2695 Alliance solvent manager (Waters, Milford, MA) equipped with a Waters 996 photodiode array detector. Chromatographic separations were performed on an ACE C_{18} analytical column (250 mm × 4.6 mm, 5 μm) with guard column ACE C_{18} 5-μm (Aberdeen, Scotland). The chromatographic separation was performed using 1% (v/v) formic acid solution in pure water (solvent A) and acetonitrile (solvent B). The solvent composition for the linear gradient elution was as follows: 10–22% solvent B over 30 min; followed by 22–80% solvent B from 30 to 45 min The mobile phase flow rate in all analyses was set at 1 mL/min, and the injection volume of all samples was 10 μL. The confirmation of the chromatographic peaks identity was achieved by comparing the retention times and spectral characteristics (λ = 200–600 nm) of the eluting peaks with those of reference compounds. All samples were run in triplicate.

HPLC post-column addition of ABTS and FRAP reagent was performed using a continuously working Waters Reagent Manager (Milford, MA) pump. The flow rate of the individual reagents was set at 0.5 mL/min. The mobile phase with separated analytes and ABTS or FRAP reagents flowed through a mixing tee to the reaction coil. The reaction coil was made of TFE (Teflon) tubing of the following size: 15 m × 0.3 mm i.d., 1.58 mm o.d., ∼1 mL. The product chromatograms after ABTS and FRAP post-column reaction were registered at 650 and 593 nm respectively, using Waters 2487 dual λ absorbance (UV/Vis) detector (Milford, MA). Data received from experimental research was processed by Waters Empower software (Milford, MA).

The stock ABTS solution was prepared by dissolving ABTS in aqueous potassium persulfate (0.7 mM) to obtain the concentration of 2 mM. The mixture was stored for 16–17 h in the dark at room temperatures before use [26]. Acetate buffer was prepared of sodium acetate trihydrate, acetic acid and water up to the concentration of 300 mM (pH 3.6). The working solution of ABTS was prepared by diluting with acetate buffer up to the concentration of 0.11 mM.

The working FRAP solution comprised TPTZ (10 mM dissolved in 40 mM HCl), $FeCl_3{\cdot}6H_2O$ (20 mM in water) and acetate buffer (300 mM, pH 3.6) in the ratio of 1:1:25.

2.4. Antioxidant activity assessment

The antioxidant activity of sample compounds was assessed by standard antioxidant Trolox. Calibration curves of Trolox (concentrations 5–400 μM) were made in ABTS (R^2 = 0.999) and FRAP (R^2 = 0.999) post-column assays. The radical scavenging and ferric reducing capacities of antioxidant active compounds in strawberry extracts were expressed as Trolox equivalent antioxidant capacity (TEAC). TEAC corresponds to Trolox quantity (μmol), which at the equal conditions has the same antioxidant activity as the sample compound in 1 g of strawberries. TEAC was calculated according to the formula:

$$\mathrm{TEAC} = \frac{S_{\mathrm{comp.}} - b}{a}(\mu\mathrm{M}) \times \frac{V_{\mathrm{sampl.}}(\mathrm{L})}{m_{\mathrm{sampl.}}(\mathrm{g})}(\mu\mathrm{mol/g})$$

$S_{\mathrm{comp.}}$ is the peak area of antioxidant active compound in the post-column chromatogram; a is the slope, and b is the y-intercept from Trolox calibration curve regressive equation; $V_{\mathrm{sampl.}}$ is the volume of herbal raw material extract; $m_{\mathrm{sampl.}}$ is the weighed (precise) quantity of herbal raw material.

$\mathrm{TEAC_{rel}}$ value, that shows how many times the researched known antioxidant is more active than standard antioxidant Trolox. $\mathrm{TEAC_{rel}} = a_{\mathrm{sample}}/a_{\mathrm{trolox}}$, a is the slope of the sample compound and Trolox of the calibration curves [16].

2.5. Statistical analysis

Statistical analysis was performed using SPSS version 11.0 (Chicago, USA) and Microsoft Excel. All determinations were done in triplicate, and results were calculated as mean ± standard error (SE). Linear regression model was analyzed. For the suitability of each regression model determination coefficient R^2 and p-value obtained by checking hypothesis on non-linear regression were

used. The Mann–Whitney *U* test was performed for the hypothesis concerning equality of distributives. Level of significance $\alpha = 0.05$.

3. Results and discussion

3.1. Assessment of ABTS and FRAP post-column assay characteristics

ABTS and FRAP post-column assays evaluate the qualitative characteristics of sample compounds with specific reagents (ABTS and FRAP). ABTS characterize the ability to scavenge free radicals by electron-donating antioxidants, resulting in the absorbance decrease of the chromophoric radical at 415, 650, 734 and 815 nm [26–28]. FRAP is based on the reduction of Fe(III)–tripyridyltriazine (Fe(III)–TPTZ) complex to Fe(II)–tripyridyltriazine (Fe(II)–TPTZ) at low pH by electron-donating antioxidants, resulting in the absorbance increase at 593 nm [28,29]. While ABTS method measures the active compound capacity against an oxidant, the FRAP assay directly measures the substance's reducing capacity, which is an important parameter for a compound to be a good antioxidant [22,30]. Since the antioxidant activity of a substance is usually correlated directly to its reducing capacity, the FRAP assay provides a reliable method to study the antioxidant activity of various compounds [11].

Antioxidant activity of sample compound is quantitatively assessed by TEAC value. For the objective and comprehensive comparison of ABTS and FRAP post-column assays at the equal experimental conditions, the assays need to be validated. Only the developed and validated method confirms that the analytical procedure employed for a specific test is suitable for its intended use [31]. ABTS and FRAP post-column assays were evaluated according to the following validation parameters: specificity, precision, limit of detection (LoD), limit of quantitation (LoQ) and linearity.

Reaction kinetics between antioxidant active compound and ABTS or FRAP reagent is distinct, depending on the concentration of reagent, pH in the reactor's medium, reaction time and temperature [12]. FRAP method was modified from Benzie and Strain [32] and was adapted for post-column assay. Optimization of working FRAP solution was performed by adjusting the concentration of TPTZ/$FeCl_3 \cdot 6H_2O$ in the reactor. Fixed concentration of Trolox (400 μM and 20 μM) was used for peak area assessment at different TPTZ/$FeCl_3 \cdot 6H_2O$ concentrations in the reactor (Fig. 1). At the concentration range of 123/246–278/556 μM no significant differences ($p > 0.05$) in Trolox peak area were detected (4,268,514 ± 1017 and 275,210 ± 930 for 400 μM and 20 μM Trolox respectively). When the concentration of TPTZ/$FeCl_3 \cdot 6H_2O$ reached 104/208, peak area of Trolox significantly ($p < 0.05$) decreased. TPTZ/$FeCl_3 \cdot 6H_2O$ concentration of 123/246 μM in the reactor has been selected for the further research. Optimization of ABTS post-column assay (ABTS concentration in the reactor, reaction time, flow rate and reaction coil size) was performed in our previous study [12]. The comparison of ABTS and FRAP post-column assays was performed at optimal concentrations of ABTS (35 μM ABTS radical cation) and FRAP (123 μM of TPTZ and 246 μM $FeCl_3 \cdot 6H_2O$) in the reactor at low pH of 3.6. Reaction time depends on ABTS and FRAP reagents (0.5 mL/min) and HPLC mobile phase (1 mL/min) flow rates and volume of reaction coil. Reaction coil (TFE) of fixed size 15 m × 0.3 mm i.d., 1.58 mm o.d. volume ~1 mL, was used for investigations. Reaction between antioxidant and ABTS or FRAP reagents lasts for ~40 s in reactor at room temperature. Two phenolic acids (chlorogenic acid, caffeic acid), three flavanols ((+)-catechin, (−)-epicatechin, epigallocatechin gallate), five flavonols (quercetin, rutin, isoquercitrin, quercitrin, hyperoside), ellagic acid, and Trolox were chosen as reference compounds for the validation.

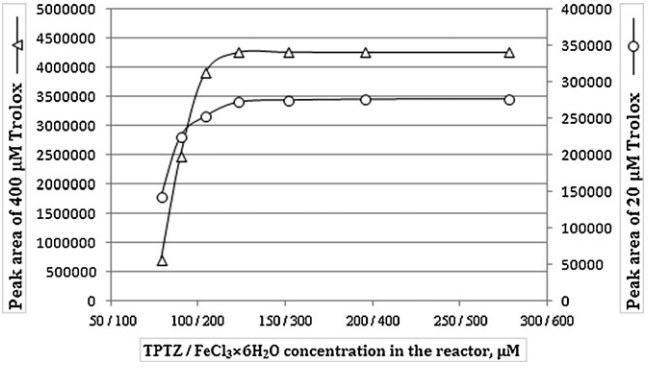

Fig. 1. Dependence of Trolox (400 μM and 20 μM) peak area on TPTZ and $FeCl_3 \cdot 6H_2O$ reagent concentration in the reactor. Reaction coil – TFE (Teflon) 15 m × 0.3 mm i.d., 1.58 mm o.d. (volume ~ 1 mL). HPLC flow rate 1 mL/min. FRAP solution flow rate 0.5 mL/min.

ABTS and FRAP post-column assay specificity was assessed by identification test discriminating of active compounds [12]. The reaction of chromophoric ABTS radical cation and particular active ingredient present in the sample results in the decrease of blue-green solution colour, conforming its radical scavenging identity [33]. The reaction of Fe(III)–TPTZ complex with antioxidant compound results in the formation of a blue-colored ferrous chelate (Fe(II)–TPTZ) [34], confirming its ferric reducing identity. All the tested reference compounds had radical scavenging and ferric reducing abilities.

The precision of the assays was evaluated by repeatability and intermediate precision by the repeated injection (Trolox 100 μM, chlorogenic acid 85 μM, caffeic acid 110 μM, (+)-catechin 100 μM, (−)-epicatechin 140 μM, epigallocatechin gallate 65 μM, quercetin 80 μM, rutin 40 μM, isoquercitrin 50 μM, quercitrin 50 μM, hyperoside 50 μM and ellagic acid 100 μM). The intraday experiment was obtained by six replicates for a day, and the interday was determined by six injections for 3 days for the post-column derivatization peak area. The mean value of relative standard deviations (RSD) of batch of samples within the same day (intraday) and at different days (interday) corresponds to repeatability and intermediate precision, respectively. The experimental values obtained for the determination of ABTS and FRAP post-column assays of 12 reference compounds are presented in Table 1. The ABTS post-column assay repeatability RSD ranged from 1.18 (ellagic acid) to 3.21% (chlorogenic acid), FRAP from 0.33 (quercitrin) to 2.88% (caffeic acid). The ABTS post-column assay repeatability RSD values were greater than of FRAP method, but did not exceed 5%. RSD values of intermediate precision were <10% (maximum RSD was chlorogenic acid equals to 6.84% of ABTS and to 5.78% of FRAP method) suggesting that the post-column methods exhibited satisfactory results. The precision values are influenced by the instability of baseline, which is expressed as S/N (signal-to-noise) ratio. The instability of baseline increases with the decreasing S/N ratio and thus accuracy of compound determination decreases. Baseline stability is important in ABTS post-column assay, as ABTS is a colorful reagent [35]. This problem is absent in FRAP post-column assay, as Fe(II)–TPTZ converts into colorful complex after reaction with active compound and therefore its baseline is stable. The same thing occurs with the limits of detection and quantitation that define the sensitivity of the assays, as the instability of the baseline makes the negative impact.

The limit of detection was calculated as $LoD = 3.3\sigma/a$ and the limit of quantitation as $LoQ = 10\sigma/a$, where σ is the standard deviation of the response, a is the slope of the calibration curve [31]. The lowest LoD and LoQ in ABTS post-column assay was of hyperoside 1.47 μM and 4.46 μM, respectively, while hyperoside in FRAP post-column assay accounted for 1.12 μM and 3.40 μM, respectively. The lowest LoD and LoQ in FRAP post-column assay was of quercetin 0.91 μM and 2.74 μM, respectively, while quercetin in ABTS

Table 1
Validation characteristics of ABTS and FRAP post-column assays.

Reference compound	LoD[a] (μM)	LoQ[b] (μM)	Intraday[c] RSD (%)	Interday[d] RSD (%)	Linear range (μM)	Calibration curve[e]	R^2 (n)[f]
ABTS post-column assay							
Trolox	1.68	5.09	1.53	3.99	5–400	$y = 6793.9x - 7302.9$	0.9998 (7)
Chlorogenic acid	2.29	6.94	3.21	6.84	5–285	$y = 3394.4x - 1735.1$	0.9978 (6)
Caffeic acid	2.22	6.73	1.28	3.16	5–555	$y = 6414.6x - 6228.6$	0.9994 (6)
Ellagic acid	3.28	9.94	1.18	4.56	5–200	$y = 897.7x - 2061.7$	0.9994 (6)
Rutin	2.02	6.11	1.22	3.83	4–80	$y = 3570.4x + 1113.5$	0.9994 (6)
Quercetin	1.55	4.69	2.68	5.30	4–330	$y = 6471.9x + 1917.3$	0.9989 (6)
Quercitrin	1.83	5.56	2.11	4.73	5.5–110	$y = 2106.8x + 4838.5$	0.9987 (6)
Isoquercitrin	2.37	7.18	2.41	4.97	5.5–110	$y = 3625.4x + 1267.1$	0.9982 (6)
Hyperoside	1.47	4.46	1.63	3.07	2.75–110	$y = 4842.7x + 1178.4$	0.9998 (6)
(+)-Catechin	1.71	4.93	1.35	4.11	4–410	$y = 5503.1x + 7823.6$	0.9997 (6)
(−)-Epicatechin	2.28	7.19	1.42	4.39	5–550	$y = 4484.2x + 1193.7$	0.9988 (6)
Epigallocatechin gallate	2.12	5.37	1.89	5.03	5–260	$y = 10{,}190.0x - 6715.2$	0.9991 (6)
FRAP post-column assay							
Trolox	1.88	5.69	1.01	2.17	5–400	$y = 10{,}984.1x + 18{,}323.7$	0.9997 (7)
Chlorogenic acid	2.17	6.58	2.59	5.78	5–340	$y = 9165.2x - 2426.8$	0.9985 (6)
Caffeic acid	1.97	5.98	2.88	5.55	5–667	$y = 13{,}244.8x - 16{,}185.5$	0.9978 (6)
Ellagic acid	1.20	3.63	2.65	4.99	2.5–400	$y = 15{,}987.3x + 96{,}401.2$	0.9982 (6)
Rutin	1.21	3.66	0.47	2.10	2–160	$y = 9894.6x - 2812.1$	0.9999 (6)
Quercetin	0.91	2.74	1.60	3.67	2–330	$y = 14{,}759.7x + 65{,}949.3$	0.9988 (6)
Quercitrin	1.01	3.07	0.33	2.41	2.75–220	$y = 11{,}397.1x + 2638.6$	0.9999 (6)
Isoquercitrin	1.91	5.78	0.62	1.75	5.5–220	$y = 10{,}879.4x + 5093.3$	0.9998 (6)
Hyperoside	1.12	3.40	0.94	3.12	2.75–220	$y = 14{,}625.2x + 16{,}564.5$	0.9995 (6)
(+)-Catechin	1.08	3.26	0.90	2.58	2–410	$y = 14{,}303.2x + 16{,}836.9$	0.9998 (6)
(−)-Epicatechin	1.82	5.51	0.88	1.93	5–550	$y = 11{,}355.7x + 41{,}959.4$	0.9998 (6)
Epigallocatechin gallate	1.24	3.77	1.36	3.65	2.5–260	$y = 16{,}987.1x - 2621.7$	0.9996 (6)

[a] Limit of detection.
[b] Limit of quantitation.
[c] Repeatability.
[d] Intermediate precision.
[e] In the calibration curve, x stands for the concentration of the antioxidant compound and y is the peak area.
[f] Determination coefficient (data points in linear range).

post-column assay accounted for 1.55 μM and 4.69 μM, respectively. Differences in sensitivity occur due to the affect of the baseline as explained hereinbefore. The LoD and LoQ values in Table 1 demonstrate that the ABTS and FRAP post-column assays proposed can be used for the evaluation of antioxidant active compounds as well as for their quantification according to TEAC.

The linearity was tested by measuring the change of absorption in both post-column assays of each antioxidant compound at known concentrations. Each measurement was repeated three times and the mean value was used for calculation of the regression line (Table 1). The ABTS post-column assay lacked linearity for the higher concentrations of certain reference compounds. The linearity ranges can be expanded by increasing the ABTS concentration in the reactor. This ABTS assay uses fixed concentrations of ABTS radical cation (35 μM) and therefore greater concentrations of compounds reach the limits of detection. All 12 antioxidant compounds showed significant ($p < 0.0001$) linear regression with a determination coefficient higher than 0.99 for both post-column assays. The ABTS and FRAP post-column assays were fully linear over the concentration range that were tested. The data obtained are presented in Table 1.

3.2. Analysis of reference compound antioxidant activity

In order to compare the efficacy of antioxidant activity evaluation using ABTS and FRAP post-column assays, 12 samples of antioxidant compound were analyzed. The $TEAC_{rel}$ values of reference compounds from both of the post-column assays are presented in Table 2.

Various studies have determined that antioxidant activity of active compounds is pH dependent [36–39]. Experiments by Lemanska et al. show that the radical scavenging ability increases with the increasing pH values [39]. The ionization potential decreases with the increasing pH values, which reflects the higher electron-donating capacity with deprotonation [8]. FRAP assay is established in pH of 3.6. Therefore in order to compare the antioxidant activity of the tested compounds ABTS assay was performed at same pH. Consequently, all the reference compounds in ABTS post-column assay possess lower radical scavenging activity than Trolox, except epigallocatechin gallate ($TEAC_{rel}$ 1.50 ± 0.07). Scientific studies propose that flavanols and flavonols have greater activity compared to Trolox [16,22,37,39,11]. Inconsistent results may occur due to different experimental pH values.

In order to evaluate the radical scavenging and ferric reducing ability of antioxidant compound ABTS/FRAP $TEAC_{rel}$ ratio (A/F ratio) was estimated. If the ratio of sample antioxidant compound is less than 1, the compound possesses greater ferric reducing abilities; if the ratio is greater than 1, radical scavenging abilities are more expressed (Table 2).

Our results demonstrate that quercetin possesses greater radical scavenging activity ($TEAC_{rel}$ 0.95 in ABTS post-column assay)

Table 2
Comparison of relative Trolox equivalent antioxidant capacities ($TEAC_{rel}$) of reference compounds in ABTS and FRAP post-column assays.

Reference compound	$TEAC_{rel}$ in ABTS	$TEAC_{rel}$ in FRAP	A/F ratio[a]
Trolox	1.00	1.00	1.00
Chlorogenic acid	0.42 ± 0.03	0.83 ± 0.05	0.50
Caffeic acid	0.83 ± 0.06	1.21 ± 0.06	0.69
Ellagic acid	0.13 ± 0.01	1.46 ± 0.08	0.09
Rutin	0.53 ± 0.04	0.90 ± 0.05	0.58
Quercetin	0.95 ± 0.07	1.34 ± 0.07	0.71
Quercitrin	0.31 ± 0.01	1.04 ± 0.06	0.30
Isoquercitrin	0.53 ± 0.02	0.99 ± 0.04	0.54
Hyperoside	0.71 ± 0.04	1.33 ± 0.06	0.54
(+)-Catechin	0.81 ± 0.05	1.30 ± 0.05	0.62
(−)-Epicatechin	0.66 ± 0.03	1.03 ± 0.05	0.64
Epigallocatechin gallate	1.50 ± 0.07	1.55 ± 0.08	0.97

[a] Ratio of $TEAC_{rel}$ value in ABTS post-column assay and $TEAC_{rel}$ value in FRAP post-column assay.

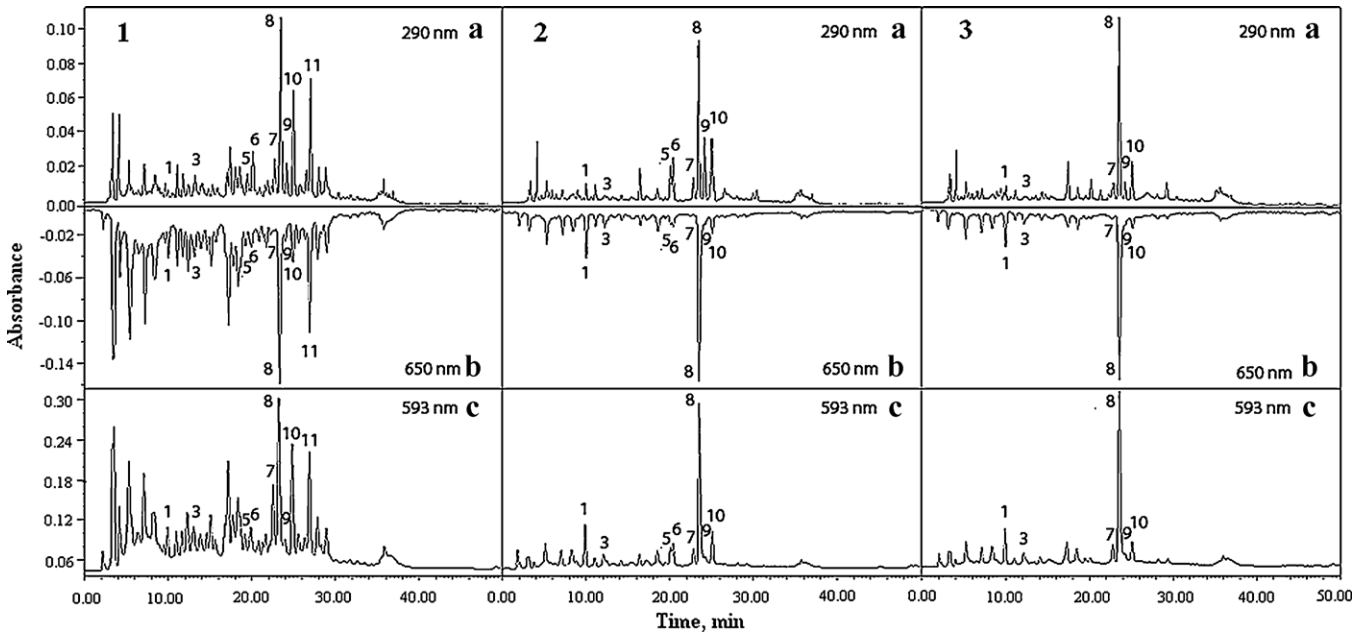

Fig. 2. Combined chromatograms of *F. viridis* (1), *F. vesca* (2) and *F. moschata* (3) leaf extracts: chromatographic elution (a) and post-column reaction with ABTS (b) or FRAP (c) reagents. Numbers refer to identified antioxidant compounds: 1 – (+)-catechin, 3 – (−)-epicatechin, 5, 6, 11 – quercetin derivatives, 7 – ellagic acid, 8 – epigallocatechin gallate, 9 – hyperoside, 10 – isoquercitrin.

and reduction power ($TEAC_{rel}$ 1.34 in FRAP post-column assay) than its derivatives (rutin, quercitrin, isoquercitrin, hyperoside (Table 2)) in both ABTS and FRAP post-column assays. This is in agreement with other scientific studies, showing that sugar moiety reduces the activity [22,40]. Activity of quercetin derivatives analyzed with ABTS post-column assay had the following rank order: quercetin > hyperoside > isoquercitrin ≈ rutin > quercitrin. In FRAP post-column the rank order is slightly different: quercetin > hyperoside > quercitrin > isoquercitrin > rutin. Isoquercitrin and hyperoside have the same ABTS/FRAP $TEAC_{rel}$ ratio (0.54), but different $TEAC_{rel}$ values ($p < 0.05$) in ABTS and FRAP post-column assays (0.53 ± 0.02, 0.71 ± 0.04 in ABTS and 0.99 ± 0.04, 1.33 ± 0.06 in FRAP, respectively). The ratio demonstrates that radical scavenging properties and ferric reducing abilities of both glycosides are proportional, while quercetin with galactosyde moiety (hyperoside) is more active in both assays. Antioxidant activity of quercetin (flavonol) has been confirmed in many studies [15,34,41,42] and its structure–activity relationship has been determined [34]. The high activity of flavonols with catethol group can be explained by the bielectronic oxidation and formation of two highly stable quinonic structures [11].

Flavanol compound, epigallocatechin gallate, has strong ferric reducing power ($TEAC_{rel}$ 1.55 ± 0.08). This compound was determined as the most active in both assays. Other flavanols – (+)-catechin and (−)-epicatechin have lower $TEAC_{rel}$ (in both assays) than flavonol quercetin due to the lack of 2,3-double bond present in quercetin. Hydroxyl groups at C3, 2,3-double bond and 4-oxo group are necessary for the antioxidant activity [14,15]. These structural peculiarities are essential for both radical scavenging activity [14] and reducing activity [11].

Ellagic acid was determined as a weak radical scavenger in our study ($TEAC_{rel}$ 0.13 ± 0.01), but it possesses a strong ferric reducing ability – $TEAC_{rel}$ 1.46 ± 0.08. Literature data on ellagic acid $TEAC_{rel}$ values in ABTS and FRAP systems are scarce. Most commonly the content of ellagic acid is being correlated with total antioxidant activity of complex extract test in ABTS, DPPH or FRAP systems [25,43–45].

3.3. Analysis of antioxidant activity of Fragaria species

Ethanolic extracts of *F. viridis*, *F. vesca*, *F. moschata* leaves and fruits were analyzed in order to evaluate the efficacy of antioxidant determination using both ABTS and FRAP post-column assays. To the best of our knowledge the antioxidative activity of raw materials of *F. viridis* and *F. moschata* was analyzed for the first time. In *Fragaria* leaf extracts six phenolic compounds – (+)-catechin, (−)-epicatechin, ellagic acid, epigallocatechin gallate, hyperoside, isoquercitrin and three quercetin derivatives were identified (Fig. 2). In *Fragaria* fruit extracts, besides previously mentioned compounds, two anthocyanins – cyanidin-3-O-glucoside and pelargonidin-3-O-glucoside were determined (Fig. 3). TEAC (μmol/g) values of principal compounds and total of all quantitated compounds of *F. viridis*, *F. vesca* and *F. moschata* leaf and fruit extracts were assessed and presented in Table 3. The results demonstrate that *Fragaria* leaf extracts possesses stronger antioxidant properties (range of total TEAC values 191.23–609.36 μmol/g and 178.63–642.20 μmol/g of ABTS and FRAP respectively) than *Fragaria* fruit extracts (8.24–25.11 μmol/g and 10.82–24.82 μmol/g of ABTS and FRAP respectively). Greater TEAC values represent the greater amounts of bioactive compounds. The main qualitative differences between leaves and fruits of *Fragaria* species are the anthocyanins. Pelargonidin and cyanidin glycosides are regarded as antioxidant active compounds [46]. In our study these compounds possessed lower activities due to low pH of the experimental medium.

F. viridis (609.36 μmol/g and 642.20 μmol/g of ABTS and FRAP respectively) leaf extracts were the most active ($p < 0.05$) among all the investigated species. *F. vesca* fruit extracts (18.05 μmol/g and 18.36 μmol/g of ABTS and FRAP respectively) showed significantly ($p < 0.05$) higher activity when compared to *F. viridis* and *F. moschata*. The calculated TEAC values of bioactive compounds in *Fragaria* leaf and fruit extracts confirmed that epigallocatechin gallate was the predominant radical scavenger and ferric reducer (Table 3).

Table 3
Comparison of radical scavenging activity and ferric reducing power of individual compounds in *Fragaria*[a] leaf and fruit extracts.

No.	Antioxidant compound	RT (min)	*Fragaria* leaves extracts									*Fragaria* fruits extracts								
			$TEAC_{ABTS}$[b]			$TEAC_{FRAP}$[c]			A/F ratio[d]			$TEAC_{ABTS}$			$TEAC_{FRAP}$			A/F ratio		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
–	Unknown	4.04	44.89	5.71	7.10	44.60	5.23	6.65	1.01	1.09	1.07	0.97	1.25	0.85	0.95	1.26	0.80	1.02	0.99	1.06
–	Unknown	5.97	52.83	12.36	10.22	41.25	9.81	7.40	1.28	1.26	1.38	1.28	4.79	0.40	0.99	4.06	0.30	1.29	1.18	1.35
–	Unknown	7.81	40.97	7.75	7.31	26.65	4.80	5.15	1.54	1.62	1.42	2.26	6.11	0.29	1.38	3.67	0.17	1.64	1.67	1.73
–	Unknown	8.92	22.95	8.75	10.30	17.55	6.62	7.74	1.31	1.32	1.33	0.27	0.55	0.13	0.22	0.46	0.09	1.23	1.20	1.44
1	(+)-Catechin	10.64	13.97	11.02	10.63	22.97	19.62	19.34	0.61	0.56	0.55	0.21	0.77	0.26	0.38	1.26	0.52	0.56	0.61	0.50
2	Cyanidin-3-O-glucoside	10.89	–	–	–	–	–	–	–	–	–	2.97	1.12	1.09	3.68	1.42	1.34	0.81	0.79	0.82
–	Unknown	11.73	14.28	3.84	2.69	10.04	2.53	1.84	1.42	1.52	1.46	–	–	–	–	–	–	–	–	–
–	Unknown	12.46	10.79	4.50	4.33	9.50	3.76	3.53	1.14	1.20	1.23	0.34	0.12	–	0.27	0.09	–	1.26	1.27	–
3	(−)-Epicatechin	13.17	17.46	1.49	1.50	26.61	2.61	2.62	0.66	0.57	0.57	0.29	0.10	–	0.42	0.15	–	0.69	0.66	–
4	Pelargonidin-3-O-glucoside	13.41	–	–	–	–	–	–	–	–	–	0.16	0.29	0.14	0.18	0.33	0.15	0.89	0.88	0.93
–	Unknown	16.01	14.13	2.49	2.01	14.14	2.55	1.91	1.00	0.97	1.05	0.19	0.30	0.13	0.18	0.28	0.12	1.03	1.09	1.08
–	Unknown	18.11	39.91	2.94	8.75	18.18	1.32	3.83	2.20	2.22	2.28	0.93	1.56	0.50	0.47	0.94	0.24	1.97	1.66	2.08
–	Unknown	19.26	22.24	5.91	5.02	21.55	5.46	5.09	1.03	1.08	0.99	0.10	0.11	–	0.09	0.12	–	1.11	0.92	–
5	Quercetin derivative	20.79	8.61	2.17	–	18.48	3.99	–	0.47	0.54	–	–	–	–	–	–	–	–	–	–
6	Quercetin derivative	21.04	5.58	3.04	–	10.76	5.08	–	0.52	0.60	–	–	–	–	–	–	–	–	–	–
7	Ellagic acid	23.43	2.50	0.52	0.87	24.48	4.02	6.00	0.10	0.13	0.15	0.08	0.18	0.15	0.70	1.72	1.14	0.11	0.10	0.13
8	Epigallocatechin gallate	24.15	49.62	51.45	58.50	50.37	52.11	55.55	0.99	0.99	1.05	2.15	2.89	2.53	2.19	2.88	2.61	0.98	1.00	0.97
9	Hyperoside	24.86	8.25	2.29	2.69	13.38	4.52	4.01	0.62	0.51	0.67	–	–	–	–	–	–	–	–	–
10	Isoquercitrin	25.68	16.38	4.59	3.84	32.84	8.63	6.79	0.50	0.53	0.57	0.20	0.15	0.16	0.28	0.27	0.22	0.71	0.55	0.73
11	Quercetin derivative	27.69	31.58	–	–	32.76	–	0.09	0.96	–	–	0.87	0.21	–	0.75	0.20	–	1.16	1.03	–
Total of all quantitated compounds			609.36	191.23	221.24	642.20	178.63	206.15	0.95	1.07	1.07	18.05	25.11	8.24	18.36	24.82	10.82	0.98	1.01	0.76

[a] 1 – *Fragaria viridis*; 2 – *Fragaria vesca*; 3 – *Fragaria moschata*.
[b] Trolox equivalent antioxidant capacity values (μmol/g) in ABTS post-column assay.
[c] Trolox equivalent antioxidant capacity values (μmol/g) in FRAP post-column assay.
[d] Ratio of $TEAC_{ABTS}$ and $TEAC_{FRAP}$ values.

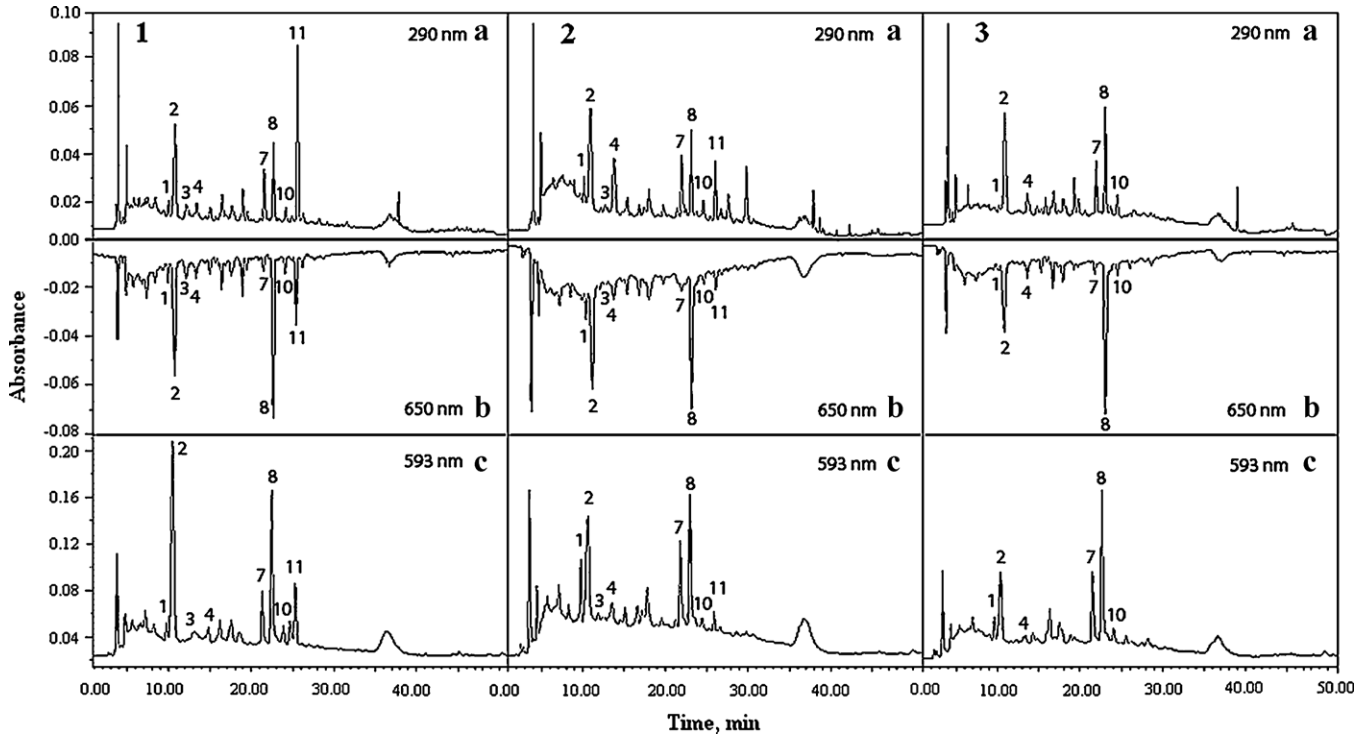

Fig. 3. Combined chromatograms of *F. viridis* (1), *F. vesca* (2) and *F. moschata* (3) fruit extracts: chromatographic elution (a) and post-column reaction with ABTS (b) or FRAP (c) reagents. Numbers refer to identified antioxidant compounds: 1 – (+)-catechin, 2 – cyanidin-3-O-glucoside, 3 – (−)-epicatechin, 4 – pelargonidin-3-O-glucoside, 7 – ellagic acid, 8 – epigallocatechin gallate, 10 – isoquercitrin, 11 – quercetin derivative.

Epigallocatechin gallate antioxidant activity comprises about 26% of total activity in leaf extracts of *F. vesca* and *F. moschata* and only about 8% of total in leaf extract of *F. viridis* (in both assays) due to higher amounts of other bioactive compounds present in the raw material (Fig. 2). In fruit extracts of *F. viridis* and *F. vesca*, the epigallocatechin gallate comprised only about 11% of total antioxidant activity, whereas in fruits of *F. moschata* this bioactive compound determined about 30% of the radical scavenging activity and 24% of ferric reducing ability. Extracts of *F. moschata* fruits contain lower amounts of bioactive compounds (Fig. 3).

The assessed ABTS/FRAP TEAC ratio (A/F ratio) of the identified compounds in the extracts of leaves and fruits of *Fragaria* species (Table 3) was similar to ABTS/FRAP TEAC ratio of the reference compounds (Table 2). This confirms the identity of the determined compounds. However, structure elucidation of the active unidentified compounds still needs to be performed in order to evaluate the full complex of radical scavengers or ferric reducers. The obtained results confirm the reliability of ABTS and FRAP post-column assays for screening of antioxidants in complex mixtures and the determination of radical scavenging and ferric reducing ability by their TEAC values.

4. Conclusions

ABTS and FRAP post-column assays meet all validation criteria, are specific, repeatable and sensitive, and thus can be used for the evaluation of antioxidant active compounds as well as for their assessment according to TEAC. Both assays were performed at the same experimental conditions, therefore the obtained results are comparable. The assays revealed that the radical scavengers and compounds with expressed ferric reducing abilities can be discriminated and their antioxidant potential can be evaluated. Optimized and validated ABTS and FRAP post-column assays can be applied for the scientific research in food chemistry, agricultural and pharmaceutical industries. Assays enable monitoring of food supplements quality and the stability of their active compounds during the manufacturing and storage processes. Fingerprinting of herbal raw materials and phytopreparations are also the area of application of the post-column assays. Research of natural antioxidants is comprised of screening, identification and evaluation processes that can be performed using the post-column assays.

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