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A New Test Method for the Evaluation of Total Antioxidant Activity of Herbal Products

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A new test method for measuring the antioxidant power of herbal products, based on solid-phase spectrophotometry using tetrabenzo-[b,f,j,n][1,5,9,13]-tetraazacyclohexadecine−Cu(II) complex immobilized on silica gel, is proposed. The absorbance of the modified sorbent (λ_{max} = 712 nm) increases proportionally to the total antioxidant activity of the sample solution. The method represents an attractive alternative to the mostly used radical scavenging capacity assays, because they generally require complex long-lasting stages to be carried out. The proposed test method is simple ("drop and measure" procedure is applied), rapid (10 min/sample), requires only the monitoring of time and absorbance, and provides good statistical parameters ($s_r \leq 0.2$). The method was approved in the analysis of the most popular herbal beverages (black and green teas) and drugs (*Echinacea* products). The results obtained were compared with the content of total flavonoids and tannins of teas and total caffeic acid derivatives of *Echinacea*, determined spectrophotometrically.

INTRODUCTION

Nowadays, the fact of harmful effect of reactive oxygen species on human health is well-known. The capability of natural defense systems of living organisms against excess production of these species decreases when influenced with negative environmental factors or aging. As a result, different cellular and extracellular components, and especially nucleic acids, are damaged, causing or enhancing a number of degenerative diseases. Therefore, antioxidants that scavenge free radicals are of great value in preventing such "oxidative" pathologies. That is why natural products with antioxidant properties become more and more popular all over the world.

In recent years, many different methods have been proposed for the evaluation of antioxidant power. Most of them are based on the measurement of the relative abilities of antioxidants to scavenge radicals in comparison with the antioxidant potency of a standard antioxidant compound. Generally, synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid − the water soluble vitamin E analogue) is used as a standard. Instead, common bioantioxidants such as ascorbic acid (*1*) or quercetin (*2*) may be used.

One of the most frequently used radical scavenging methods is that of Miller (*1, 3−10*). The highly stable 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical (ABTS$^{+•}$), produced by the ferrylmyoglobin radical generated from metmyoglobin and H_2O_2 in the presence of the peroxidase, is a blue/green chromogen with characteristic absorption at 734 nm. The ability of the analyzed antioxidant sample either to delay its appearance or to capture it and diminish its absorption, is measured spectrophotometrically (ABTS assay). The evaluation of the total antioxidant activity according to this method may be carried out using the RANDOX kit (Randox Laboratories Ltd., Ardmore, U.K.). Flow-injection electrochemical method based on the measurement of the amount of current necessary to generate ABTS$^{+•}$ in the presence or absence of the sample has also been developed (*11*).

The DPPH test is based on the bleaching of the stable radical of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (*12*). This method was applied to determine the antioxidant power of wines (*13*), orange juices (*5*), fruits (*1*), and apple extracts (*14*).

The scavenging abilities of oxygen radical (ORAC test) (*15−17*), peroxy-radical (*18*), hydroxy-radical (*19*), Fremy's and galvinoxyl radical (*2*) or total radical-trapping antioxidant parameters (TRAP assay) (*20*) were measured by fluorimetry (*18, 20*), chemiluminescence (*19*), electron spin resonance spectroscopy (*2*), and other techniques.

The ABTS assay and similar methods require complex stages over different prolonged periods of time. For this reason, these methods are not suitable for application when a large number of samples need to be tested in a short time, especially when

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Scheme 1
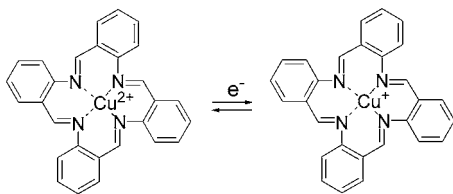
the samples are of different types. The semi-automated methods enable the simultaneous analysis of a series of samples, but in this case, special equipment and highly skilled personnel are required.

Another group of methods of antioxidant activity evaluation is based on redox-reactions. In the ferric reducing/antioxidant assay (FRAP assay), antioxidants of the sample reduce Fe(III)/tripyridyltriazine complex to the blue colored ferrous form, with an increase in absorbance at 593 nm. The ΔA is proportional to the antioxidant power of the sample. The FRAP assay was applied to the analysis of teas (*21*) and wines (*22*). In the electrochemical redox method, electrogenerated bromine is used as an oxidant (*23*). The main disadvantage of the method is caused by the bromine ability to take part not only in redox or radical reactions but also in the addition and substitution reactions.

For all these reasons, a direct, rapid, simple and reliable method is of great need for the determination of antioxidant power. Test methods using analytical reagents, immobilized on matrixes are known to be easy-to-handle and sufficiently sensitive; they do not require additional reagent solutions or toxic organic solvents, and therefore, may be performed not only under laboratory conditions but also at the site of sampling (*24*). For antioxidant power evaluation, no test method reported was found.

The redox reaction of tetrabenzo-[b,f,j,n][1,5,9,13]-tetraaza-cyclohexadecine copper(II) complex immobilized on silica gel (CuTAAB−SG) (**Scheme 1**) was earlier proposed by the authors for sorption-spectrophotometric determination of individual organic reductants (ascorbic acid and analgin) in synthetic drugs (*25*).

This modified sorbent is stable during storage, easily obtained by adsorption from aqueous solutions (*26*), and has a contrasting color change; the modifier synthesis is performed in one stage (*27*). CuTAAB−SG has not been applied to the analysis of herbal or other natural materials. Our preliminary studies have shown that this immobilized reagent does not react with inorganic reductants, and thus, may be a specific reagent for organic antioxidants. The present study was aimed to develop a simple and reliable test method for antioxidant power evaluation using CuTAAB−SG.

For the approvement of the proposed method the most popular herbal beverages (black and green teas) and drugs (*Echinacea* extracts) have been chosen. Tea (*Camellia sinensis*) is the most widely consumed beverage worldwide, and so is an important agriculturul product. Tea containes different components possessing P-vitamin and antioxidant activity, among which flavonoids, tannins, and catechins are the most considerable. The beneficient action of these phenolic compounds is due to the inhibition of low-density lipoprotein peroxidation and the protection of blood-vessels. Regular intake of tea is known to improve antioxidant status in vivo, and thereby help lower risk of certain types of cancer and coronary disease (*28*). *Echinacea* is widely accepted for its immunostimulant medicinal usage. Extracts of the dried roots and the upper plants are known to show bacteriostatic, virusstatic, and immunostimulating activity

and to cause general beneficial therapeutical effect at infections and chronic inflammations (*29*). Despite the numerous chemical and pharmacological studies devoted to medicinal action of *Echinacea* species, there is little research on the antioxidant activity of these phytoceuticals.

The results of the antioxidant power evaluation using CuTAAB−SG were compared with the content of total flavonoids and tannins of teas and total caffeic acid derivatives of *Echinacea*, determined by spectrophotometry.

MATERIALS AND METHODS

Chemicals. CuTAAB−SG ($a = 5 \cdot 10^{-6}$ mol/g) was obtained by the reagent adsorption from its aqueous solution on silica gel according to (*26*) as follows: 100 mL of 0.75 mmol/L CuTAAB(NO$_3$)$_2$ solution was stirred with 15 g of silica gel L100/250 (Chemapol, Czech Republic) during 1 h. Then the sorbent was filtered and air-dried. CuTAAB(NO$_3$)$_2$ was synthesized by tetramerization of *o*-aminobenzaldehyde in the presence of Cu(II) nitrate, according to (*27*). Caffeic acid, quercetin, tannin (molecular weight 1700, extracted from *Cotinus coggygria Scop.*) and rutin were obtained from Sichuan Xieli Pharmaceutical. Co. Ltd. (Korea).

Eight *Echinacea purpurea* medicines and eleven teas were all purchased locally. All solutions were prepared using fresh-boiled and cooled distilled water to decrease the content of dissolved oxygen.

Apparatus. Absorbance spectra were registered using UV−vis spectrophotometer Specord M-40 (Carl Zeiss Jena, Germany). A potentiometer model EV-74 with glass electrode (Gomel, Belarus) was used for pH measurements.

Sample Preparation. Water-ethanol *Echinacea purpurea* medicines (storaged at 4 °C) and freshly prepared, filtered, and cooled tea infusions (1.00−2.00 g of dry tea leaves/100 mL of boiling distilled water, $t = $ 5 min) were diluted as appropriate with distilled water.

Antioxidant Power Test Procedure. A portion of CuTAAB−SG (0.070 ± 0.001 g) was put into the cell and treated with 1 drop (40 μL) of carbonate buffer solution (pH 10.5 ± 0.1) and 1 drop (40 μL) of antioxidant sample solution or standard solution and kept covered for 10 min at room temperature. Then, absorbance at 712 and 870 nm was measured. The antioxidant activity is proportional to the value of $\Delta A = (A'_{712} - A'_{870}) - (A''_{712} - A''_{870})$ where A'_{712} and A''_{712} is absorbance at λ_{max}, respectively, with and without sample addition, A'_{870} and A''_{870} is absorbance at λ_{min}, respectively, with and without sample addition.

Due to its reducing properties and availability, quercetin was chosen as a standard for the antioxidant activity of teas and *Echinacea* medicines. The use of a common standard makes it possible to compare the antioxidant properties of various herbal products with different bioactive compounds. The antioxidant power of *Echinacea* and tea samples was expressed as mg/mL of quercetin and g of (quercetin)/100 g of tea leaves, respectively.

Determination of Total Content of Caffeic Acid Derivatives in *Echinacea* and of Flavonoids and Tannins in Tea Samples. Total caffeic acid derivatives of *Echinacea* medicines were determined by the spectrophotometric method based on the complexation with Al-(III) (*30*) as follows: Into the 25-mL flask, an aliquot of *Echinacea* water−alcohol medicine and 2.5 mL of 0.5 mol/L AlCl$_3$ solution (pH 2.0) were placed; pH was adjusted to pH 4.8 with 10% NH$_4$Cl solution, and distilled water was added up to the mark. Total caffeic acid derivative content (expressed as mg/mL of caffeic acid) was calculated using the calibration graph A_{355} − caffeic acid concentration.

In line with Abdullin et al. (*23*), we have chosen AlCl$_3$ as a complexing agent for the spectrophotometric determination of total flavonoids of teas. The determination was performed under optimum conditions (pH 3.1 ± 0.1, 0.045 M AlCl$_3$). To avoid the interference of tea infusion self absorbance, all the spectra were measured against blank solutions, containing all the same components as analyzed solution except AlCl$_3$. It was found that the spectra of AlCl$_3$ solutions in the presence of tea samples have two peaks (**Figure 1**, curve 3−5): the first (323 nm) corresponding to the maximum absorbance of Al(III) complexes with tannins (**Figure 1**, curve 2), the second (407
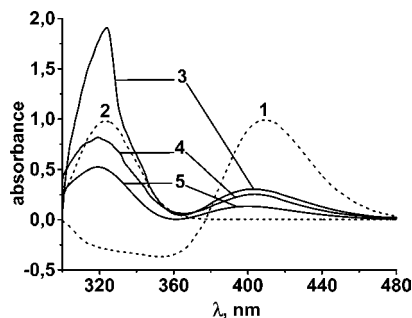
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Figure 1. Absorption of Al(III) solutions in the presence of rutin (1), tannin (2), and tea samples (3–5). C(rutin) = C(tannin) = 40 μg/mL, C(tea infusion) = 10% (v/v), $C_{Al(III)}$ = 0.045 M; pH 3.1 ± 0.1; l = 1 cm. Teas: Ahmad Green Tea (3), Princess Candy (4), Maisky Chai Chinese Dragon (5).

Table 1. The Equations of the Calibration Graphs (Absorbance – Concentration of a Standard Phenolic Compound) for the Spectrophotometric Determination of Total Flavonoids and Tannins in Tea Infusions[a]

standard cpd	λ, nm	$A = (a \pm \Delta a) + (b \pm \Delta b) C(\mu g/mL)$	r
rutin	407	$(0.014 \pm 0.007) + (0.0207 \pm 0.0003)C$	0.999
rutin	323	$(-0.010 \pm 0.006) + (-0.0062 \pm 0.0003)C$	0.995
tannin	323	$(0.03 \pm 0.02) + (0.0240 \pm 0.0006)C$	0.999

[a] $C_{Al(III)}$ = 0.045 M, pH 3.1±0.1, l=1 cm. Calibration curves are linear up to 50 μg/mL rutin and tannin.

nm) to the maximum absorbance of Al(III)-flavonoid complexes (**Figure 1**, curve 1). Tannin complexes with Al(III) do not absorb at 407 nm, enabling the simultaneous determination of tea flavonoids and tannins as follows: The concentration of total flavonoids (μg/mL rutin) was determined by measuring the absorbance at 407 nm and using the corresponding equation of the calibration graph (**Table 1**). Total flavonoid content was calculated according to the following:

$$\text{total flavonoids (g of rutin/100 g of tea leaves)} = 0.0001 \cdot C \cdot D \cdot V/m$$

where C is the concentration of total flavonoids in the analyzed solution (μg/mL rutin), D is the dilution of a tea infusion, and V and m are the volume of a tea infusion and the weight of tea leaves, respectively.

Total tannins were determined by measuring the absorbance at 323 nm; their content was calculated as described above, taking into account the absorption of flavonoid−Al(III) complexes at this wavelength (**Table 1**).

RESULTS AND DISCUSSION

Radical scavenging capacity and antioxidant activity of *Echinacea* root extracts were investigated recently (*4*, *31*). Caffeic acid and its derivatives make considerable contributions to the immunostimulatory action of alcohol extracts of *Echinacea* (*31*). We have chosen this group of bioactive compounds to study the correlation between their content and the antioxidant power of *Echinacea* products.

Total caffeic acid derivative concentration and total antioxidant activity of eight *Echinacea purpurea* water−alcohol medicines are presented in **Table 2**.

The present results indicate that *Echinacea* medicines have widely different both antioxidant power and caffeic acid derivative content, even when produced by the same manufacturer. Thus, the problem of their quality control during manufacturing and storage is of great importance. Strong correlation

Table 2. Total Caffeic Acid Derivatives and in Vitro Antioxidant Power of Water−Alcohol Root Tinctures (1–5) and Extract (6) of *Echinacea purpurea*, and Immunal (Contains *Echinacea* Juice) (7–8) (n = 3, P = 0.95)

Echinacea medicine	manufacturer[a] (batch no.; expiry date)	total antioxant activity (quercetin, mg/mL)	total caffeic acid derivatives (caffeic acid, mg/mL)
1	I (05.03.96; III 1997)	0.16 ± 0.02	0.095 ± 0.006
2	I (21.10.00; X 2002)	0.09 ± 0.01	0.065 ± 0.005
3	I (03.02.01; II 2003)	0.43 ± 0.05	0.407 ± 0.009
4	II (25.10.01; XI 2003)	0.24 ± 0.03	0.229 ± 0.007
5	II (18.08.01; IX 2003)	0.30 ± 0.05	0.361 ± 0.004
6	III (21100; XII 2002)	1.7 ± 0.2	1.80 ± 0.02
7	IV (4304509A; IX 2001)	0.24 ± 0.01	0.170 ± 0.009
8	IV (1102810B; X 2002)	0.36 ± 0.03	0.369 ± 0.008

[a] Kyiv (I) and Ternopil (II) pharmaceutical plants, Lubnypharm (III), Ukraine; Lek Pharmaceutical and Chemical Company, Ljubljana, Slovenia (IV).
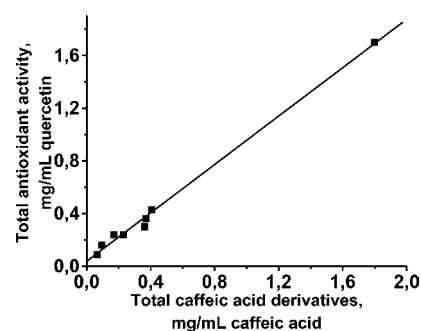

Figure 2. Total antioxidant activity and total content of caffeic acid derivatives of eight water-alcohol *Echinacea* medicines. Results show strong correlation (r = 0.998) between the two parameters. Each point represents the mean of triplicate measurement.
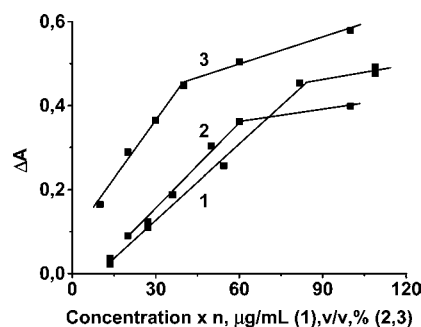

Figure 3. CuTAAB−SG absorption as a function of the concentration of quercetin (1), black (2), and green (3) tea infusions. (100% corresponds to 2.00 (2) and 1.00 (3) g of tea leaves/100 mL of distilled boiling water). n = 0.3 (1); 1(2,3).

(r = 0.998) is observed between the antioxidant activity and total caffeic acid derivatives of *Echinacea* (**Figure 2**), in good agreement with reported studies (*31*). For all these reasons, the developed test method for the evaluation of the antioxidant power may be proposed for the quality screening of *Echinacea* products.

In vitro antioxidant activity of different types of tea has been studied by the FRAP assay (*21*), peroxy and hydroxy-radical scavenging capacity (*32*), electrochemically by an oxygen electrode method (*33*) and by the titration with electrogenerated bromine (*23*).

The dependence of CuTAAB−SG absorption on quercetin concentration and the strength of green and black tea infusions were studied. These curves may be approximated by two linear

Table 3. The Equations of the Calibration Graphs ($\Delta A_{\text{CuTAAB-SG}}$ − Concentration of the Antioxidant)

antioxidant	$\Delta A = (a \pm \Delta a) + (b \pm \Delta b)C$ (μg/mL)	range of linear response μg/mL	r
quercetin	$(-0.05 \pm 0.10)+(0.00200 \pm 0.00009)C$	40–240	0.996
green tea infusion	$(0.09 \pm 0.02)+(0.0092 \pm 0.0008)C^a$	10–40[a]	0.993
black tea infusion	$(-0.05 \pm 0.01)+(0.0035 \pm 0.0002)C^a$	10–100[a]	0.998

[a] v/v, % (100% correspond to 1.00 g of tea leaves/100 mL of boiling distilled water).

Table 4. Total Flavonoids, Total Tannins, and in Vitro Antioxidant Power of Green and Black Teas ($n = 3$, $P = 0.95$)

tea trade mark and name	total antioxant activity (g of quercetin/ 100 g of tea leaves)	total flavonoids (g of rutin/ 100 g of tea leaves)	total tannins (g of tannin/ 100 g of tea leaves)
Nonfermented green teas			
Ahmad Green Tea	7.1 ± 0.6	1.40 ± 0.02	8.22 ± 0.02
Hyleys English Green Tea	5.3 ± 0.5	1.12 ± 0.02	6.83 ± 0.03
Monomah No.29	3.9 ± 0.3	0.51 ± 0.03	1.6 ± 0.1
Maisky Chai Chinese Dragon	3.6 ± 0.3	0.56 ± 0.01	2.18 ± 0.03
Jingxuantieguanyin	3.1 ± 0.5	0.48 ± 0.02	1.33 ± 0.01
Japanese Tea	1.7 ± 0.2	0.24 ± 0.01	0.58 ± 0.02
Fermented black teas			
Hyleys English Aristocratic Tea	2.2 ± 0.2	1.46 ± 0.01	5.96 ± 0.06
Lipton	1.7 ± 0.1	1.75 ± 0.02	5.57 ± 0.04
Maisky Chai The Tzar Crown	1.6 ± 0.2	1.64 ± 0.01	4.45 ± 0.04
Nestea	1.6 ± 0.1	1.21 ± 0.02	3.72 ± 0.06
Princess Candy	1.5 ± 0.3	1.19 ± 0.02	3.67 ± 0.03

Figure 4. The relationship between the total antioxidant activity and total flavonoid (A) and tannin (B) content of green (1) and black (2) teas. Results show strong correlation between the antioxidant activity and total flavonoids ($r = 0.974$) and tannins ($r = 0.942$) of nonfermented green teas. Green teas: Japanese Tea (*a*), Jingxuantieguanyin (*b*), Monomah No.35 (*c*), Maisky Chai Chinese Dragon (*d*), Hyleys English Green Tea (*e*), Ahmad Green Tea (*f*). Black teas: Princess Candy (*g*), Nestea (*h*), Hyleys English Aristocratic Tea (*i*), Maisky Chai The Tzar Crown (*j*), Lipton (*k*). Each point represents the mean of triplicate measurement.

parts (**Figure 3**, curves 1−3). The relationship between the strength of green and black tea infusion and the ΔA value is linear (**Table 3**) up to 40 and 100%(v/v), respectively (100% correspond to the strength of 1.00 g of tea leaves/100 mL of water). For quercetin, used as a standard antioxidant, the relationship is linear at the wide range of concentrations (up to 240 μg/mL) (**Table 3**).

Total flavonoid and tannin content, measured spectrophotometrically, and total antioxidant activity values of eleven teas are presented in **Table 4**.

The antioxidant power of green teas was found to be considerably higher than that of black teas (in good agreement with the results obtained by the FRAP assay (*21*)). The comparison of different types of tea, produced by the same manufacturer, has revealed that the ratio between green and black tea antioxidant activity makes about 2.2 (Maisky chai)−2.4 (Hyleys). This fact is probably related to the processes of tea manufacturing. During green tea producing, tea leaves do not undergo any oxidation, and therefore, they contain large amounts of non-oxidized phenolic compounds with antioxidant properties. On the contrary, black tea manufacturing include a range of biochemical oxidative processes referred to as fermentation. During the fermentation, flavanols in green tea leaves, mainly catechins and their gallic esters, undergo a polyphenol oxidase catalyzed oxidative polymerization, which turns the leaves black. As the content of catechins, which make considerable contribution to the antioxidant capacity (*34*), decreases 7−10 times, lower antioxidant power of fermented teas can be easily explained. For the same reason, the antioxidant activity of black teas is not closely related to the initial content of polyphenols in tea leaves, and so, it has little difference, ranging mostly from 1.5 to 1.7 g of quercetin/100 g of tea leaves for
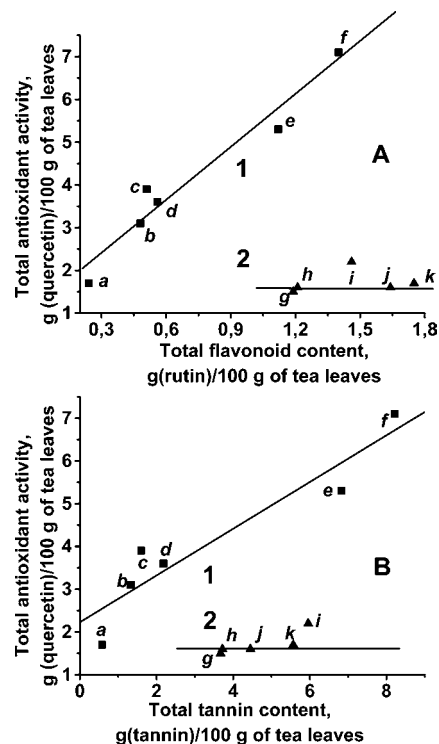
analyzed samples (**Table 4**). Flavonoids and tannins are also partly oxidized during fermentation, but the rest of their phenolic hydroxyl groups enables the complexation with Al(III). That is why total flavonoid and tannin contents, measured spectrophotometrically using Al(III), have no correlation with the antioxidant activity of black teas (**Figure 4A**, curve 2 and **Figure 4B**, curve 2, respectively). For green, nonfermented teas, the antioxidant power is correlated with total flavonoids ($r = 0.974$) and tannins ($r = 0.942$) (**Figure 4A**, curve 1 and **Figure 4B**, curve 1, respectively).

It can be concluded that the proposed test method has been successfully employed for the direct monitoring of the antioxidant power of herbal products. Strong correlation was found between the antioxidant activity and total caffeic acid derivative content of *Echinacea* medicines, in agreement with previous studies on the role of these bioactive compounds in *Echinacea* antioxidant status. The antioxidant power of green teas is significantly higher than that of black teas. It was found that strong correlation exists between the antioxidant activity and total content of flavonoids and tannins of nonfermented green teas.

The developed test method provides reliable results with the measuring of only the time and absorbance, without the need for complicated instrumentation. It is time-saving (when a series of 10 samples is analyzed, the time of one determination is 4−5 min); the use of organic solvents and additional reagent solutions is eliminated. CuTAAB−SG used as a solid-phase reagent is easily obtained by adsorption from aqueous solutions and stable during storage. These advantages make the new method an

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attractive alternative to the normally used methods of antioxidant power evaluation.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); ABTS$^{+•}$, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) cation radical; CuTAAB−SG, silica gel modified with tetra-benzo-[b,f,j,n][1,5,9,13]−tetraazacyclohexadecine copper(II) complex; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing/antioxidant power; ORAC, oxygen radical absorbance capacity; TRAP, total radical-trapping antioxidant parameter.

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