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Antioxidant Capacity: Experimental Determination by EPR Spectroscopy and Mathematical Modeling

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ABSTRACT: A new method of determining antioxidant capacity based on a mathematical model is presented in this paper. The model was fitted to 1000 data points of electron paramagnetic resonance (EPR) spectroscopy measurements of various food product samples such as tea, wine, juice, and herbs with Trolox equivalent antioxidant capacity (TEAC) values from 20 to 2000 μmol TE/100 mL. The proposed mathematical equation allows for a determination of TEAC of food products based on a single EPR spectroscopy measurement. The model was tested on the basis of 80 EPR spectroscopy measurements of herbs, tea, coffee, and juice samples. The proposed model works for both strong and weak antioxidants (TEAC values from 21 to 2347 μmol TE/100 mL). The determination coefficient between TEAC values obtained experimentally and TEAC values calculated with proposed mathematical equation was found to be $R^2 = 0.98$. Therefore, the proposed new method of TEAC determination based on a mathematical model is a good alternative to the standard EPR method due to its being fast, accurate, inexpensive, and simple to perform.

KEYWORDS: *antioxidant capacity, EPR spectroscopy, mathematical modeling, Trolox equivalent antioxidant capacity (TEAC)*

INTRODUCTION

In recent years, there has been growing interest in the antioxidant properties of food products.[1−5] Large variations in the structure, function, and origin of antioxidant substances have given rise to a number of research methods.[6−9] So far, there is not one universal method that would allow for estimation of antiradical and antioxidant properties of foods. Furthermore, antioxidants interact in a variety of ways with different radicals and oxidizing agents, making it difficult to study their antioxidant properties. Moreover, the reaction environment and the presence of other chemicals in the foods studied have a significant impact on the determination of antioxidant capacity.[9,10] As a consequence of the above, the existence of different reaction mechanisms as well as variations in the environment of the processes taking place have resulted in the proposal of several methods allowing for the study of the antioxidant properties of food products.[9,10] The most frequently used method for determining antioxidant capacity is a method allowing for the determination of total antioxidant capacity, which indicates the sum of components with antioxidant properties, and points to their ability to deactivate free radicals.[8−17] Antioxidant capacity is determined by measuring the change in concentration of a test reagent, for example, a stable free radical, as a result of its direct reaction with the antioxidant undergoing testing. Free radicals used for determining antioxidant capacity are 2,2′-azinobis(3-ethylbenzothiazoline)-6 sulfonic acid (ABTS),*N* ,*N*-dimethylphenylenediamine (DMPD), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) via ultraviolet−visible (UV−vis) spectroscopy.[9,10,15] The most popular screening assays are the following: oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter, (TRAP) photochemiluminescence (PCL), chemiluminescence (CL), total oxidant scavenging capacity (TOSC), total antioxidant capacity (TAC), and total antioxidant potential using Cu(II) (CUPRAC).[9,11−13]

The results obtained by the above methods are often converted to equivalents of Trolox (TE), gallic acid, or ascorbic acid.[8,9,14] Each of the methods mentioned for determining total antioxidant capacity requires the conduction of 5−10 individual measurements in at least three measurement series.[4,18] In the literature, various modifications of the aforementioned methods can be found; however, one recommended universal method for determining antioxidant capacity does not exist.

EPR spectroscopy is a method that is used relatively rarely in comparison with UV−vis spectroscopy for determining antioxidant capacity, although it is the only analytical method that specifically detects compounds having unpaired electrons.[4,7,17−20] Only a few research groups have used EPR spectroscopy for monitoring antioxidant behavior of tea leaves,[17,21,22] coffee,[23] wine,[5,24,25] honey,[19] beer,[7,26] alcoholic beverages,[4] and fruits and vegetables.[17] The method is based on the detection of molecules endowed with at least one unpaired electron, otherwise known as free radicals. The determination of antioxidant capacity is based on measuring changes in the intensity of the spectrum of stable radicals, for example, DPPH, through the reaction of those radicals with antioxidants added in various volumes. The resulting spectrum is compared with an external standard solution with a constant concentration of free radicals.[4,7,17−20] To determine a singular value of the total antioxidant capacity TEAC through EPR spectroscopy, it is necessary to perform 15−30 measurements of the EPR spectra of the DPPH radical with the antioxidant.[4,18]

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Table 1. Calculated $TEAC_{calcd}$ and Experimental $TEAC_{exptl}$ Values Obtained for the Studied Samples by EPR Spectroscopy[a]

no.	sample	V (mL)	%I	I_{100}	$TEAC_{calcd}$ (μmol TE/100 mL)	$TEAC_{exptl}$ (μmol TE/100 mL)	relative error (%)
S1	lemon balm 1	0.002	8.22	638596.26	646.6	688	6.4
S2	lemon balm 1′	0.014	97.1	680166.58	688.7	688	0.1
S3	lemon balm 2	0.002	14.1	697610.64	706.3	738	4.5
S4	lemon balm 2′	0.012	86.1	683262.10	691.8	738	6.7
S5	heartsease 1	0.120	85.0	64917.08	65.7	66	0.4
S6	heartsease 1′	0.140	95.1	64932.30	65.7	66	0.4
S7	heartsease 2	0.080	69.6	69461.10	70.3	64	8.9
S8	heartsease 2′	0.120	82.7	61563.65	62.3	63	2.7
S9	menthe 1	0.015	25.8	177667.15	179.9	177	1.6
S10	menthe 1′	0.0525	94.0	178924.16	181.1	177	2.3
S11	menthe 2	0.0225	47.6	182095.76	184.4	172	6.7
S12	menthe 2′	0.045	80.2	170238.13	172.4	172	0.2
S13	horsetail 1	0.025	50.0	170440.20	172.6	156	9.6
S14	horsetail 1′	0.040	73.3	169399.4	171.5	156	9.0
S15	horsetail 2	0.015	33.9	212671.4	215.3	186	13.6
S16	horsetail 2′	0.040	72.3	165871.96	167.9	186	10.7
S17	nettle 1	0.040	44.7	77608.82	78.6	75	4.5
S18	nettle 1′	0.060	59.9	73743.99	74.6	75	0.5
S19	nettle 2	0.030	40.3	103106.19	104.4	89	14.7
S20	nettle 2′	0.100	97.2	95252.86	96.4	89	7.7
S21	chamomile 1	0.040	46.6	82944.04	84.0	86	2.4
S22	chamomile 1′	0.070	71.9	87678.43	88.8	86	3.1
S23	chamomile 2	0.060	63.8	83641.46	84.7	91	7.5
S24	chamomile 2′	0.080	76.4	84040.21	85.1	91	6.9
S25	eyebright 1	0.008	76.0	855266.69	866.0	1007	16.3
S26	eyebright 1′	0.01	89.4	844233.56	854.8	1007	17.8
S27	eyebright 2	0.008	74.0	830029.33	840.4	917	9.1
S28	eyebright 2′	0.012	96.8	783472.58	793.3	917	15.6
S29	hypericum 1	0.006	59.7	844269.16	854.8	823	3.7
S30	hypericum 1′	0.009	78.8	802565.39	812.6	823	1.3
S31	hypericum 2	0.003	27.4	755117.08	764.6	746	2.4
S32	hypericum 2′	0.007	59.9	743393.45	752.7	746	0.9
S33	fennel 1	0.350	87.9	19028.01	19.3	23	19.5
S34	fennel 1′	0.400	96.3	19102.52	19.3	23	19.0
S35	fennel 2	0.350	90.2	20443.73	20.7	21	1.5
S36	fennel 2′	0.400	94.6	18756.50	19.0	21	10.7
S37	ground coffee a1	0.007	71.2	892645.69	903.8	963	6.5
S38	ground coffee a1′	0.008	81.0	919585.92	931.1	963	3.4
S39	ground coffee a2	0.003	35.9	891823.22	903.0	919	1.8
S40	ground coffee a2′	0.004	44.4	877871.08	888.9	919	3.4
S41	ground coffee b1	0.004	48.0	938364.31	950.1	1058	11.3
S42	ground coffee b1′	0.007	69.8	873475.20	884.4	1058	19.6
S43	ground coffee b2′	0.005	52.9	869463.2	880.4	915	3.9
S44	ground coffee b2′	0.008	73.9	827889.0	838.3	915	9.1
S45	ground coffee c1	0.007	83.9	1077659.5	1091.2	1239	13.5
S46	ground coffee c1′	0.008	93.5	1088833.2	1102.5	1239	12.4
S47	ground coffee c2	0.006	76.7	1107222.8	1121.1	1240	10.6
S48	ground coffee c2′	0.007	85.9	1106820.7	1120.7	1240	10.6
S49	instant coffee a1	0.005	47.9	782747.1	792.6	830	4.7
S50	instant coffee a1′	0.007	65.2	811268.02	821.4	830	1.0
S51	instant coffee a2	0.006	65.1	923779.7	935.4	948	1.3
S52	instant coffee a2′	0.008	79.0	893824.31	905.0	948	4.8
S53	instant coffee b1	0.004	70.1	1375626.50	1392.9	1239	11.0
S54	instant coffee b1′	0.007	96.6	1274621.34	1290.6	1239	4.0
S55	instant coffee b2	0.004	70.5	1385570.666	1402.9	1283	8.5
S56	instant coffee b2′	0.007	96.6	1274621.342	1290.6	1283	0.6
S57	instant decaffeinated coffee a1	0.0015	69.1	2317997.892	2347.1	2147	8.5
S58	instant decaffeinated coffee a1′	0.0025	78.0	2134273.302	2161.1	2147	0.6
S59	instant decaffeinated coffee a2	0.0025	77.2	2108618.061	2135.1	2073	2.9
S60	instant decaffeinated coffee a2′	0.0035	83.8	1871337.538	1984.9	2073	9.4

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Table 1. continued

no.	sample	V (mL)	%I	I_{100}	$TEAC_{calcd}$ (μmol TE/100 mL)	$TEAC_{exptl}$ (μmol TE/100 mL)	relative error (%)
S61	instant decaffeinated coffee b1	0.001	18.1	1246402.553	1262	1496	18.5
S62	instant decaffeinated coffee b1′	0.0025	53.2	1377434.707	1394.7	1496	7.3
S63	instant decaffeinated coffee b2	0.001	31.5	1400887.636	1418.5	1492	5.2
S64	instant decaffeinated coffee b2′	0.004	69.9	1371595.897	1388.8	1492	7.4
S65	espresso coffee a1	0.007	44.3	562630.0452	569.7	649	14
S66	espresso coffee a1′	0.013	87.1	642252.5487	650.3	649	0.2
S67	espresso coffee a2	0.011	78.1	661043.0	669.3	690	3.1
S68	espresso coffee a2′	0.012	87.1	692086.3584	700.8	690	1.5
S69	espresso coffee b1	0.014	64.4	422755.2747	428	458.1	7.0
S70	espresso coffee b1′	0.018	80.9	432031.9939	437.4	458.1	4.7
S71	espresso coffee b2	0.014	76.9	517972.1808	524.5	461.4	12
S72	espresso coffee b2′	0.019	89.8	464136.8967	469.9	461.4	1.8
S73	black currant nectar 1	0.014	66.8	440522.7068	446	436.4	2.2
S74	black currant nectar 1′	0.02	85.6	417322.0496	422.5	436.4	3.3
S75	black currant nectar 2	0.008	57.6	635511.3281	643.5	686.6	6.7
S76	black currant nectar 2′	0.01	78.0	720621.496	729.6	686.6	5.9
S77	orange juice 1	0.01	81.2	754783.6141	764.2	765.4	0.2
S78	orange juice 1′	0.0105	82.6	736282.0769	745.5	765.4	2.7
S79	orange juice 2	0.006	52.8	749176.354	758.6	727.5	4.1
S80	orange juice 2′	0.0105	86.7	779067.9429	788.8	727.5	7.8

[a]Abbreviations: %I, inhibition (%) of the EPR signal of the DPPH free radical; I_{100}, value calculated from the equation describing the relationship between %I and V corresponding to a 100 mL sample; $TEAC_{calcd}$, Trolox equivalent antioxidant capacity calculated by model; $TEAC_{exptl}$, Trolox equivalent antioxidant capacity determined by EPR spectroscopy; a,b,c, various manufacturers.

Such studies are very time-consuming and costly. To reduce the cost and time of measurement, attempts have been made to determine the antioxidant capacity on the basis of empirical or semiempirical mathematical models.[27] This new method allows researchers to reduce the time and costs associated with determining the TEAC value more than five times. In accordance with the above, the goal of this study was to develop and assess an empirical model allowing for an estimation of total antioxidant capacity, along with conversion into Trolox equivalents, among others, depending on the standard curve utilized, and using EPR spectroscopy and DPPH as the free radical standard for a variety of food products.

MATERIALS AND METHODS

Chemicals and Samples. DPPH (Sigma-Aldrich, Poznań, Poland) was used as the source of free radicals. To quantify the antioxidant capacity of the tinctures, Trolox (Acros Organics, Geel, Belgium) was used. All other chemicals and solvents were of analytical grade and used without further purification.

The model was constructed on the basis of the antioxidant capacity, determined by EPR spectroscopy, for a wide range of food products available on the market and in pharmacies with varied antioxidant capacity (substances with both strong and weak antioxidant properties): tea, wine, juice, and herbs.

The study was performed on 20 samples of white wines and 40 red wine samples measured immediately after opening. The following were tested: sweet wines (10 samples), semisweet wines (10 samples), semidry wines (10 samples), and dry wines (30 samples), with differing antioxidant properties. The second group of products represented 24 sample juices. The juices differed in the type of fruit that was used for their production, color, percent fruit content, and turbidity. The following beverages were included in the study: 12 juices (orange, apple, grapefruit, citron, raspberry), 10 drinks (red grape, orange, apple, grapefruit, black currant), and 8 nectars (cherry, plum, apple, black currant). All commercial juices, drinks, and nectars were used immediately after opening. Fresh juice obtained by squeezing also was measured immediately after preparation.

Thirty-six samples of tea produced by various manufacturers were also used: black tea (12 samples), green tea (12 samples), and Earl Gray tea (12 samples). An infusion was prepared from each tea by pouring 200 mL of boiling water over one tea bag.

Moreover, 24 samples of herbs (thyme, lemon balm, sage, heartsease, menthe, chamomile) were used. One hundred milliliters of boiling water was poured over teabags filled with herbs, and the tea was infused for a length of time suggested by the manufacturer.

Tests of the model were performed on samples of herbs, coffees, and juices available on the market and in pharmacies. Thirty-six samples of herbs (lemon balm (S1−S4), heartsease (S5−S8), menthe (S9−S12), horsetail (S13−S16), nettle (S17−S20), chamomile (S21−S24), eyebright (S25−S28), hypericum (S29−S32), fennel (S33−S36)) were used. One hundred milliliters of boiling water was poured over tea bags filled with herbs, and the tea was infused for a length of time suggested by the manufacturer. Thirty-two samples of coffee, ground coffee (S37−S48), instant coffee (S49−S56), instant decaffeinated coffee (S57−S64), and espresso coffee (S65−S72), were also used. The coffee differed in species and manufacturer. An infusion was prepared from each coffee by pouring 150 mL of boiling water over 2 g of coffee. The last group of products used for testing included 12 samples of juices produced by various manufacturers (black currant juices (S73−S76) and orange juices (S77−S80)) (Table 1).

Determination of Antioxidant Capacity. Trolox equivalent antioxidant capacity ($TEAC_{exptl}$) was determined using the method described previously.[4,7,20] The regression equation for the linear relationship between the percent inhibition of EPR signal intensity and the mole number of Trolox was assessed as $\%I = 987.60V + 16.36$, where %I is the inhibition (%) and V is the volume of the sample (mL). This equation was used to calculate the antioxidant capacity of the studied samples in micromoles of TE per 100 mL of the studied sample. The percent inhibition of the EPR spectrum was calculated according to the following equation: $\%\ \text{inhibition} = [(I_0 I)/I_0] \times 100\%$, where I_0 is the area of the EPR spectrum of DPPH (control sample) and I is the area of the EPR spectrum of DPPH with sample.

A typical reaction mixture contained 1 mL of 200 μmol/L DPPH solution in ethanol together with 0.002−0.4 mL of sample, depending on the manifested antioxidant properties. For all samples, the regression equation of the linear relationship between the percent

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inhibition (%I) of the EPR signal intensity and the volume of sample (V) was determined. On the basis of this equation the %I corresponding to 100 mL of the studied sample was calculated (I_{100}). Then, from the standard curve obtained for Trolox, the antioxidant capacity given in micromoles of TE per 100 mL of sample was defined. To indicate antioxidant capacity measured using the DPPH by EPR spectroscopy $TEAC_{exptl}$ is used.

EPR spectra were obtained with a Bruker EMX EPR spectrometer (Bruker-Biospin, Karlsruhe, Germany) operating at the X-band frequency at room temperature. The typical instrument parameters were as follows: central field, 3480 G; modulation amplitude, 2.0 G; time constant, 40.96; gain, 1×10^4 G; microwave power, 20.12 mW.

Mathematical Model. The mathematical model was based on three variables: $x = V$, $y = \%I$, and $z = I_{100}$, where V is the volume of antioxidant added to the DPPH free-radical solution (mL), %I is the inhibition (%) of the EPR signal of the DPPH free radical, and I_{100} is the value calculated from the equation describing the relationship between %I and V corresponding to a 100 mL sample. The approximation procedure starts from the variable separation $z(x,y) = f(x)g(y)$. Taking into account the qualitative behavior of the data, that is, a strong decay with respect to x, the volume of antioxidant added to the DPPH, and a boundedness with respect to y, the inhibition, the function $f(x)$ is represented as a Laurent series, which includes terms with negative degrees only

$$f(x) = \sum_{n=0}^{\infty} f_n x^{-n}$$

and $g(y)$ as the Taylor series

$$g(y) = \sum_{m=0}^{\infty} g_m y^m$$

around the origin of coordinates $(x,y) = (0,0)$ that results in the following formal double series:

$$z(x, y) = \sum_{n=0}^{\infty} \sum_{m=0}^{\infty} g_m f_n x^{-n} y^m \quad (1)$$

As the next step, the polynomial cutoff $\max(n) = N$ is reproduced. The properties of the obtained Laurent polynomial guarantee that the product eq 1 with finitely many negative terms exists.

The last step consists of the choice of the maximal polynomial index for x terms in such a way that $\max(m_n) = N - n$. Thus, the final expression takes the form

$$z(x, y) = \sum_{n=0}^{N} \sum_{m=0}^{N-n} f_n g_m x^{-n} y^m$$

For $N = 3$

$$z(x, y) = a_0 + a_1 y + a_2 y^2 + a_3 y^3 + \frac{a_4 + a_5 y + a_6 y^2}{x} + \frac{a_7 + a_8 y}{x^2} + \frac{a_9}{x^3} \quad (2)$$

where $a_0 = f_0 g_0$, $a_1 = f_0 g_1$, $a_2 = f_0 g_2$, $a_3 = f_0 g_3$, $a_4 = f_1 g_0$, $a_5 = f_1 g_1$, $a_6 = f_1 g_2$, $a_7 = f_2 g_0$, $a_8 = f_2 g_1$, and $a_9 = f_3 g_0$.

Fitting Model to Experimental Data. The model eq 2 was fitted to 1000 data points of EPR spectroscopy measurements.

For each sample, $TEAC_{exptl}$ was determined in three trials, according to the standard method of determining antioxidant capacity, described above, using EPR spectroscopy and DPPH as the free radical. Adjustable parameters a_i were estimated by a nonlinear regression procedure using the Levenberg–Marquardt algorithm in double precision. The set of a_i coefficients and the adjusted coefficient of determination for the nonlinear regression, adj-R^2, are listed in Table 2. One can see that the data satisfy the assumed function with high accuracy.

Table 2. Coefficients of Equation 2 together with the Adjusted Coefficient of Determination for the Nonlinear Regression, Adj-R^2

a_0	−50924 ± 1005
a_1	−1493 ± 110
a_2	47.23 ± 7.63
a_3	−0.277 ± 0.012
a_4	2842 ± 440
a_5	32.887 ± 8.399
a_6	0.446 ± 0.058
a_7	−4.782 ± 0.39
a_8	−0.0438 ± 0.0095
a_9	0.00329 ± 0.00047
adj-R^2	0.91

Testing of the Mathematical Model. The model was tested on 20 sample food products, that is, for 80 EPR spectroscopy measurements.

For each sample, $TEAC_{exptl}$ was determined twice, according to the standard method of determining antioxidant capacity, described above, using EPR spectroscopy and DPPH as the free radical. Next, for two randomly selected volumes of antioxidant sample added to 1 mL of DPPH solution, the EPR measurements were repeated (80 measurements) and %I of the DPPH free radical of EPR signal was determined. On the basis of the value of the V of antioxidant added to the free radical solution, as well as %I of the DPPH free radical EPR signal, the I_{100} value was determined using the model eq 2. On the basis of the I_{100} value the antioxidant capacity $TEAC_{calcd}$ was calculated according to the equation $\%I = 987.60V + 16.36$. Subsequently, the average $TEAC_{exptl}$ values were determined from the two series on the basis of seven measurements in each series, the average calculated $TEAC_{calcd}$ values were determined from the model on the basis of two measurements for each series for each studied sample, and these values were compared to each other.

Statistical Analysis. The presented data are the mean values, whereas errors were calculated as standard deviation (SD). Determination coefficients (R^2), obtained $TEAC_{exptl}$, and expected $TEAC_{calcd}$ were calculated by the Pearson test with a level of significance of $p = 0.05$.

RESULTS AND DISCUSSION

A plot of the proposed model and the observed data points is useful to determine how well the model fits the data. Model eq 2 and the observations are plotted in Figure 1. It is evident from the figure that a definite trend exists in the data and that the model eq 2 proficiently performed the task of capturing that trend. Note that the model was fitted to a larger data set than that shown.

One way of evaluating how well the model fits and predicts the data is to compute not only the deviations between the model and the actual data, analysis of the randomness of the residual plots, and normality of the errors but also deviations between the model and the new data. The proposed mathematical equation allows for a determination of the antioxidant capacity of food products based on a single EPR spectroscopy measurement. The value of the experimental TEAC antioxidant capacity for the products, selected for testing of the model, was situated in the wide range between 21 μmol TE/100 mL of the sample and 2347 μmol TE/100 mL (Table 1). The values of TEAC for the studied food samples are consistent with the TEAC values that have been determined through the use of other research methods described in the literature.[1,28,29]

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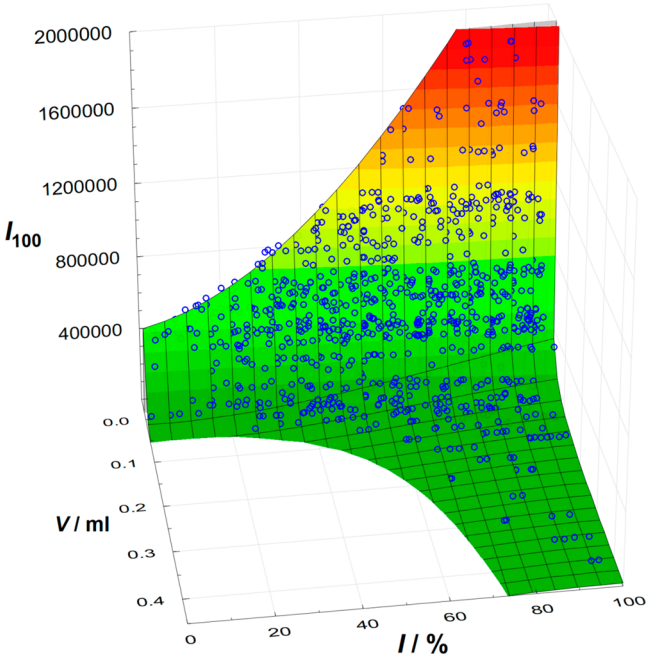

Figure 1. I_{100} as a function of the inhibition of the EPR signal, I, and the volume of antioxidant added to the DPPH solution, V. Points are experimental data on V–I surface calculated using eq 2.

The determination coefficient between TEAC $_{calcd}$ and TEAC $_{exptl}$ was found to be $R^2 = 0.98$ (Figure 2).

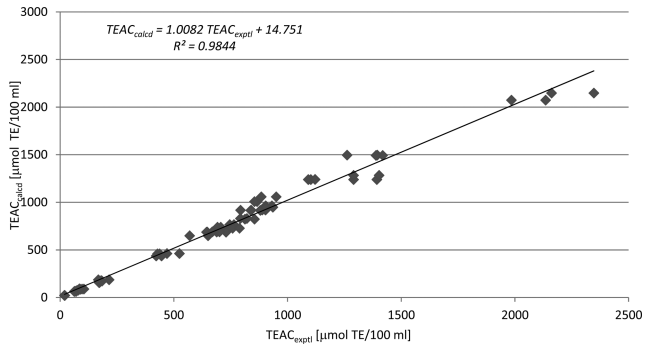

Figure 2. Regression between calculated ($TEAC_{calcd}$) and experimental ($TEAC_{exptl}$) antioxidant capacity.

On the basis of the mathematical model and test conducted, it was concluded that the average relative percentage error of $TEAC_{calcd}$ versus $TEAC_{exptl}$ was 6.6% (95% confidence intervals, CI, 5.5–7.7). It should be noted that for 40 of 80 measurements the value of the error is below 5%, and for 62 of the 80 measurements it was below 10%. The proposed model works for both strong and weak antioxidants. For strong antioxidants (TEAC > 200) the average relative percentage error was found to be 6.5% (95% CI, 5.1–7.8), whereas for weak antioxidants (TEAC < 200) it was 6.8% (95% CI, 4.5–9.0). Such an agreement between $TEAC_{exptl}$ and $TEAC_{calcd}$, as well as an agreement with values found in the literature, indicates the possibility of using this method, which connects mathematical modeling with EPR spectroscopy, for evaluating antioxidant capacities.

For each product the average $TEAC_{exptl}$ and $TEAC_{calcd}$ values from the two series in Figure 3 are presented.

The average relative error for the determined average TEAC calculated values amounted to only 5.25%, which indicates a high utility of the proposed model. To obtain the average antioxidant capacity value via the standard method for determining antioxidant capacity using EPR spectroscopy and DPPH free radicals, it is necessary to perform 22 separate EPR measurements. With the proposed model it is sufficient to perform between two and four EPR experimental and calculated TEAC values to obtain comparable accuracy. Moreover, thanks to the utilization in the model of the I_{100} value, the model offers the possibility of determining the antioxidant capacity in various standards. It is sufficient to develop a standard curve for a standard other than Trolox, to obtain, from one series of measurements, the antioxidant capacities expressed in different standard equivalents.

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Notes

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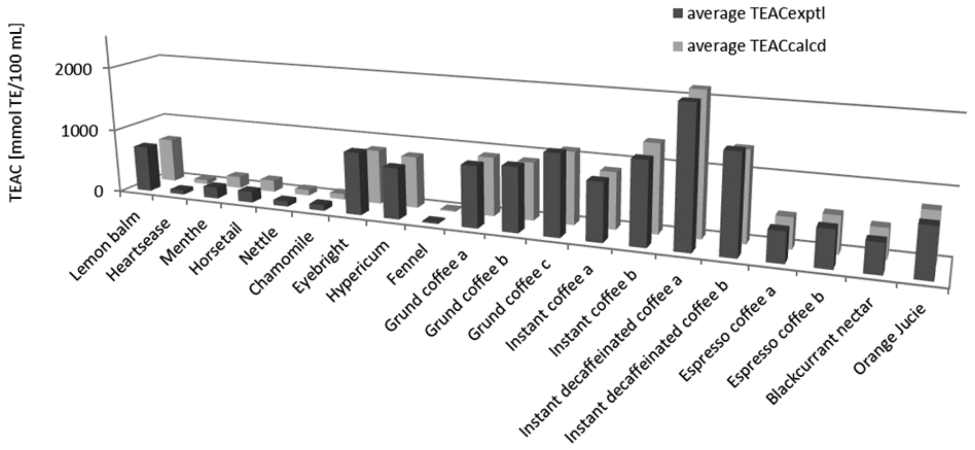

Figure 3. Average values of experimental and calculated TEAC of investigated samples (a, b, and c are various manufacturers).

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