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A Modification of the ABTS• Decolorization Method and an Insight into Its Mechanism

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Abstract: A modification of the ABTS• decolorization assay for plate readers is presented. In our modification, 200 µL of ABTS solution of absorbance 1.0 at 734 nm was added with an antioxidant and decreased absorbance resulted. For comparison of antioxidant activities in the kinetic assay of absorbance decrease, concentration dependence of absorbance decrease and of area under curve are recommended. "Fast" and "slow" antioxidants were distinguished: while the reactions of "fast" antioxidants ABTS• were completed within seconds, the reactions of "slow" antioxidants were not finished after 6 min. We recommend reaction time of 60 min for assays of such antioxidants, blood plasma and plant extracts. Sub-additive interactions between some antioxidants (ascorbate and Trolox, hispidulin and Trolox, and glutathione and ascorbate) were found in the ABTS• decolorization; possible reasons for such interactions are discussed.

Keywords: ABTS• decolorization; antioxidant; antioxidant capacity; free radical

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1. Introduction

The interest in antioxidants observed in recent decades has resulted in proposals for several simple methods of estimating antioxidant activities of individual compounds and antioxidant capacities of complex antioxidant mixtures, such as body fluids, beverages and food samples [1–5]. While the clinical relevance of "total antioxidant capacity" (TAC) of body fluids is not always straightforward [6–8], TAC assays measuring the sum of antioxidant activities of food products have become very popular. Databases have been constructed allowing the evaluation of TAC of diet components and meals [9–12] although doubts have been raised whether the TAC values of foods can be translated to health effects provided by the food [11,12].

One group of methods assessing antioxidant activity and capacity is based on the reduction of the relatively stable ABTS• radical formed by one-electron oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Figure 1). The ABTS• radical absorbs visible light, its solutions being bluish green, and changes in the ABTS• concentration can be easily quantified by absorbance measurements. ABTS• can be generated in various ways. Oxidation of ABTS by hydrogen peroxide catalyzed by myoglobin in a pseudoperoxidase reaction [5] was the basis of a commercial "ABTS Antioxidant Assay Kit". A convenient ingenious modification of this method, using ABTS• pre-formed by ABTS oxidation with a substoichiometric amount of potassium persulfate, was proposed by Re et al. [13] over 20 years ago. This modification ("an improved ABTS radical cation decolorization assay") has become one of the most frequently used methods for estimating of total antioxidant activity. According to Web of Science, this paper has been cited 14,883 times, according to Scopus 15,913 times, and according to Google Scholar 24,373 times (data for 28 June 2022), and we are aware of papers in which this method was

used but Re et al. [13] was not cited. The popularity of the "improved ABTS radical cation decolorization assay" is due to its simplicity, low cost and low instrument requirement, and to the promiscuous reactivity of ABTS$^{\bullet}$, allowing its broad application. It should be mentioned that, as pointed out by certain authors [14], the commonly used term "ABTS cation radical" is at least questionable. ABTS, the substrate for ABTS$^{\bullet}$ formation, is a dianion and ABTS$^{\bullet}$, simply being less charged than ABTS, is still a net anion (monoanion), bearing two negative charges and one positive charge (Figure 1). Therefore, the symbol ABTS$^{\bullet}$ and not ABTS$^{\bullet+}$ will be used in this paper.

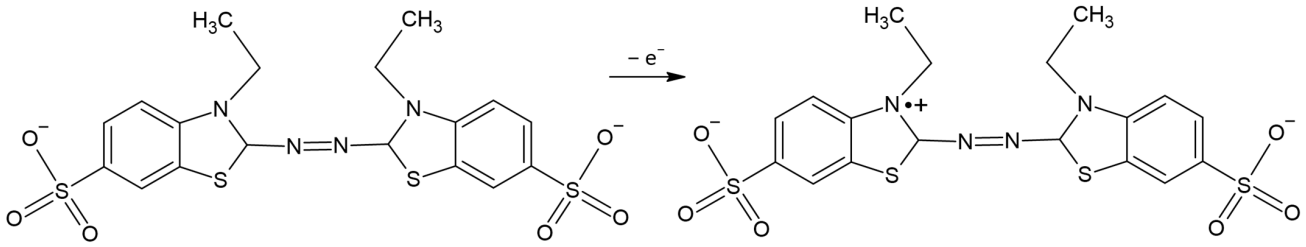

Figure 1. Formation of ABTS$^{\bullet}$ by one-electron oxidation of ABTS.

The antioxidant activity measured by ABTS$^{\bullet}$ reduction is usually referred to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard antioxidant. It allows expression of results in Trolox equivalents (TE), which is a quite useful way comparing antioxidant activities, and is universally valid as the antioxidant activity determined by this assay is proportional to the concentration or volume of a sample. For this reason, the assay based on ABTS$^{\bullet}$ reduction is often referred to as assessment of "Trolox Equivalent Antioxidant Capacity" (TEAC), although there are no substantial reasons for restricting this term to the ABTS$^{\bullet}$ assays only.

Originally, the "improved ABTS radical cation decolorization assay" was proposed for a spectrophotometer, but nowadays plate readers are more commonly used. In this study, we present a modification of the method for use with a plate reader. There are several facets of the ABTS$^{\bullet}$ decolorization method that require closer insight. They include the dependence of the ABTS$^{\bullet}$ decolorization on pH and ionic strength, the rate of ABTS$^{\bullet}$ reactions with various substances, and interactions between antioxidants in the ABTS$^{\bullet}$ decolorization reaction. These questions are addressed in the present study.

2. Materials and Methods

2.1. Reagents, Materials and Equipment

All the reagents used were obtained from Merck (Poznań, Poland) except for phosphate-buffered saline (PBS) purchased from Lab Empire (Rzeszów, Poland). Extract of *Boletus edulis* was prepared by homogenization of the fresh fruiting bodies in 10 mM buffer, pH 7.4 (1:4, *w*/*v*) and centrifugation (10 min, 12,100× *g*). The supernatant diluted 10 times was used for the reaction with ABTS$^{\bullet}$. Human blood plasma obtained from blood anticoagulated with citrate was diluted 5 times for the measurements. Absorbance measurements were carried out using a Spark multimode microplate reader (Tecan Group Ltd., Mannedorf, Switzerland).

2.2. Modified ABTS$^{\bullet}$ Decolorization Assay

The stock ABTS$^{\bullet}$ solution was obtained by oxidation of 7 mM 2,2′-azinobis(3-ethylbenz othiazoline-6-sulfonic acid) diammonium salt with 2.45 mM (final) potassium persulfate [13]. In our modification of the ABTS$^{\bullet}$ decolorization assay, we diluted the stock ABTS$^{\bullet}$ solution with PBS, pH 7.4 (unless stated otherwise), to a concentration providing absorbance of a 200-µL aliquot of 1.0 at the wavelength of 734 nm in one well of a Greiner 96-well plate. The optical path of such an aliquot is 6.125 mm and the ABTS$^{\bullet}$ concentration of the solution is 106.7 µM, assuming a micromolar absorption coefficient of 0.015 μM^{-1} cm^{-1} [15,16]. A 200 µL volume of this solution contains 21.34 nmoles of ABTS$^{\bullet}$.

2.3. EPR Measurements

Electron paramagnetic resonance (ESR) measurements were performed in a Bruker multifrequency and multiresonance FT-EPR ELEXSYS E580 spectrometer (Bruker Analytische Messtechnik, Rheinstetten, Germany) operating at the X-band (9.837530 GHz). The following settings were used: central field, 3505.6 G; modulation amplitude, 1 G; modulation frequency, 100 kHz; microwave power, 94.64 mW; power attenuation, 10 dB; scan range, 80 G; conversion time, 25 ms; and sweep time, 25.6 s.

2.4. Statistical Analysis and Data Presentation

All measurements were performed in triplicate and repeated at least three times on different preparations. Statistical significance of differences was evaluated using the paired Student's *t*-test. *p*-values of <0.05 were considered significant.

The magnitude of absorbance decrease (initial value of 1.0 minus the actual absorbance) is presented in the plots. Such plots allow for an easier estimation of the percentage of decolorization and show the reaction progress in positive values.

3. Results and Discussion

3.1. The Modified ABTS$^\bullet$ Decolorization Assay

Our modification of the method presented by Re et al. [13] employs a higher initial concentration of ABTS$^\bullet$ (106.7 µM) than that used in the original version of the method (46.7 µM). As the light path is shorter in a well containing a 200-µL sample on the multiwell plate than it is in a standard cuvette, the increased ABTS$^\bullet$ concentration prevents a decrease in the accuracy of the absorption measurements. The modified assay allows the use of higher volumes or concentrations of extracts or pure compounds. The use of a plate reader allows simultaneous analysis of a larger number of samples. A small disadvantage is the somewhat longer lag period between the mixing of a sample with the ABTS$^\bullet$ solution and starting the measurement, so we routinely filled only one row of wells in a single measurement or used a multichannel pipette.

3.2. pH Dependence of ABTS$^\bullet$ Reactivity

Using a series of 0.1 M buffers (phosphate, pH 7.4 and 6.0; acetate, pH 5.0 and 6.0; glycine/HCl, pH 3.0, phosphate/HCl, pH 2.0), we studied the pH dependence of ABTS$^\bullet$ self-decay, and its reaction with Trolox in the pH range of 2.0–7.4.

The self-decay of ABTS$^\bullet$ was lower at lower pH (Figure 2), confirming the reported higher stability of ABTS$^\bullet$ under acidic conditions [17].

The reaction of ABTS$^\bullet$ with the standard antioxidant Trolox is believed to proceed in two steps (Reactions (1) and (2)):

$$\text{ABTS}^\bullet + \text{Trolox-OH} \rightarrow \text{ABTS} + \text{Trolox-O}^\bullet + \text{H}^+ \quad (1)$$

$$\text{ABTS}^\bullet + \text{Trolox-O}^\bullet \rightarrow \text{ABTS} + \text{Trolox} = \text{O} \quad (2)$$

where Trolox-OH, Trolox-O$^\bullet$ and Trolox = O represent Trolox phenol, Trolox semiquinone radical and Trolox quinone, respectively (Figure 3).

Lowered pH did not affect significantly the reaction of ABTS$^\bullet$ with Trolox; there was a tendency for somewhat lower reactivity when pH was lowered but the differences were not statistically significant (Figure 4). A modification has been proposed for the original method presented by Re et al. [13], in which the assay was run at pH 5.8. The author claimed higher stability for ABTS$^\bullet$ prepared at pH 3.6 [17]. In our opinion, however, the measurement of antioxidant reaction at physiological pH optimally reflects its reactivity in vivo and prevents undesired reactions (e.g., precipitation) among components of complex samples.

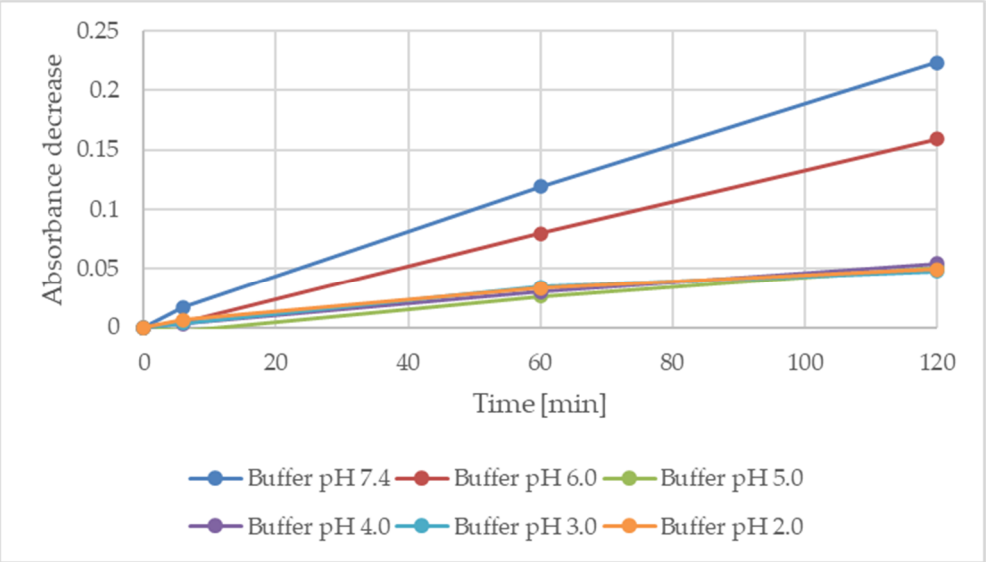

Figure 2. Self-decay of ABTS• in buffers (100 mM) of various pH.

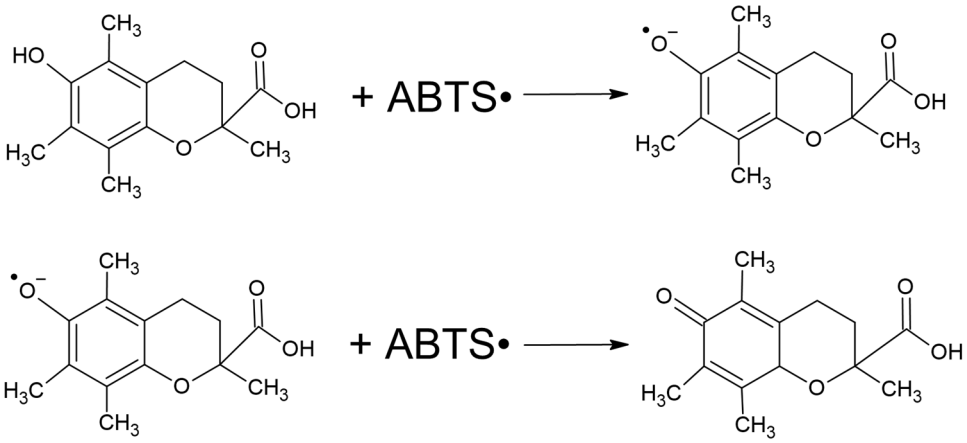

Figure 3. Reaction of ABTS• with Trolox.

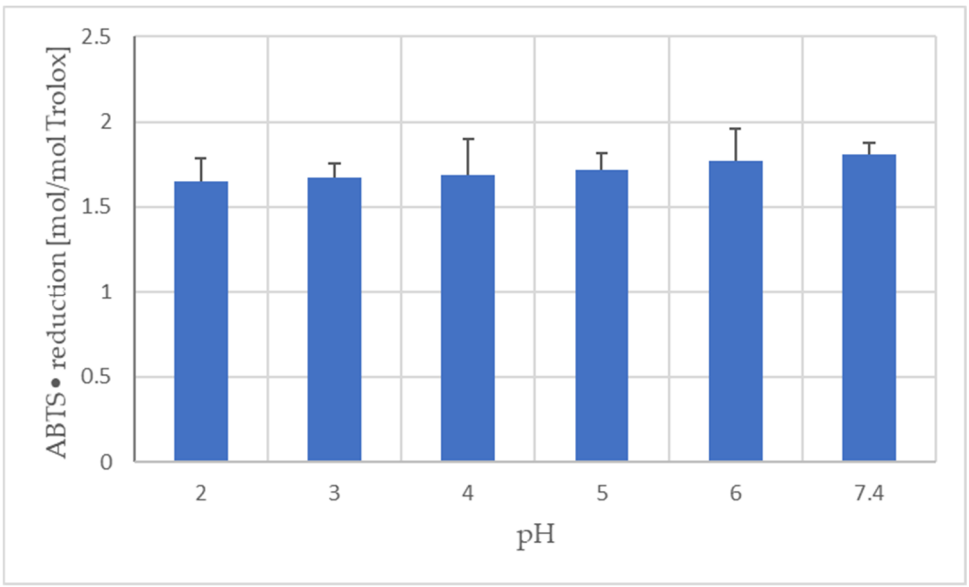

Figure 4. Reactivity of Trolox (5 nmoles) with ABTS• in buffers (100 mM) of various pH.

3.3. *Effect of Ionic Strength of the Reactivity of ABTS•*

In order to check the effect of ionic strength on the reactivity of ABTS•, we monitored the reactions of ABTS• with Trolox and blood plasma in sodium phosphate buffer, pH 7.4, at various concentrations from 10 to 500 mM. The self-decay of ABTS• showed a small decrease with increasing ionic strength (Figure 5), while the reactions with Trolox (Figure 6) and with blood plasma (Figure 7) were moderately inhibited by increasing ionic strength.

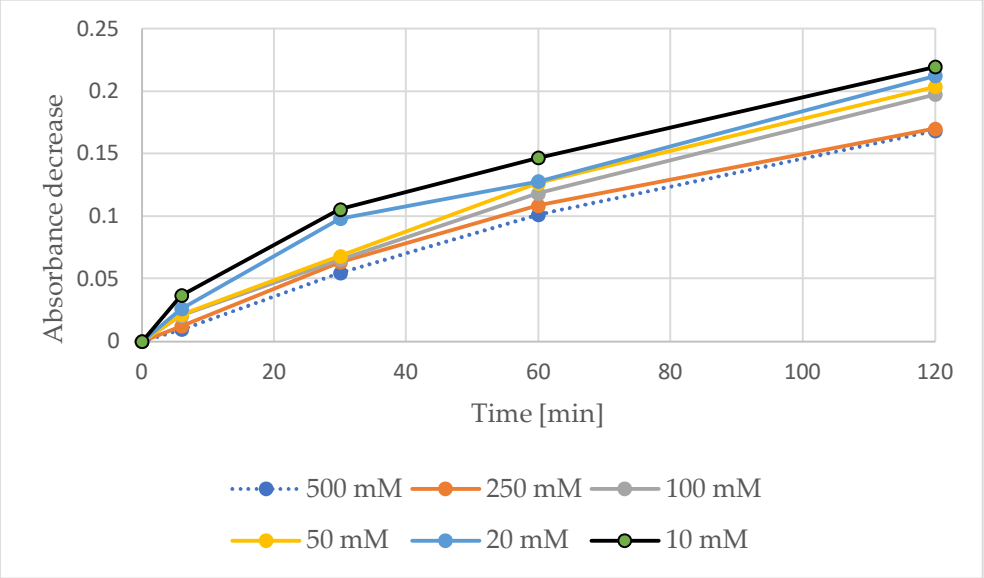

Figure 5. Effect of ionic strength on the self-decay of ABTS•. Decrease in ABTS• absorbance was monitored in phosphate buffer, pH 7.4, at various concentrations (10–500 mM).

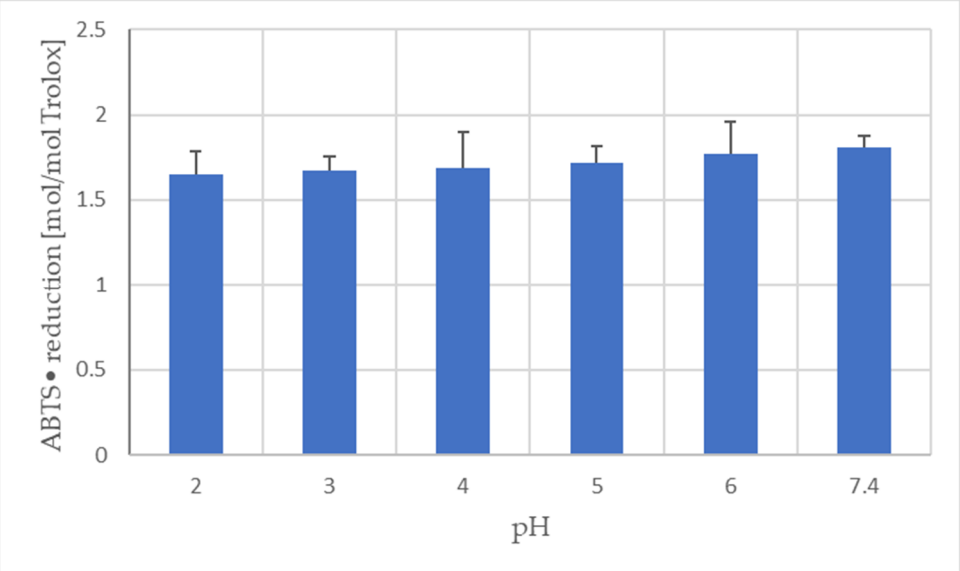

Figure 6. Effect of ionic strength on the reaction of ABTS• with Trolox (5 nmol). ABTS• reduction was measured in phosphate buffer at various concentrations (10–500 mM).

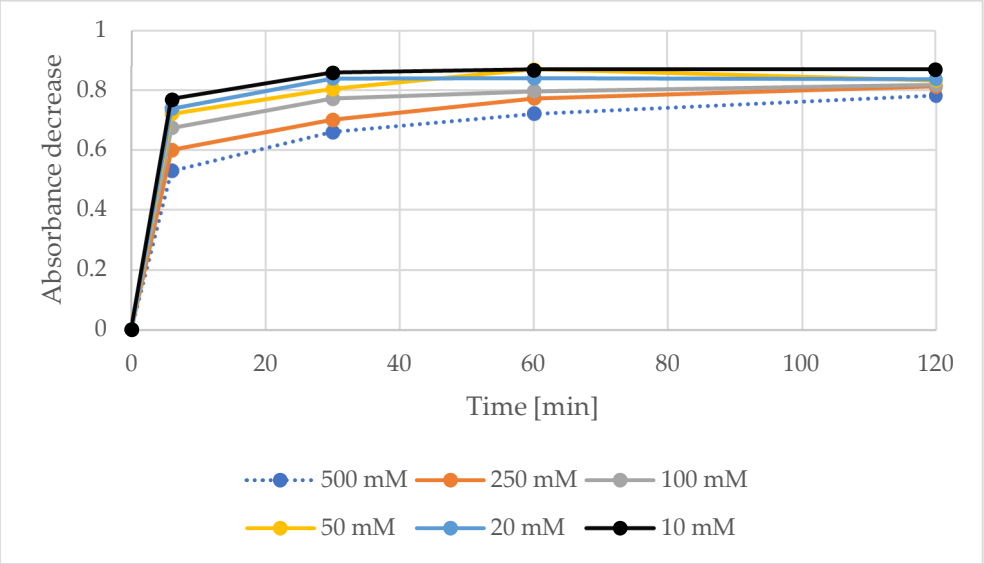

Figure 7. Effect of ionic strength on the reaction of ABTS• with human blood plasma. Decrease of ABTS• absorbance was monitored in phosphate buffer, pH 7.4, at various concentrations (10–500 mM).

3.4. ABTS• Self-Quenching

In the absence of added reductants, absorbance of ABTS• solutions decreased slowly. The self-decay of ABTS• could be expected to be due mainly to disproportionation of ABTS• radicals to ABTS and ABTS biradical

$$ABTS^{\bullet} + ABTS^{\bullet} \rightarrow ABTS + ABTS^{2\bullet} \quad (3)$$

However, this was not the case since the ABTS biradical absorbed light with a maximum in the range of 513–520 nm [15] and upon ABTS• decomposition we did not observe the appearance of an absorption peak in this range. Rather, formation of degradation products which do not absorb in the visible range [18] probably occurred.

Even this reaction is not simple and does not follow second-order kinetics (not shown). Moreover, the rate of absorbance decrease was not proportional to the initial concentration of ABTS• (Figure 8).

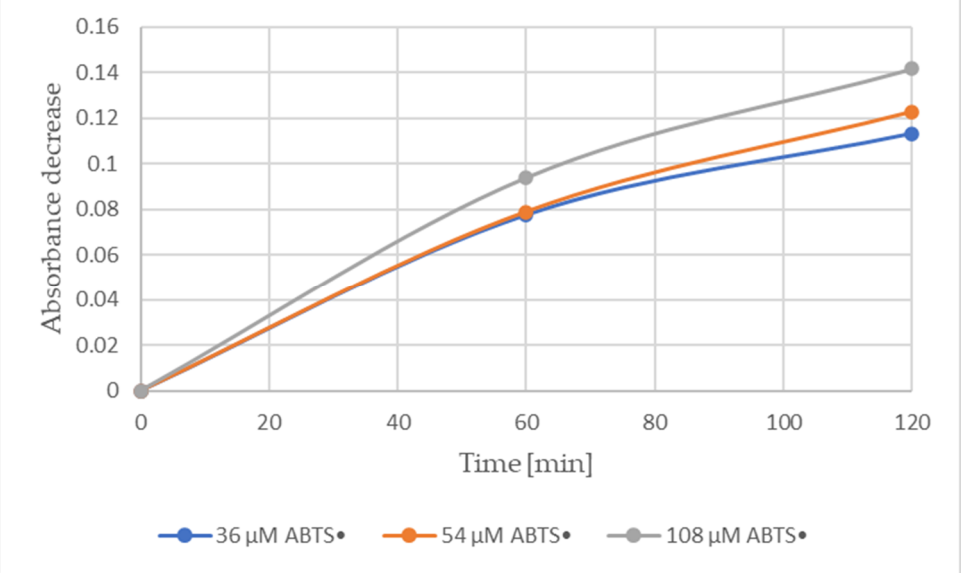

Figure 8. Absorbance decrease of ABTS• solutions with various initial concentrations.

A correction for ABTS• self-decay should be done when studying slowly reacting antioxidants, and it should take into account the decrease of ABTS• concentration during the course of reaction. However, the lack of linear dependence of the decay rate on the concentration of ABTS• makes this task non-trivial.

3.5. "Slow" and "Fast" Antioxidants

In previous studies, we observed that while some antioxidants reacted almost instantaneously with ABTS• (within less of a second or few seconds), others required more time, with reactions proceeding over a time scale of minutes. In order to obtain a more detailed view of this problem, we studied the reactions of various antioxidants with ABTS• over a prolonged time, up to 180 min. The results, presented in Table 1, point to long reaction times of various antioxidants in the reduction assay. In this experiment, concentrations of antioxidants were chosen that provided final ABTS• reduction in the range of 80–95% after 180 min.

Table 1. Time course of ABTS• reduction by various antioxidants. Initial value of absorbance: 1.0 in all cases.

Compound	$A_{0.25\ min}$	$A_{6\ min}$	$A_{30\ min}$	$A_{60\ min}$	$A_{120\ min}$	$A_{180\ min}$	$t_{1/2}$
ABTS• only	0.999	0.025	0.064	0.101	0.142	0.171	-
Ascorbic acid, 10 nmol	0.843	0.853	0.865	0.877	0.896	0.908	ca 9 s
Caffeic acid, 7 nmol	0.458	0.492	0.550	0.646	0.789	0.878	9.84 min
Capsaicin, 10 nmol	0.386	0.549	0.628	0.669	0.702	0.730	3.03 min
Capsaicin, 14 nmol	0.444	0.639	0.770	0.823	0.891	0.911	1.14 min
Carnosine, 50 nmol	0.225	0.399	0.674	0.836	0.904	0.941	12.51 min
Cyanidin, 10 nmol	0.374	0.594	0.767	0.842	0.891	0.910	2.07 min
Cysteine, 24 nmol	0.448	0.556	0.708	0.767	0.803	0.824	2.65 m
EGCG, 2 nmol	0.506	0.654	0.768	0.830	0.887	0.921	ca 15 s
Ergothionein, 50 nmol	0.504	0.574	0.707	0.834	0.954	0.955	ca 15 s
Ethoxyquin, 22 nmol	0.648	0.684	0.713	0.737	0.801	0.826	ca 12 s
Ferulic acid, 10 nmol	0.653	0.768	0.800	0.821	0.840	0.858	ca 11 s
Gallic acid, 3 nmol	0.561	0.699	0.791	0.844	0.880	0.900	ca 13 s
Genistein, 6 nmol	0.471	0.745	0.867	0.873	0.939	0.941	ca 24 s
Glutathione, 10 nmol	0.505	0.627	0.797	0.854	0.885	0.901	ca 15 s
Histidine, 6.5 µmol	0.167	0.411	0.698	0.866	0.887	0.948	10.73 min
Mohr salt, 32 nmol	0.693	0.742	0.770	0.793	0.799	0.804	ca 11 s
Trolox, 10 nmol	0.947	0.947	0.948	0.948	0.948	0.948	Fast reaction
Tryptophan, 10 nmol	0.324	0.730	0.856	0.886	0.942	0.948	55 s
Tyrosine, 10 nmol	0.202	0.662	0.826	0.863	0.930	0.940	2.50 min

EGCG, epigallocatechin gallate; Fast reaction, completed before starting the measurement (ca 15 s). Stoichiometry of ABTS• reduction by Trolox: 1.90 mol ABTS•/mol Trolox.

Ilyasov et al. distinguished three classes of antioxidants with respect to the rate of reaction with ABTS•, on the basis of kinetic measurements and a visual- spectrophotometric titration assay. The group of fast-reacting antioxidants consisted of Trolox and α-tocopherol, the slow-reacting group included naringenin and apigenin, while the group of moderate-reacting antioxidants contained dihydroquercetin, quercetin, rutin, morin, and glutathione [19]. A group of antioxidants reacting at an intermediate rate could be also distinguished on the basis of our results (Table 1). However, since we used various concentrations of antioxidants in order to provide comparable final percentages of reduction, and reaction half-times depended on antioxidant concentration, that classification would be arbitrary. Moreover, the time course of reactions was different for various antioxidants: some antioxidants of relatively short half-time continued to react for a long time. The data summarized in Table 1 indicate that most of common antioxidants are "slow" antioxidants, which must be taken into account when setting the time of the assay. Generally, the fast reactions with ABTS• were ascribed to single electron transfer (SET) reactions, and slow ones to hydrogen atom transfer (HAT) reactions. However, steric hindrance may slow down SET

reactions, and mixed reactions can occur, including HAT reactions at low concentrations of a reductant and SET reactions dominating at high concentrations [20,21].

It is evident from Table 1 that for most antioxidants, the reduction was not complete after 6 min, and in many cases not even after 30 min. A time of 60 min allowed a reasonable estimation of total reduction of ABTS• by "slowly reacting" antioxidants. Even this time was not sufficient to complete the reactions of certain slow antioxidants (e.g., the extent of reaction after 60 min was 94%, 93% and 90% of that after 180 min for gallic acid, glutathione and EGCG, respectively), but the time of 60 min seems to be a reasonable compromise between obtaining maximum accuracy of measurement and the convenience of the assay.

Various authors have used different times in ABTS• decolorization assay. Some authors [22–25] employed the reaction time of 6 min, as proposed by Re et al. [13], but others used shorter or longer reaction times. For example, in the analysis of fruit extracts, reaction times of 2 min [26], 5 min [27] 10 min [28,29], 15 min [30] 20 min [31], 30 min [32], 60 min [33], up to 90 min [34], 120 min [35] and 300 min [36] have been employed. Some authors measured ABTS decolorization immediately ("without incubation time") [37], while there are other papers which do not report the reaction time. Although comparisons between various matrices within a study are fully legitimate, comparison of data obtained with different reaction times is hardly possible. Magalhães et al. proposed a "kinetic matching approach" for ABTS• assay. This approach is based on selecting a standard compound that presents a kinetic profile similar to the sample. It allows prediction of a sample's total reactivity on the basis of a short (5–15 min) time measurement [36]. However, because various antioxidants that may be present in a sample show different time courses in their reactions with ABTS•, the validity of such predictions may be limited.

Taking into account the long reaction times of some "slow" antioxidants, the 60 min assay time can provide more accurate estimates of their reactivities than a short-time assay. E.g., comparison of reactivities of capsaicin and Trolox, shown in Table 1, provides the capsaicin reactivities of 0.71 and 0.74 mol TE/mol for reaction times of 60 and 180 min, respectively, while a value of 0.029 was reported on the basis of 6 min reactivity [38]. Similarly, we found genistein reactivity of 0.92 and 0.99 mol TE/mol, respectively, while a value reported from 5 min measurement was 0.45 mol TE/mol [39]. Immediate measurement of the reactivity of gallic acid with ABTS• brought a value of 0.85 mol TE/mol [40], while our results corresponded to values of 2.97 and 3.16 TE/mol after 60 and 180 min, respectively.

3.6. Alternative Parameters to Measure Antioxidant Activity

A simple and straightforward way to determine antioxidant activity is to measure the decrease of ABTS• absorbance after a specified time; 6 min as recommended by Re et al. [13] or a different time, as used by some authors (we would recommend 60 min for "slow" antioxidants, blood plasma or plant extracts). However, other parameters can also be used.

One such parameter is the dependence of the slope of the ABTS• absorbance decrease on the concentration of a compound or the amount of an extract. Comparison of such a slope with the slope obtained for Trolox or another standard antioxidant allows determination of Trolox equivalent antioxidant activity (mol TE/mol of a compound) or Trolox equivalent antioxidant capacity (mol TE/l of extract or mol TE/kg material). This approach partly compensates the effects of errors of individual measurements (Figure 9 top; Table 2).

Another approach is based on the comparison of "area under curve" i.e., the sum of ABTS• absorbance values read in successive measurements during the course of reduction, if the measurements are taken in the kinetic loop mode (Figure 9 bottom, Table 2). This approach is less dependent on errors of individual measurements. It is a standard method in the quantification of "oxygen radical absorbance capacity" (ORAC) [9,41,42]. As shown in Table 2, the two approaches brought concordant results.

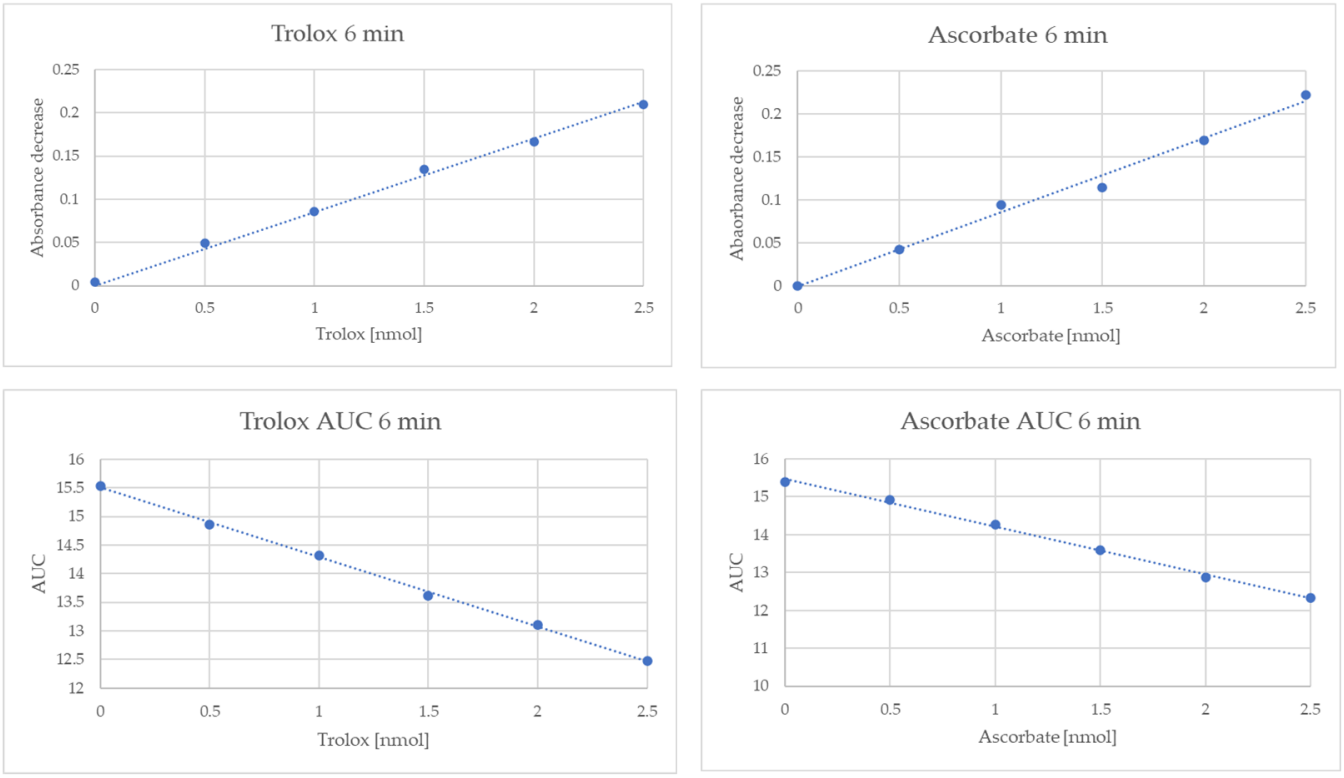

Figure 9. Concentration dependence of ABTS• reduction by ascorbic acid and Trolox (absorbance decrease and AUC after 6 min; absorbance measured every 30 s).

Table 2. Antioxidant activity of ascorbic acid with respect to Trolox, based on the decrease of absorbance and slope of dependence of absorbance decrease on the amount of compound.

Parameter Amount [nmol]	Trolox	Ascorbic Acid	Ratio of Absorbance Decrease = Activity Ratio
Absorbance decrease			
0	0	0	-
0.5	0.043	0.042	0.977
1.0	0.079	0.094	1.190
1.5	0.124	0.115	0.927
2.0	0.159	0.170	1.069
2.5	0.199	0.222	1.116
Mean			1.056 ± 0.105
Slope of absorbance decrease	0.07947 ± 0.00110	0.08313 ± 0.00414	1.046 ± 0.043
Slope AUC	−1.2154 ± 0.0212	−1.26113 ± 0.0346	1.041 ± 0.040

The agreement between the calculations based on the dependence of absorbance decrease and AUC on the extract volume was confirmed for the measurement of the antioxidant capacity of *Boletus edulis* extract. In this case ABTS• reduction was monitored up to 60 min and was significantly different for 6 and 60 min (Figure 10; Table 3).

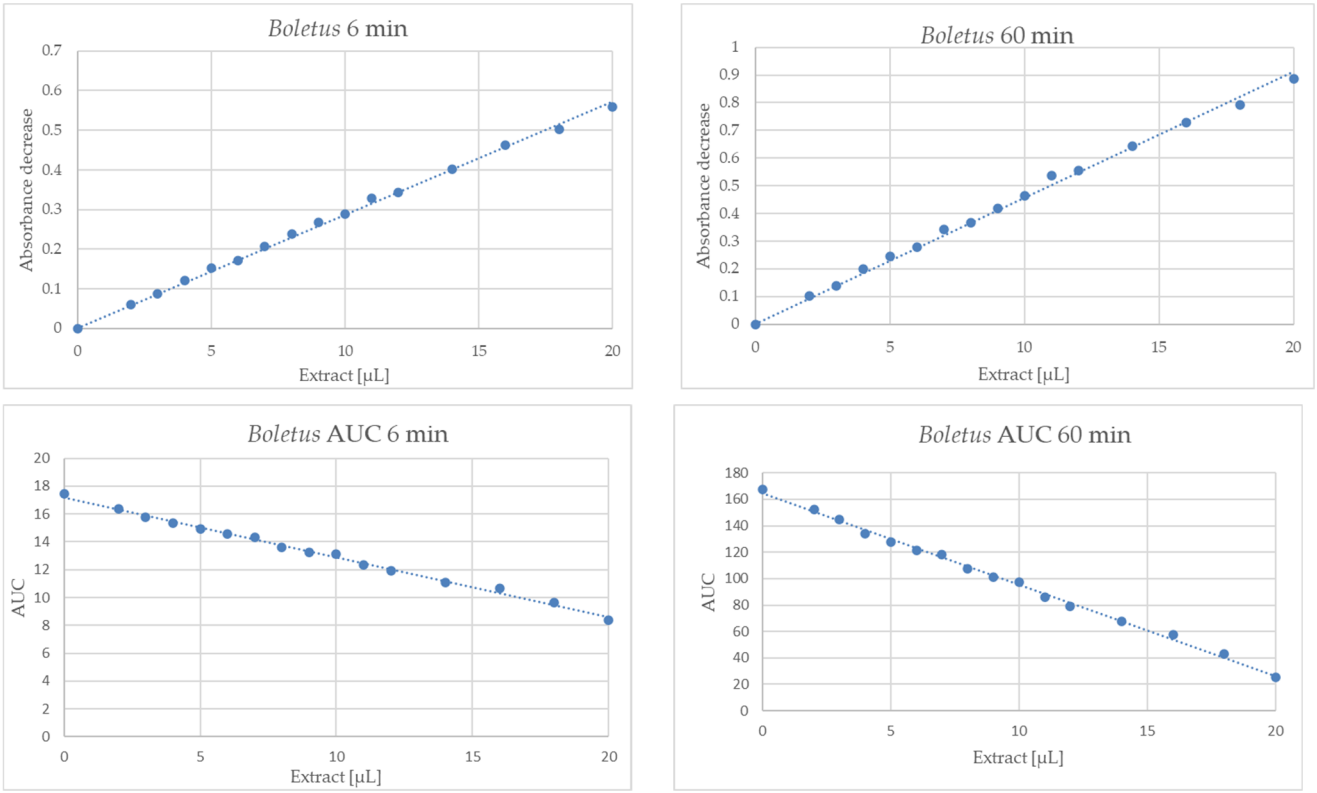

Figure 10. Dependence of absorbance decrease and AUC on the volume of *Boletus* extract after 6 min and after 60 min. Measurements were taken every 30 s.

Table 3. Calculation of the antioxidant capacity of *Boletus* extract, based on the dependence of absorbance decrease and AUC on extract volume.

Parameter	Trolox [nmol^{-1}]	*Boletus* Extract [µL^{-1}]	Antioxidant Capacity [nmol TE/µL]
6 min			
Slope of the line of dependence of absorbance decrease on the concentration or volume	0.07947 ± 0.00110	0.02797 ± 0.00029	0.352 ± 0.006
Slope of the line of dependence of AUC on the concentration or volume	−1.2154 ± 0.0212	−0.42900 ± 0.00787	0.353 ± 0.068
60 min			
Slope of the line of dependence of absorbance decrease on the concentration or volume	0.07947 ± 0.00110	0.04414 ± 0.00611	0.555 ± 0.077
Slope of the line of dependence of AUC on the concentration or volume	−12.547 ± 0.17923	−6.8960 ± 0.0983	0.545 ± 0.008

3.7. Reactivity of ABTS•

ABTS• reacts with many substances, its reactivity being higher than that of DPPH and Fe^{3+} in the FRAP assay. For example, we found that the reactivity of hispidulin with ABTS• was 2.82 mol TE/mol hispidulin, while its reactivity with DPPH and Fe^{3+} (FRAP assay) was negligible (0.019 and 0.09 mol TE/mol hispidulin, respectively) [43]. Similarly, capsaicin showed a reactivity with ABTS• of 0.74 (Table 1), a reactivity in the FRAP assay of 1.103 mol TE/mol, and only 0.079 mol TE/mol with DPPH (submitted).

The thermodynamic condition for a reduction reaction to occur requires lower redox potential of a reductant than that of an oxidant. Thus, ABTS• can be reduced by compounds of one-electron redox potential lower than of the ABTS•/ABTS redox couple (standard redox potential $E^{o'}$ of 0.68 V). This value is similar to the redox potential of the Fe^{3+}/Fe^{2+} couple ($E^{o'}$ of 0.70 V) [44] and much higher than that of the DPPH•/$DPPH_2$ redox couple, for which the cathodic and anodic peaks the in electrochemical reduction of DPPH are 251 mV and 310 mV, respectively [45]. Thus, some compounds are unable to reduce DPPH but able to reduce ABTS• or Fe^{3+} if their one-electron redox potential is higher than that of DPPH but lower than 0.68. There are no good reasons to assume that the "more selective" DPPH reduction test is more biologically relevant than the "more promiscuous" ABTS• reduction test, since the most relevant biological oxidants have one-electron redox potentials higher than that of the ABTS•/ABTS redox couple (HO•, H^+/H_2O, $E^{o'}$ of 2.31 V; RO•, H^+/ROOH, $E^{o'}$ of 1.60 V; allyl•, H^+/allyl-H, $E^{o'}$ of 0.96 V; $O_2{}^{-}$•, 2 H^+/H_2O_2, $E^{o'}$ of 0.94 V; RS•/RSH^-, $E^{o'}$ of 0.92 V) [46]. By steric hindrance, this affects the possibility and rate of reaction with DPPH and ABTS•, so the reactivity is impossible to predict on a thermodynamic basis alone.

3.8. ABTS• Reduction Generates Free Radicals of Reducing Compounds

An obvious consequence of one-electron reaction with the ABTS• radical is the formation of a free radical of the reducing compound, which further reacts to form a fully oxidized non-radical form of this compound. When using excess reducing compound with respect to ABTS• (mixing ca 3.5 mM ABTS• solution with an equal volume of 5 mM Trolox or ascorbic acid) and immediately measuring ESR spectra, free radicals of Trolox and ascorbate were detected (Figure 11).

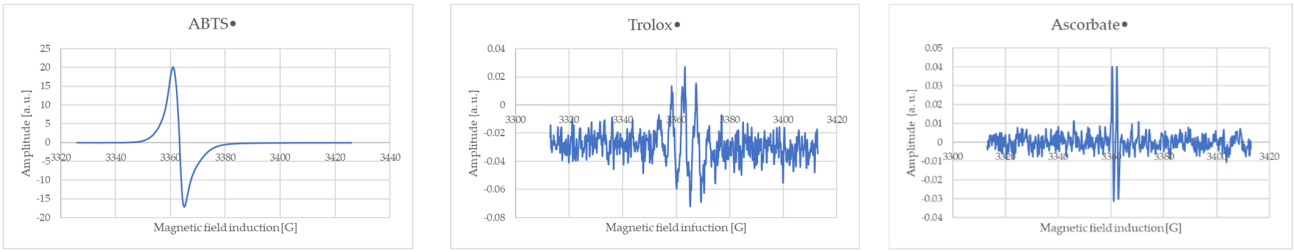

Figure 11. ESR spectra of ABTS•, Trolox and ascorbate radicals recorded after treatment of ABTS• solution with excess of Trolox and ascorbic acid, respectively.

3.9. Interaction between Antioxidants

Interactions between low-molecular antioxidants leading to subadditive behavior in antioxidant activity assays have been reported [47–49]. In this study, we checked the interactions between Trolox, ascorbate and hispidulin as model compounds by comparing ABTS• reduction by single antioxidants and their combination (slopes of the lines of concentration dependence of absorbance decrease or AUC).

Interaction coefficient (IC) (expressed in %) was calculated as

IC = 100% × [(Calculated sum of values for a chosen parameter) − (Value of the parameter measured for the sum of compounds)]/(Calculated sum of values for the parameter)

So defined interaction coefficient (IC) has positive values if the interaction between antioxidants is subadditive (antagonistic), zero if the interaction is additive, and negative values if the interaction is superadditive (synergistic). Statistically significant subadditive interactions in ABTS$^{\bullet}$ decolorization were found to occur between ascorbic acid and Trolox, hispidulin and Trolox, and glutathione and ascorbic acid, but not between glutathione and Trolox, nor hispidulin and ascorbic acid. The interactions were observed when studying concentration dependence of absorbance decrease and AUC (Table 4).

Table 4. Interaction between compounds in the ABTS$^{\bullet}$ decolorization assay as estimated by concentration dependence of absorbance decrease after 1 min and concentration dependence of AUC after 60 min. IC, interaction coefficient.

Concentration Dependence of:	Compound 1 [nmol^{-1}]	Compound 2 [nmol^{-1}]	Calculated Sum [nmol^{-1}]	Value Measured for Sum of Compounds >[nmol^{-1}]	IC [%]
Absorbance decrease 1 min	Ascorbic acid 0.1107 ± 0.0188	Trolox 0.1058 ± 0.0158	0.2165 ± 0.0246	0.0927 ± 0.0157 ***	57.2
AUC 60 min	Ascorbic acid −10.207 ± 0.304	Trolox −9.664 ± 0.265	−19.871 ± 0.812	−7.916 ± 0.458 ***	60.2
Absorbance decrease 1 min	Glutathione 0.0931 ± 0.0265	Ascorbic acid 0.1079 ± 0.0047	0.2010 ± 0.0269	0.1086 ± 0.0017 **	46.0
AUC 60 min	Glutathione −14.172 ± 2.932	Ascorbic acid −10.312 ± 0.344	−24.484 ± 2.952	−16.477 ± 0.917 **	32.7
Absorbance decrease 1 min	Glutathione 0.09376 ± 0.00490	Trolox 0.10623 ± 0.00475	0.1999 ± 0.0068	0.2198 ± 0.0161 ^{NS}	−0.1
AUC 60 min	Glutathione −14.708 ± 1.353	Trolox −9.613 ± 0.530	−24.321 ± 1.453	−24.977 ± 2.336 ^{NS}	−0.3
Absorbance decrease 1 min	Hispidulin 0.22107 ± 0.03192	Trolox 0.10626 ± 0.00201	0.3273 ± 0.0320	0.1728 ± 0.0318 **	47.2
AUC 60 min	Hispidulin −22.804 ± 2.104	Trolox −9.670 ± 0.723	−32.474 ± 2.225	−25.455 ± 2.885 *	21.6
Absorbance decrease 1 min	Hispidulin 0.22764 ± 0.004037	Ascorbic acid 0.10942 ± 0.004084	0.3371 ± 0.0057	0.3287 ± 0.0074 ^{NS}	2.5
AUC 60 min	Hispidulin −16.920 ± 1.372	Ascorbic acid −8.060 ± 0.541	−24.980 ± 1.475	−25.137 ± 2.062 ^{NS}	−0.01

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ^{NS} not significant (measured parameter vs. parameter calculated as a sum of parameters for individual compounds).

These subadditive effects in ABTS$^{\bullet}$ scavenging might be ascribed to the reported interactions between vitamin E and ascorbate, vitamin E and flavonoids [50,51], and ascorbate and glutathione [52,53]. These interactions were postulated to consist in the reduction of tocopheryl radicals by ascorbate or flavonoids, and reduction of ascorbate free radical and ascorbate by glutathione. However, such reactions are unlikely to account for the subadditive effect in ABTS$^{\bullet}$ decolorization, because reduction of a radical by a non-radical molecule leads to formation of a new free radical, leaving the number of radicals in the system unchanged. The apparent mechanism of subadditive interactions between antioxidants in ABTS$^{\bullet}$ scavenging should involve reactions between radicals of antioxidants, formed upon reaction with ABTS$^{\bullet}$, involving or not involving ABTS$^{\bullet}$, i.e., a significant contribution of Reactions (6)–(8) in the set of reactions proceeding in a sample:

$$Ant_1 + ABTS^{\bullet} \rightarrow ABTS + Ant_1^{\bullet} \quad (4)$$

$$Ant_2 + ABTS^{\bullet} \rightarrow ABTS + Ant_2^{\bullet} \quad (5)$$

$$Ant_1^{\bullet} + ABTS^{\bullet} \rightarrow \text{non-radical products} \quad (6)$$

$$Ant_2^{\bullet} + ABTS^{\bullet} \rightarrow \text{non-radical products} \quad (7)$$

$$Ant_1^{\bullet} + Ant_2^{\bullet} \rightarrow \text{non-radical products} \quad (8)$$

The non-radical products formed in this reaction are mostly reduced ABTS and the oxidized form of an antioxidant. However, adducts of Ant$^\bullet$ and ABTS$^\bullet$ can be also formed, and their formation (6) and (7) and further reactions (9) between these adducts and Ant$^\bullet$ [54] may affect the stoichiometry of ABTS$^\bullet$ reduction:

$$\text{Ant-ABTS} + \text{Ant}^\bullet \rightarrow \text{Ant-Ant} + \text{ABTS}^\bullet \quad (9)$$

In some cases, the product(s) of the reaction of an antioxidant with ABTS$^\bullet$ may itself be reactive; such a situation was reported for chrysin [16]. In studies of single compounds such a situation would lead to a higher apparent reactivity of the parent compound, but in a mixture of compounds it can contribute to a synergic effect of antioxidant interaction, e.g., initiating one-electron reactions with another antioxidant, thus increasing the quantity of radicals in the system:

$$\text{Ant}_1 + \text{Ant}_2 \rightarrow \text{Ant}_1^\bullet + \text{Ant}_2^\bullet \quad (10)$$

However, the situation may be more complex as ABTS$^\bullet$ may also undergo self-cleavage and degradation [14,18], so Reactions (4)–(10) do not account for all the reactions occurring in the system. There are further reasons that account for deviations of stoichiometry in the ABTS$^\bullet$ decolorization assay.

Non-additivity effects in the binary mixtures of flavonoids with Trolox and ascorbic acid were revealed by ORAC and square-wave voltammetry methods. In mixtures of *O*-glucosylated flavonoids with Trolox or ascorbic acid, a negative non-additive effect (antagonism) was seen for quercetin and morin, while a synergistic interaction was found for rutin and naringin [48]. Synergistic and antagonistic interactions between components of *Gingko biloba* leaf extracts have been reported using the ABTS$^\bullet$ reduction assay [49]. Thus, it cannot always be expected that ABTS$^\bullet$ reduction by a summed compound will be equal to the sum of ABTS$^\bullet$ reductions by individual compounds, and ABTS$^\bullet$ reduction by a complex extract does not necessarily reflect the sum of the ABTS$^\bullet$-reducing abilities of its components.

4. Conclusions

A modification of the ABTS$^\bullet$ decolorization assay for use with plate readers is presented. The assay is applicable in a broad pH range (2.0–7.4). Increase in ionic strength decreases ABTS$^\bullet$ reactivity. Reactions of "fast" antioxidants with ABTS$^\bullet$ are completed within seconds, while the reactions of "slow" antioxidants are not finished after 6 min (as proposed in the original method); we suggest a reaction time of 60 min for assays including antioxidants, blood plasma and plant extracts. For comparison of antioxidant activities in a kinetic assay, as well as direct decrease of absorbance, concentration dependence of absorbance decrease or of AUC is recommended.

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