

Research Article

Relevance and Standardization of *In Vitro* Antioxidant Assays: ABTS, DPPH, and Folin–Ciocalteu

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Trolox, gallic acid, chlorogenic acid, caffeic acid, catechin, epigallocatechin gallate, and ascorbic acid are antioxidants used as standards for reaction with chromogenic radicals, 2,2-diphenyl-1-picrylhydrazyl (DPPH') and 2,2'-azino-bis-3-ethylbenzotiazolin-6-sulfonic acid (ABTS⁻⁺), and Folin–Ciocalteu (FC) reagent. The number of exchanged electrons has been analyzed as function of method and solvent. A majority of compounds exchange more electrons in FC assay than in ABTS and DPPH assays. In reaction with chromogenic radicals, the largest number of electrons was exchanged in buffer (pH 7.4) and the lowest reactivity was in methanol (DPPH) and water (ABTS). At physiological pH, the number of exchanged electrons of polyphenols exceeded the number of OH groups, pointing to the important contribution of partially oxidized antioxidants, formed in the course of reaction, to the antioxidant potential. For Trolox, small impact on the number of exchanged electrons was observed, confirming that it is more suitable as a standard compound than the other antioxidants.

1. Introduction

Numerous methods can be applied for determining the in vitro antioxidant potential (AOP) of single compounds or their mixtures. Due to their simplicity, the spectrophotometric methods based on reaction of the antioxidants with chromogenic radicals such as 2,2'-azino-bis-3-ethylbenzotiazolin-6-sulfonic acid (ABTS⁺) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) or with phospho-tungsto-molybdate in Folin-Ciocalteu (FC) reagent are widely applied. Recent papers nevertheless point to the major drawbacks of such methods as no relation to the in vivo antioxidant efficacy was observed [1-3]. In AOP determinations, the endpoint measurements in the in vitro assays correspond to the amount of hydrogens/electrons which certain compound or mixture can exchange in the reaction with the oxidant probe, rather than to the actual efficiency in the reaction with radicals, which is crucial for in vivo function of this compound.

All three methods are based on the transfer of an electron from the deprotonated antioxidant to the probe when AOP is determined in protic solvents [4], and the mechanism is described as sequential proton loss electron transfer (SPLET). The fact that the overall reaction rate depends on the proportion of ionized molecules of the antioxidant (typically the phenolate group) [5] implies that, apart from the structure of the antioxidant molecule, the type of solvent and the pH of the assay solution in particular have a large influence on the reactivity of antioxidants [6–10]. Solvent composition influences not only the rate of initial oxidation steps but also the degree of secondary reactions of partially oxidized antioxidants that contribute significantly to the number of exchanged electrons [6, 11, 12].

A serious disadvantage and shortcoming of the above methods is that they are poorly standardized. A survey of the literature reveals that the reactions with a given probe are performed in different solvents and for different periods of time [13]. Additionally, the AOPs of samples are normalized to different standard antioxidants. The AOP determined by the ABTS or the DPPH method is mostly expressed as molar equivalent of (\pm) -6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) [14], but normalization to ascorbic acid (AA) [15], catechin (CTH) [16], chlorogenic

(CGA) [17], caffeic acid (CA) [18], gallic acid (GA) [19], and other antioxidants is not uncommon.

AOP of food samples is usually evaluated by more than one method, and correlation analysis of AOPs obtained by DPPH, ABTS, and FC assays is often performed. The correlations can be high and significant or weak [20-22], reflecting the lack of consistency of the results of such analyses. The large influence of the experimental parameters on the reactivity of antioxidants in the samples undoubtedly contributes to the observed discrepancies. These are potentiated when AOPs of samples with different composition and reactivity of antioxidants in particular assays are compared. The fact that AOP of the samples determined by DPPH, ABTS, and FC assays is rarely normalized to the same model antioxidant contributes additionally to the ambiguity in this area of research. It is almost impossible to compare the AOP of samples determined by different methods on a quantitative basis. To enable a relevant comparison between the results of studies carried out with different methods under various experimental conditions, the uniform reactivity of the compound used as standard is of great importance.

We report here how the method applied for AOP estimation, length of the assay, and the composition of the solvent affects the reactivity of selected antioxidants frequently used as standard compounds. The aim of the study was to find the standard compound with reactivity that is the least affected by experimental conditions and could be therefore applied as a general standard for DPPH, ABTS, and FC assays.

2. Materials and Methods

2.1. Materials. Trolox, caffeic acid, gallic acid, chlorogenic acid, L-ascorbic acid, dehydroascorbic acid (DHA), catechin hydrate, iron(II) sulphate heptahydrate (FeSO₄ × 7H₂O), Folin–Ciocalteu reagent, DPPH, and ABTS were from Sigma-Aldrich (Steinheim, Germany). Acetic acid, sodium hydroxide, sodium carbonate (Na₂CO₃), sodium hydrogen phosphate dihydrate, and methanol were from Merck (Darmstadt, Germany). Manganese(IV) oxide (MnO₂) was from Kemika (Zagreb, Croatia). Epigallocatechin gallate (EGCG) was from DSM Food Specialities BV (Delft, Netherlands). Water was purified using a MilliQ system (resistivity >18 MΩ·cm; Millipore).

Stock solutions (10 mM) of Trolox, CTH, CA, CGA, GA, and EGCG were prepared in MeOH, AA, and FeSO₄ in MilliQ water and DHA in acetate buffer (50 mM, pH 5.0). All further dilutions were made in the solvents used for particular assay.

2.2. The Folin–Ciocalteau Assay. The FC assay was performed according to a modified method of Gutfinger [23]. An appropriate volume of the antioxidant or FeSO₄ solution (50 μ L) was added into a 1.5 mL microcentrifuge tube, mixed with MilliQ water (700 μ L) and FC reagent (125 μ L, previously diluted 1:2 (v/v) with MilliQ water). After 5 min at 25°C, a solution of Na₂CO₃ (125 μ L, 20%, w/w) was added, and the sample was mixed again and incubated for an additional 55 min. The absorbance at 765 nm (A_{765}) was measured with a Varian Cary 100 BIO UV-VIS spectrophotometer in a 1 cm cell. The concentration range of antioxidants and FeSO₄ in the assay solution is given in Table 1. The measurements were made in triplicate, including the preparation of sample solutions and reagents.

2.3. The DPPH and ABTS Assays. The DPPH assay was performed according to a modified method of Brand-Williams et al. [24] and the ABTS assay according to a modified method of Re et al. [25]. The DPPH solution was prepared in MeOH and diluted to the concentration that would give an absorbance of 2.4 at 520 nm in a cuvette with 1 cm path length. ABTS⁺⁺ was produced by reaction of ABTS in aqueous solution with MnO₂ followed by centrifugation and filtration; the solution was then diluted with MilliQ water to the concentration that would give an absorbance of 2.4 in the cuvette with 1 cm path length at 734 nm. All the solutions, buffers, and solvents were incubated at 25°C prior to analysis. The assay solutions were prepared in a 1.5 mL microcentrifuge tube by mixing DPPH⁻ or ABTS⁺⁺ solution $(500 \,\mu\text{L})$ with $450 \,\mu\text{L}$ of the solvent (MilliQ water, MeOH, and acetate buffer (250 mM, pH 5.0) or phosphate buffer (50 mM, pH 7.4) for the DPPH assay, and MilliQ water and acetate buffer (250 mM, pH 5.0), or phosphate buffer (50 mM, pH 7.4) for the ABTS assay). The reactions were started by adding 50 μ L of the antioxidant solution into the assay medium, with thorough mixing. After 60 min incubation at 25°C, the absorbance at 520 nm for DPPH^{\cdot} (A_{520}) and at 734 nm for ABTS⁺⁺ (A_{734}) was measured. The measured value was subtracted from the corresponding value for the control (the selected solvent added into the assay medium instead of the antioxidant solution) and the data expressed as ΔA_{520} or ΔA_{734} . The concentration range of antioxidants in assay solution is given in Table 1. All measurements were carried out in triplicate, including the preparation of sample solutions and reagents.

The reaction kinetics of model antioxidants CGA ($10 \mu M$), CA ($10 \mu M$), Trolox ($10 \mu M$), and GA ($7.0 \mu M$) was analyzed in 1 cm quartz cuvettes with the stopper to prevent evaporation. The solvent compositions were the same as those for endpoint measurements explained above. The A_{520} and A_{734} were continuously measured at 15 s intervals over 180 min. The first time point was measured 15 s after mixing the antioxidant with radical probe. The measured absorbances were subtracted from the corresponding absorbances of the controls at appropriate time points and obtained ΔA_{520} and ΔA_{734} values were normalized to the number of exchanged electrons per molecule as explained in Section 3.1.

2.4. Statistical Analysis. The slope of the calibration curve, obtained by linear regression analysis with program Origin (Microsoft), prepared with each model antioxidant in a particular type of assay and used to calculate the number of exchanged electrons, was the average value obtained from three independent experiments including the preparation of sample solutions and reagents. Relative standard deviation of the average slope was not larger than 5%.

Pearson correlation coefficients (*r*) were calculated with the program Excel (Microsoft).

Sample	$c_{antioxidant}$ range (μ mol/L)									
		DP	PH	$ABTS^+$			FC			
	MeOH	H_2O	pH 5	pH 7.4	H_2O	pH 5	pH 7.4	FC		
CA	8-40	8-40	8-40	3-15	8-40	8-40	8-40	3-15		
CGA	6-30	6-30	6-30	2-10	6-30	6-30	4-20	6-30		
GA	2.6-13	2.6-13	2.6-13	2.6-13	1.6-8.0	1.6-8.0	1.6 - 8.0	6-30		
CTH	1.6-6.4	3-15	3-15	1.6-8	1.6-6.4	1.6-6.4	1.6-6.4	3-15		
EGCG	1.4-7	1.4-7	1.4-7	0.8 - 4	1.4-7	0.8 - 4	0.8 - 4	3-15		
AA	4-40	4-40	4-40	4-40	4-40	4-40	4-40	4-40		
DHA	100-500	100-500	100-500	20-200	100-500	100-500	20-200	10-100		
Trolox	5-35	5-35	5-35	5-35	5-35	5-35	5-35	5-35		
FeSO ₄	—	—	—	—	—	—	_	20-120		

TABLE 1: The range of concentration of antioxidants in assay solution ($c_{antioxidant}$).

Mean absolute errors (MAE) and mean absolute percent errors (MAPE) in the number of exchanged electrons (n) for seven model antioxidants (a) were calculated (Equations (1) and (2)) for each pair of antioxidant assays (X, Y). Data for DHA were not included in the calculation since the low reactivity of DHA in all conditions except in the FC assay would distort MAPE values. The antioxidant assay which gave the lowest *n* value for the particular antioxidant is designated as *Y*:

MAPE =
$$\frac{100}{j} \sum_{a=1}^{j} \left| \frac{nX_a - nY_a}{nY_a} \right|,$$
 (1)

MAE =
$$\frac{1}{j} \sum_{a=1}^{j} |nX_a - nY_a|.$$
 (2)

3. Results and Discussion

3.1. Quantification of Exchanged Electrons in the Reaction of Antioxidants with the Oxidant Probe. The AOP of tested compounds, quantified as the number of exchanged electrons (*n*) for the reaction between antioxidants and DPPH⁻ or ABTS⁻⁺ radical, was estimated as the molar ratio of the quenched radical to the tested antioxidant after 60 min incubation. It was calculated considering molar absorption coefficients (ε) of the radical in the solvent used and the slope of the calibration curve ($k_{antioxidant}$) prepared with model antioxidants (dependence of ΔA_{520} and ΔA_{734} on antioxidant concentration in assay solution ($c_{antioxidant}$)) as shown in Equations (3)–(5). For all antioxidants analyzed, the dependence of ΔA_{520} and ΔA_{734} was linear in the concentration range, as shown in Table 1:

$$\Delta A_{\lambda} = \varepsilon_{\lambda} \times \Delta c_{\text{radical}} \times l, \tag{3}$$

$$\Delta A_{\lambda} = k \times \Delta c_{\text{antioxidant}},\tag{4}$$

$$n = \frac{\Delta c_{\text{radical}}}{\Delta c_{\text{antioxidant}}} = \frac{k}{\varepsilon_{\lambda} \times l},$$
(5)

where c_{radical} represents the concentration of the radical in the assay solution and *l* represents the cuvette path length. In calculations of *n* for the reaction between DPPH and antioxidants in MeOH, water, or buffer (pH 5.0), we used the ε value of 12000 L·mol⁻¹·cm⁻¹ [26]. The corresponding *n* value for reaction with the ABTS⁺ radical in the tested solvents was calculated, taking into account the ε value of (15000 ± 549) L·mol⁻¹·cm⁻¹ [25].

The contribution of the reaction product, DPPH₂, to the A_{520} in MeOH or in the mixture of MeOH and acetate buffer can be neglected, and therefore, a change in absorbance can be attributed solely to the change in concentration of DPPH. At a neutral pH, the absorbance of formed DPPH₂ should however be considered in calculations as observed by other authors [27]. Indeed, when we recorded the absorption spectrum for the DPPH solution with large molar excess of Trolox in the test in phosphate buffer (pH 7.4), we found that the DPPH₂ absorbs at 520 nm and significantly contributes to the measured value. The absorbance of DPPH₂ contributes 41% of the absorbance of a DPPH radical at 520 nm. It is important to emphasize that the actual pH in the mixture of buffer and MeOH can be higher than in the pure aqueous buffer [28]. The number of exchanged electrons was calculated by considering the contribution of DPPH₂ to the measured value of A_{520} and of ε for DPPH⁻ at neutral and basic pH of 10700 L·mol⁻¹·cm⁻¹ [27] and applying Equation (6) instead of Equation (3):

$$\Delta A_{\lambda} = \varepsilon_{\lambda} \times \Delta c_{\text{radical}} \times l \times 0.59.$$
(6)

For the FC assay, the AOP, i.e., the value of *n* in the oxidation of antioxidants with phospho-tungsto-molybdate cannot be calculated from the corresponding molar absorption coefficient, as ε of the products is not known. In order to evaluate the reactivity of the investigated compounds, a calibration curve with FeSO₄ was prepared, in which Fe²⁺ ions in the reaction with FC are oxidized to Fe³⁺ (one electron exchange). It was previously shown that Fe²⁺ reacts in the FC assay [29]:

$$\Delta A_{765} = k_{\mathrm{Fe}^{2+}} \times \Delta c_{\mathrm{Fe}^{2+}},\tag{7}$$

$$\Delta A_{765} = k_{\text{antioxidant}} \times \Delta c_{\text{antioxidant}}, \tag{8}$$

$$n = \frac{\Delta c_{\rm Fe^{2+}}}{\Delta c_{\rm antioxidant}} = \frac{k_{\rm antioxidant}}{k_{\rm Fe^{2+}}}.$$
 (9)

The *n* value in reaction of investigated antioxidants with FC reagent was estimated with Equations (7)–(9) using the slopes of the calibration curves for antioxidants ($k_{antioxidant}$) and for FeSO₄ ($k_{Fe2+} = 0.0034 \pm 3 \times 10^{-5} L \cdot \mu mol^{-1}$).

3.2. Number of Exchanged Electrons Greatly Varies with Solvent and Type of AOP Assay. The AOPs for each of the 8 model antioxidants in 4 variants of DPPH assay, 3 variants of ABTS assay, and 1 variant of the FC assay are shown in Figure 1. Large variations are observed in different types of assay and also within subvariants of ABTS and DPPH assays. In reaction with chromogenic radicals, the largest number of electrons are exchanged in buffer (7.4), while the lowest one was observed in MeOH (DPPH) and in water (ABTS⁺). It is evident that, for the majority of antioxidants, higher *n* values were observed in the FC assay than in ABTS and DPPH assays. The exceptions are phenolic compounds with pyrogallol group, GA and EGCG.

Large variations in the number of exchanged electrons for different antioxidants are to be expected since antioxidants differ in the number of OH groups bound to aromatic rings or to an unsaturated carbon atom. Typical oxidation of polyphenols is best depicted by the oxidation of a diphenolic compound (catechol) into the corresponding quinone (Equation (10)) and of enediol (ascorbic acid) into vicinal diketone (Equation (11)):



Oxidation of one OH group on an aromatic ring/enole therefore involves transfer of one electron on the radical (DPPH and ABTS⁺) or other oxidants (phospho-tungstomolybdate). The results shown in Table 2 nevertheless reveal that *n* per OH can be substantially higher than 1. For GA, the value of n per OH group in the FC test and with chromogenic radicals ranged from 1.7 to 3.5, being highest for ABTS⁺ at pH 7.4. For the compound bearing two pyrogallol groups in its structure, EGCG, *n* per OH group ranged from 1.1 to 2.0, being the highest for DPPH and ABTS at pH 7.4. CA, CGA, and CTH, the phenolic compounds with a catechol group, also exchanged more than 1 electron per OH group under the majority of assay conditions, being the highest for DPPH at pH 7.4 and with the FC assay where, for CGA, *n* per OH group amounted to almost 4. DHA reacts only in FC assay where AA also shows higher AOP, most likely as a result of formation of hydroxy furanones [30]

which are redox active substances [31]. In the FC assay that is performed at basic pH, a sufficient amount of secondary antioxidants, which contribute to AOP, is formed.

All the polyphenolic antioxidants analyzed exchanged more than 1 electron per OH group, which confirmed that secondary reactions under certain conditions contribute even more to AOP than primary oxidations of the phenolics. A relevant question is whether such reactions can be related to the efficiency of the antioxidants in food matrices and, potentially, in vivo where antioxidants are enzymatically and nonenzymatically modified. In the light of the fact that the high rate is crucial for the efficiency of the antioxidant reacting with the radical [2], one could argue that analysis of AOP, which is based on oxidation in the second phase, is irrelevant in this respect. There are serious and justified concerns about the current practice that assays with radicals are allowed to proceed for long reaction periods [9]. However, a slow reaction rate in the second phase does not necessarily mean that compounds that are formed from partially oxidized phenolic compounds react at slow rates per se if we assume that the rate-limiting step is the formation of these compounds. It was previously shown that the product formed from partially oxidized chrysin in reaction with ABTS⁺ reacts faster with the radical than the parent molecule [12]. The lack of relevant information related to this topic means that these secondary reactions have to be considered relevant. Additionally we have shown that, at neutral pH which is encountered in large proportion of the gastrointestinal tract, body fluids, and cellular compartments, reactivity of antioxidants in the second phase is increased (Section 3.3).

Of all the standard antioxidants analyzed, the number of exchanged electrons for Trolox was the least dependent on the type of assay, solvent composition, and pH. The *n* value determined in all conditions, regardless of the method, was in the range of 1.75 to 2.25 (Table 2). For the reaction between DPPH^{\cdot} and Trolox, the highest *n* (1.92) was observed in buffer at pH 7.4, followed by buffer solution at pH 5 and water, with the lowest one in MeOH. The oxidation of Trolox by ABTS⁺⁺ in water resulted in n = 1.79. Comparable values were also obtained for the reaction at pH 5 and at pH 7.4. In the FC assay, Trolox exchanged 2.25 electrons. Thus, the average value of *n* in all tests was 1.88 ± 0.17 and, taking into consideration Trolox purity (97%), it amounted to 1.94, which is comparable with literature values of between 1.9 and 2 [5, 8, 24]. Analysis of oxidation in aqueous and alcoholic solvents revealed that the chromane ring is broken upon oxidation, and Trolox quinone with two C=O groups is formed (Equation (12)), resulting in two electron oxidation [32]:





FIGURE 1: The number of exchanged electrons per molecule of caffeic acid (CA), chlorogenic acid (CGA), gallic acid (GA), catechin (CTH), epigallocatechin gallate (EGCG), ascorbic acid (AA), dehydroascorbic acid (DHA), and Trolox in DPPH, ABTS, and FC assays. Numbers of exchanged electrons were determined after 60 min incubation of antioxidant with the probe at 25°C in the appropriate solvent.

TABLE 2: Number of exchanged electrons (n) per OH group in different antioxidant assays.

	n/OH									
Sample		PH	ABTS			ГC				
	MeOH	H_2O	pH 5	pH 7.4	H_2O	pH 5	pH 7.4	FC		
CA	1.1	1.1	1.1	3.1	1.2	1.1	1.2	2.9		
CGA	1.0	1.1	1.2	3.1	1.0	1.1	1.5	3.8		
GA	1.8	2.1	2.1	1.7	2.1	2.7	3.5	1.9		
CTH	0.8	1.0	1.4	2.1	1.4	1.7	2.0	2.3		
EGCG	1.2	1.3	1.4	2.0	1.1	1.4	1.9	1.5		
AA	0.9	0.9	0.9	1.0	0.9	0.9	0.9	2.1		
Trolox	1.8	1.9	1.9	1.9	1.8	1.8	1.7	2.2		

Trolox is formally a compound with one OH group; however, when hydrolysis of the ether bond in the chromane ring resulting in the formation of 1,4-hydroquinone is taken into the account, it is oxidized as a typical diphenolic compound (Equation (10)) with one electron exchanged per OH group. The extent of additional oxidation reactions is therefore relatively small, as only in the FC assay, there are slightly more than two electrons exchanged.

Large difference in AOP determined at 60 min can be exemplified by mean absolute errors and mean absolute percent errors in the n for model antioxidants for each pair of the antioxidant assays (Supplementary file).

Since there are considerable differences in n values already between variants of a particular assay (Table 2), control of experimental parameters such as pH and solvent composition is therefore particularly important. Often those parameters are not controlled and even not reported in the papers, which complicates the comparison of obtained AOP for similar samples in different studies. Especially DPPH assay is often performed in the absence of buffer, and there is large probability that, only with the added sample, the conditions regarding water content and pH can be drastically changed resulting in changed reactivity [6]. To increase the robustness of the assays with chromogenic radicals, they should be preferentially performed in buffered solvents.

3.3. The Influence of Solvent on the Kinetics of Reaction of Antioxidants with ABTS⁺ and DPPH Radicals. We have analyzed the kinetics of reaction of CGA, CA, GA, and Trolox with chromogenic radicals in solvents giving the lowest (MeOH for DPPH and water for ABTS⁺⁺) and the highest (buffer (pH 7.4) for both) AOP after 60 min. The results shown in Figure 2 reveal that the solvent and type of the assay have large influence on the amplitude and the kinetics of the reaction. Additionally, it is clearly shown that secondary reactions of partially oxidized antioxidants contribute significantly to the number of exchanged electrons already at the minute time scale. Accordingly, it is not possible to discriminate the contribution of primary oxidation of polyphenols to quinones from the contribution of secondary antioxidants to the AOP without stopped-flow machine.

For GA, secondary reactions are quantitatively relevant under all conditions analyzed already at the subminute range. Caffeic acid and its ester (CGA) have similar yet kinetically different profiles. On the contrary to GA, contribution of secondary reactions in DPPH assay at pH 7.4 is



FIGURE 2: The number of exchanged electrons per molecule as a function of incubation time at 25°C for caffeic acid (CA), chlorogenic acid (CGA), gallic acid (GA), and Trolox in DPPH assay in methanol (thin purple) or DPPH assay in the mixture of methanol and buffer (pH 7.4) (thick purple) and ABTS assays in MilliQ water (thin green) or buffer (pH 7.4) (thick green). Black lines correspond to the number of OH groups in the molecule. (a) Caffeic acid (CA), (b) chlorogenic acid (CGA), (c) gallic acid (GA), (d) trolox.

the most pronounced. For ABTS assay of CA at physiological pH, the rate of reaction at prolonged incubation time is increased, which cannot be explained only by the reaction of an antioxidant with the ABTS⁺. It is possible that H_2O_2 that is formed in the course of CA oxidation [33] reoxidizes ABTS into ABTS⁺ and therefore results in overall smaller $d\Delta A/dt$ at shorter incubation times, until it is used up. Trolox quinone (Equation (12)) or its degradation products do not react further with ABTS⁺ or DPPH as approximately two electrons are exchanged per molecule of Trolox over 180 min.

We have observed the sample specific kinetic profiles also for different food matrices [22]. The $d\Delta A/dt$ on the time scale of few tens of minutes when samples as tea, coffee, cranberry, and apple juice were analyzed was even larger than observed for antioxidant compounds in this study. As routine AOP measurements are often performed without strict temperature and time control, more reproducible results can be obtained, if longer incubation times (60 min) are applied when $d\Delta A/dt$ is smaller. Still, we have to be aware that AOP values are not the measure of the antioxidant properties of the molecules (in the kinetic term) but rather reflect the capacity of molecules to exchange certain amount of electrons in the reaction with oxidants under chosen conditions.

3.4. Correlation Analysis of AOP of Model Antioxidants Determined by DPPH, ABTS, and FC Assays. The results of correlation analysis between the n values for the eight model compounds obtained by different assays are shown in Table 3. All correlation coefficients are significant at $\alpha = 0.05$. The highest correlations are observed between different variants within DPPH or ABTS assays. The notable exception is DPPH assay at pH 7.4 with weaker correlations. When variants of DPPH and ABTS assays are compared to each other the Pearson correlation coefficients are lower than within each type of assay but are still significant at α = 0.001. When the results of FC assay are correlated to AOP obtained by DPPH and ABTS assays weaker correlations, most of them significant at $\alpha = 0.05$, are observed. The exception is again DPPH assay at pH 7.4 which shows the highest correlation with FC assay ($\alpha = 0.001$).

In general, the correlation between determined AOP of investigated antioxidants (Table 4) which are structurally different compounds such as hydroxycinnamic acids, trihydroxybenzoic acid, flavanols and their derivatives, vitamers of vitamin C, and synthetic chromanol, is much poorer than correlation between the types of assays. For GA, even negative correlation coefficients are observed with practically all other antioxidants. A weak positive correlation between GA and its derivative, EGCG, could be attributed to

			DP	ABTS ⁺⁺				
		MeOH	H_2O	pH 5	pH 7.4	H_2O	pH 5	pH 7.4
	H ₂ O	0.997***						
DPPH'	pH 5	0.982***	0.993***					
	pH 7.4	0.877**	0.887**	0.909***				
	H ₂ O	0.950***	0.968***	0.985***	0.872**			
ABTS ⁺⁺	pH 5	0.954***	0.972***	0.985***	0.853**	0.997***		
	pH 7.4	0.964***	0.979***	0.987***	0.858*	0.990***	0.997***	
FC	-	0.796*	0.816*	0.856**	0.958***	0.847**	0.821*	0.820^{*}

TABLE 3: Values of the Pearson correlation coefficient for correlation between AOPs determined in different antioxidant assays.

***Values are significant at the α =0.001 level; **values are significant at the α =0.01; *values are significant at the α =0.05 level.

TABLE 4: The values of Pearson correlation coefficient for correlation among AOPs of investigated antioxidants determined in all variants of ABTS, DPPH, and FC assays.

Sample	CA	CGA	GA	CTH	EGCG	AA	DHA
CGA	0.96***						
GA	-0.43	-0.32					
CTH	0.72^{*}	0.77^{*}	0.26				
EGCG	0.56	0.55	0.35	0.74^{*}			
AA	0.67	0.81^{*}	-0.31	0.55	0.07		
DHA	0.79*	0.90**	-0.37	0.61	0.18	0.98***	
Trolox	0.76*	0.83*	-0.51	0.52	0.15	0.90**	0.91**

*** Values are significant at the α =0.001 level; ** values are significant at the α =0.01; *values are significant at the α =0.05 level.

the similar chemical structure. In the same way, statistically significant correlation of EGCG is found with CTH that is another structural element of EGCG. The important role of compound's structure on correlation strength is particularly confirmed in the case of AA and its oxidized form, DHA. Similar considerations can be applied to the correlation between the CA and its ester, CGA, both significant at α = 0.001. Lower *r* values obtained for correlation among AOPs of investigated compounds (Table 4) than for correlation among AOPs determined in different tests and different solvents (Table 3) indicate that, despite similar influence of solvents, the AOP still greatly depends on the structure of the compounds.

4. Conclusions

Regardless of persistent critiques of the *in vitro* antioxidant assays and lack of correlations that would exist with *in vivo* antioxidant properties, this research area is lively as ever. In the year 2017, 0.17 % of all manuscripts published in the SCI journal (based on Web of Science database) contained DPPH, ABTS, or Folin in the abstracts. However, in the large proportion of those manuscripts, the methodology is poorly described and comparison with work of others is practically impossible. Due to inconsistency of published results, the adequacy of *in vitro* antioxidant assays is becoming questionable [34].

The measured AOP should in principle give the estimation of the amount of the compounds that can be oxidized under conditions of the assays. The number of exchanged electrons in the reactions with chromogenic radicals is dependent on solvent composition, pH of reaction media, length of assay, and chemical structure of the antioxidant. Secondary reactions of partially oxidized antioxidants contribute significantly to the number of exchanged electrons. We have found that the only exception is Trolox, compound with uniform number of electrons exchanged in applied assays, which is therefore the most suitable compound as standard for AOP determination of single compounds or their mixtures in ABTS, DPPH, and Folin–Ciocalteu assays. For practically all antioxidants, with the exception of Trolox, the number of exchanged electrons under the most favorable conditions, typically the FC assay, is more than two-fold higher than under the least favorable conditions in the majority of cases, the DPPH assay in MeOH.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Mean absolute errors and mean absolute percentage errors in the number of exchanged electrons of model antioxidants in different antioxidant assays. (*Supplementary Materials*)

References

- R. G. Berger, S. Lunkenbein, A. Ströhle, and A. Hahn, "Antioxidants in food: mere myth or magic medicine?," *Critical Reviews in Food Science and Nutrition*, vol. 52, no. 2, pp. 162–171, 2012.
- [2] M. C. Foti, "Use and abuse of the DPPH radical," Journal of Agricultural and Food Chemistry, vol. 63, no. 40, pp. 8765– 8776, 2015.
- [3] R. Apak, M. Mustafa Özyürek, K. Kubilay Güçlü, and E. Esra Çapanoğlu, "Antioxidant activity/capacity measurement. 1.

Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays," *Journal of Agricultural and Food Chemistry*, vol. 64, no. 5, pp. 997–1027, 2016.

- [4] D. Huang, B. Ou, and R. L. Prior, "The chemistry behind antioxidant capacity assays," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 6, pp. 1841–1856, 2005.
- [5] M. Musialik and G. Litwinienko, "Scavenging of DPPH" radicals by vitamin E is accelerated by its partial ionization: the role of sequential proton loss electron transfer," *Organic Letters*, vol. 7, no. 22, pp. 4951–4954, 2005.
- [6] L. Bertalanič, T. Košmerl, N. Poklar Ulrih, and B. Cigić, "Influence of solvent composition on antioxidant potential of model polyphenols and red wines determined with 2,2diphenyl-1-picrylhydrazyl," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 50, pp. 12282–12288, 2012.
- [7] A. L. Dawidowicz, D. Wianowska, and M. Olszowy, "On practical problems in estimation of antioxidant activity of compounds by DPPH[•] method (Problems in estimation of antioxidant activity)," *Food Chemistry*, vol. 131, no. 3, pp. 1037–1043, 2012.
- [8] T. Prevc, N. Šegatin, N. Poklar Ulrih, and B. Cigić, "DPPH assay of vegetable oils and model antioxidants in protic and aprotic solvents," *Talanta*, vol. 109, pp. 13–19, 2013.
- [9] J. Xie and K. M. Schaich, "Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity," *Journal of Agricultural and Food Chemistry*, vol. 62, no. 19, pp. 4251–4260, 2014.
- [10] H. Abramovič, T. Košmerl, N. Poklar Ulrih, and B. Cigić, "Contribution of SO₂ to antioxidant potential of white wine," *Food Chemistry*, vol. 174, pp. 147–153, 2015.
- [11] P. Goupy, C. Dufour, M. Loonis, and O. Dangles, "Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 3, pp. 615–622, 2003.
- [12] M. J. Arts, G. R. Haenen, H. P. Voss, and A. Bast, "Antioxidant capacity of reaction products limits the applicability of the trolox equivalent antioxidant capacity (TEAC) assay," *Food and Chemistry Toxicology*, vol. 42, no. 1, pp. 45–49, 2004.
- [13] L. M. Magalhães, M. A. Segundo, S. Reis, and J. L. F. C. Lima, "Methodological aspects about in vitro evaluation of antioxidant properties," *Analytica Chimica Acta*, vol. 613, no. 1, pp. 1–19, 2008.
- [14] K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, and D. H. Byrne, "Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts," *Journal of Food Composition and Analysis*, vol. 19, no. 6-7, pp. 669–675, 2006.
- [15] A. K. C. Saw, W. S. Yam, K. C. Wong, and C. S. Lai, "A comparative study of the volatile constituents of southeast asian *Coffea arabica*, *Coffea liberica* and *Coffea robusta* green beans and their antioxidant activities," *Journal of Essential Oil Bearing Plants*, vol. 18, no. 1, pp. 64–73, 2015.
- [16] J. A. Vinson, M. Mandarano, M. Hirst, J. R. Trevithick, and P. Bose, "Phenol antioxidant quantity and quality in foods: beers and the effect of two types of beer on an animal model of atherosclerosis," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 18, pp. 5528–5533, 2003.
- [17] C. Delgado-Andrade, J. A. Rufian-Henares, and F. J. Morales, "Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 20, pp. 7832–7836, 2005.

- [18] G. Spigno, L. Tramelli, and D. M. De Faveri, "Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics," *Journal of Food Engineering*, vol. 81, no. 1, pp. 200–208, 2007.
- [19] F. Shahdadi, H. O. Mirzaei, and A. Daraei Garmakhany, "Study of phenolic compound and antioxidant activity of date fruit as a function of ripening stages and drying process," *Journal of Food Science and Technology*, vol. 52, no. 3, pp. 1814–1819, 2015.
- [20] L. Fu, B. T. Xu, X. R. Xu et al., "Antioxidant capacities and total phenolic contents of 62 fruits," *Food Chemistry*, vol. 129, no. 2, pp. 345–350, 2011.
- [21] A. Floegel, D. O. Kim, S. J. Chung, S. I. Koo, and O. K. Chun, "Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods," *Journal of Food Composition and Analysis*, vol. 24, no. 7, pp. 1043–1048, 2011.
- [22] H. Abramovič, B. Grobin, N. Poklar Ulrih, and B. Cigić, "The methodology applied in DPPH, ABTS and Folin-Ciocalteau assays has a large influence on the determined antioxidant potential," *Acta Chimica Slovenica*, vol. 64, no. 2, pp. 491–499, 2017.
- [23] T. Gutfinger, "Polyphenols in olive oils," Journal of the American Oil Chemists Society, vol. 58, no. 11, pp. 966–968, 1981.
- [24] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of free radical method to evaluate antioxidant activity," *LWT-Food Science and Technology*, vol. 28, no. 1, pp. 25–30, 1995.
- [25] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radical Biology and Medicine*, vol. 26, no. 9–10, pp. 1231–1237, 1999.
- [26] P. Molyneux, "The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity," *Songklanakarin Journal of Science and Technology*, vol. 26, no. 2, pp. 211–219, 2004.
- [27] O. Friaa and D. Brault, "Kinetics of the reaction between the antioxidant Trolox[®] and the free radical DPPH⁻ in semiaqueous solution," *Organic and Biomolecular Chemistry*, vol. 4, no. 12, pp. 2417–2423, 2006.
- [28] P. R. Mussini, T. Mussini, and S. Rondinini, "Reference value standards and primary standards for pH measurements in D₂O and aqueous organic solvent mixtures: new accessions and assessments (Technical Report)," *Pure and Applied Chemistry*, vol. 69, no. 5, pp. 1007–1014, 1997.
- [29] J. D. Everette, Q. M. Bryant, A. M. Green, Y. A. Abbey, G. W. Wangila, and R. B. Walker, "Thorough study of reactivity of various compound classes toward the Folin–Ciocalteu reagent," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 14, pp. 8139–8144, 2010.
- [30] J. P. Yuan and F. Chen, "Degradation of ascorbic acid in aqueous solution," *Journal of Agricultural and Food Chemistry*, vol. 46, no. 12, pp. 5078–5082, 1998.
- [31] I. Zabetakis, J. W. Gramshaw, and D. S. Robinson, "2,5-Dimethyl-4-hydroxy-2H-furan-3-one and its derivatives: analysis, synthesis and biosynthesis – a review," *Food Chemistry*, vol. 65, no. 2, pp. 139–151, 1999.
- [32] E. N. Delicado, A. S. Ferrer, and F. G. Carmona, "A kinetic study of the one-electron oxidation of Trolox C by the hydroperoxidase activity of lipoxygenase," *Biochimica et Biophysica Acta*, vol. 1335, no. 1-2, pp. 127–134, 1997.
- [33] A. F. Hanham, B. P. Dunn, and H. F. Stich, "Clastogenic activity of caffeic acid and its relationship to hydrogen

peroxide generated during autooxidation," *Mutation Research*, vol. 116, no. 3-4, pp. 333–339, 1983.[34] D. Granato, F. Shahidi, R. Wrolstad et al., "Antioxidant ac-

[34] D. Granato, F. Shahidi, R. Wrolstad et al., "Antioxidant activity, total phenolics and flavonoids contents: should we ban in vitro screening methods?," *Food Chemistry*, vol. 264, pp. 471–475, 2018.





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