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# Rapid assessment of endpoint antioxidant capacity of red wines through microchemical methods using a kinetic matching approach

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ABSTRACT

Antioxidant capacity of food samples is usually assessed by different analytical methods, however the results attained even for the same method are strongly dependent on the selected reaction time and also on the standard compound used. To tackle this problem, we propose here a kinetic matching approach, associated to the conversion of results into equivalents of a common standard compound, as a universal way for expression of results. The methodology proposed was applied to methods based on different chemistries (Folin–Ciocalteu (F–C), CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays) and red wines ($n=40$) were chosen as a model of complex food sample. For implementation of the kinetic matching approach, the standard phenolic mixture (caffeic acid, (+)-catechin, hesperetin, morin and (−)-epigallocatechin gallate) was chosen for calibration in F–C, CUPRAC and DPPH$^{\bullet}$ assays, while tannic acid was suitable for ABTS$^{\bullet+}$ assay. Results showed that, for all methods, there was no statistical difference between results attained by the kinetic matching approach (after $<10$ min of reaction) and that at endpoint conditions (after 60 to 300 min). The repeatability and the reproducibility of the kinetic matching approach was $<4.5\%$, for all antioxidant assays. The sample throughput increases from $<18$ (endpoint measurements) to $>108\ h^{-1}$ using the proposed kinetic approach. Moreover, we have established here a way of converting results to equivalents of a common standard, providing values independent of its kinetic profile, by using the ratio between calibration sensitivities performed at endpoint conditions.



## 1. Introduction

The antioxidant capacity of food products has been assessed by different in vitro assays as no single method can accurately reflect the multiple mechanisms of antioxidant action present in complex food matrices [1–3]. These analytical methods fall into two major groups: assays based on hydrogen atom transfer and assays based on transference of electrons from antioxidant compounds to the oxidizing species [4,5].

Among the assays based on electron transfer, the Folin–Ciocalteu (F–C) and the cupric ion reducing antioxidant capacity (CUPRAC) assays as well as the radical scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS$^{\bullet+}$) are widely used in food and nutraceutical industries as first line of antioxidant measurements because they are relatively simple and feasible to conduct, relying on spectrophotometric detection, and are also cost-effective [4,5]. In these assays, the antioxidant capacity of food samples is expressed as equivalents of a given standard compound, usually gallic acid for F–C assay and Trolox (a soluble analog of vitamin E) for the other three mentioned methods. Using these standard compounds, the antioxidant capacity values determined depend on the selected reaction time because the reaction kinetics presented by these standards is frequently different from that exhibited by food matrices [4,6]. Thus, in order to attain reliable results for the total antioxidant capacity, absorbance measurements should be taken at endpoint conditions concerning the redox reaction established between sample components and oxidizing species [6,7]. The times required for reaching endpoint conditions for F–C and CUPRAC assays were established at 120 and 30 min, respectively [5], whilst for the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, reaction times over 120 min have been used [8,9]. Despite of the enormous influence of the reaction time on the antioxidant values attained [7,8], this issue has not received much attention in antioxidant related literature, since most of the assays described apply short reaction times, most of them distant from the endpoint conditions, providing underestimated values which do not correspond to the total antioxidant capacity of samples [5]. This is one of the reasons for the large diversity of results published for similar samples and consequently limits the comparison of data between works.

Another issue that hinders the homogenization of antioxidant protocols is the large diversity of compounds applied as standards [2,5]. Actually, it is difficult to interpret the overall antioxidant

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capacity of a given sample when the results obtained by several assays are expressed as equivalents of different compounds. Therefore, the choice of the standard compound should be regarded as a "critical control point" in the antioxidant capacity assessment and this issue has been addressed by recommending the use of a common standard for expression of antioxidant capacity values among different laboratories [5,10]. A standard phenolic mixture (SPM) containing multiple phytochemicals has been recently proposed for the development of a robust validation of antioxidant assays [11]. This mixture is formed by five compounds, namely caffeic acid (phenolic acid), morin (flavone), hesperetin (flavanone), (+)-catechin and (−)-epigallocatechin gallate (EGCG) (flavan-3-ol). Despite of the facts that HPLC analysis confirmed that there was no interaction between these compounds when mixed and that the mixture was stable for a time interval of three months, the reactivity towards electron transfer antioxidant assays and its usefulness as standard material have not been evaluated yet.

In this context, the main objective of this work is to establish rapid protocols for total antioxidant capacity assessment that provide "rugde" values, that are not biased by reaction time or standard applied. For this, two complementary strategies were envisioned. First, a kinetic matching approach is proposed, where the oxidation kinetic behavior of standard compounds was compared to that attained for red wines, selected here as a model of complex food matrix rich in different phenolic compounds. This comparison involves the calculation of antioxidant capacity values and, when a kinetic matching standard is found, these values are constant along reaction time. The second part of this strategy consists on converting the calculated antioxidant capacity value (expressed as equivalents of the kinetic matching standard) into an antioxidant capacity value expressed as equivalents of more commonly applied standards, namely Trolox (TE) or gallic acid (GAE), by taking into account the number of electrons transferred by each compound. To meet these aims, all antioxidant assays were performed in a microchemical format (96-well plate) to easily follow the redox reaction with high reading outputs and reduced errors on analysis time.

## 2. Materials and methods

### 2.1. Reagents and solutions

All chemicals used were of analytical reagent grade with no further purification. Ascorbic acid, caffeic acid, (+)-catechin hydrate, catechol, copper(II) chloride dihydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and rutin hydrate were purchased from Aldrich (Milwaukee, WI). (−)-Epigallocatechin gallate (EGCG), Folin–Ciocalteu reagent, morin hydrate and quercetin dihydrate were obtained from Sigma (St. Louis, MO), while 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), gallic acid, hesperetin, neocuproine hydrochloride monohydrate and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were obtained from Fluka (Buchs, Switzerland). Pyrogallol and tannic acid were obtained from Riedel-de-Haen (Seelze, Germany). Water from Milli-Q system (resistivity > 18 MΩ cm) and absolute ethanol pro analysis were used for the preparation of all solutions.

The stock solutions of ascorbic acid (2.0 mM), gallic acid (1.0 mM) and tannic acid (0.20 mM) were prepared by dissolving each compound in water. Stock solutions of Trolox (1.0 mM), caffeic acid (1.0 mM), catechol (2.0 mM), pyrogallol (2.0 mM) and (+)-catechin (2.0 mM) were prepared in ethanolic solution 10% (*v*/*v*). Stock solutions of quercetin (0.50 mM), rutin (0.50 mM), hesperetin (1.0 mM), morin (1.0 mM), EGCG (1.0 mM) and standard phenolic mixture (1.0 mM) were prepared in ethanolic solution 50% (*v*/*v*). Working standard solutions for F–C, CUPRAC and ABTS$^{\bullet+}$ assays were prepared by rigorous dilution of the respective stock solutions in water. For DPPH$^{\bullet}$ assay, all standard solutions were prepared and diluted in ethanolic solution 50% (*v*/*v*).

The standard phenolic mixture (SPM) proposed as potential standard material for assessment of antioxidant capacity was prepared as previously described [11]. Hence, 100.0 ± 0.1 mg each of caffeic acid, (+)-catechin hydrate, hesperetin and morin hydrate and 12.5 ± 0.1 mg of EGCG were weighed and the mixture was thoroughly mixed with a mortar and pestle. The SPM was kept in an amber glass vial (7 mL), flushed with nitrogen, and stored at 4 °C until analysis.

For assessment of F–C reducing capacity, commercial F–C reagent was diluted 3:10 (*v*/*v*) in water and $Na_2CO_3 \cdot 10H_2O$ 24.3% (*w*/*v*), corresponding to 9% (*w*/*v*) of sodium carbonate, was also prepared. Copper(II) solution (10 mM), neocuproine solution (7.5 mM) and ammonium acetate buffer 1.0 M (pH 7.0) were prepared for the CUPRAC assay.

For the ABTS$^{\bullet+}$ assay, the radical cation solution was prepared by mixing equal volumes of an ABTS stock solution (7 mM in water) with 2.45 mM of potassium persulfate [6]. This mixture was allowed to stand for 12–16 h at room temperature in the dark. On the day of analysis, five different concentrations of the ABTS$^{\bullet+}$ solution (between 30 and 250 μM) were prepared in acetate buffer (pH 4.6, 50 mM) and a linear relationship between the radical concentration and absorbance at 734 nm was established. Then, an ABTS$^{\bullet+}$ solution in acetate buffer that provided an absorbance value of 0.800 ± 0.020 after dilution in the microplate well was prepared. For the DPPH$^{\bullet}$ assay, a stock solution of DPPH$^{\bullet}$ in ethanol (600 μM) was prepared and kept in dark at room temperature. On the day of analysis, five different concentrations of DPPH$^{\bullet}$ (between 25 and 250 μM) were prepared in ethanolic solution 50% (*v*/*v*) in order to determine the dilution of the DPPH$^{\bullet}$ stock solution necessary to obtain a DPPH$^{\bullet}$ concentration that provided an absorbance value of 0.800 ± 0.020 at 517 nm, after dilution in the microplate well.

Red wines ($n$=40) from different origins and vintages covering several viticulture areas of Portugal were purchased at local supermarkets. The information about the origin, year and alcohol content of wine samples are presented in Table S1 (supplementary data). Samples were opened and analyzed in the same day. Red wines were diluted between 200 and 1250 times with ethanolic solution 50% (*v*/*v*) for DPPH$^{\bullet}$ assay or with water for the other three assays. The intrinsic absorption of diluted red wine samples did not contribute significantly to the measurements, since the absorbance values attained (< 0.006) represent < 5% of the analytical signal of samples for FC and CUPRAC assays and < 1% of initial absorbance of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals in the absence of antioxidant species.

### 2.2. Equipment

All antioxidant assays were performed in a microplate format (Synergy HT, Bio-Tek Instruments) using spectrophotometric detection. The microplate reader was controlled by Gen5 software (Bio-Tek Instruments). For DPPH$^{\bullet}$ assay, 96-well flat-bottom UV-transparent microplates (BD Falcon$^{TM}$, Ref. 353261,well volume 370 μL) were used, while for the other three assays flat-bottom microplates (Orange Scientific, Ref. 5530100,well volume 340 μL) were applied. The absorbance measurements were performed at room temperature at every minute beginning after the first minute of reaction.

### 2.3. Microplate protocol for F–C assay

The 96-well microplate Folin–Ciocalteu procedure using carbonate buffer as alkaline reagent was applied with some modifications

[12]. Hence, 150 μL of standard solutions or diluted red wine samples (between 1:800 and 1:200) and 50 μL of F–C reagent (3:10, *v*/*v*) were placed in each well. After that, 100 μL of carbonate buffer solution (9% (*w*/*v*)) was added. The concentrations present in reaction media were 1:20 (*v*/*v*) and 3% (*w*/*v*) of F–C reagent and carbonate buffer, respectively. The reduction at alkaline pH of phosphotungstate–phosphomolybdate heteropoly acid salts by antioxidant compounds was monitored at 760 nm every minute during 120 min. The intrinsic absorption of samples was evaluated by the addition of 50 μL of HCl (0.6 M) instead of F–C reagent, while the reagent blank was performed by the addition of 150 μL of water instead of standard compound or sample.

### 2.4. Microplate protocol for CUPRAC assay

The CUPRAC methodology described by Apak et al. [13] and further adapted by our group to a 96-well microplate format was used [14]. Hence, 50 μL of each solution were placed in each well in the following order: copper(II) solution (10 mM), neocuproine solution (7.5 mM) and ammonium acetate buffer solution (1.0 M, pH 7). After that, 100 μL of antioxidant standard solution or diluted red wine sample (between 1:1000 and 1:400) was added. The reduction kinetics of Cu(II)-neocuproine complexes to orange Cu(I)-neocuproine complexes by antioxidant compounds was monitored at 450 nm every minute during 60 min. The intrinsic absorption of red wines was evaluated by the addition of 100 μL of water instead of copper(II) and neocuproine solutions, while the reagent blank was determined by replacing the antioxidant solution by 100 μL of water.

### 2.5. Microplate protocol for DPPH$^{\bullet}$ assay

The microplate DPPH$^{\bullet}$ method previously described [15] was applied with some modifications. Thus, 150 μL of standard solutions or diluted red wine samples (between 1:1000 and 1:400) and 150 μL of DPPH$^{\bullet}$ ethanolic solution (50% *v*/*v*) were placed in each well. The DPPH$^{\bullet}$ scavenging activity of standards and samples was monitored at 517 nm every minute during 120 min. To evaluate the stability of the radical upon reaction time, the absorbance of DPPH$^{\bullet}$ in the absence of antioxidant species (control) was monitored after the addition of 150 μL of ethanolic solution 50% (*v*/*v*) instead of standard solutions. To evaluate the intrinsic absorption of red wines, 150 μL of ethanolic solution 50% (*v*/*v*) was added to 150 μL of sample. Noteworthy, the absorbance decrease of DPPH$^{\bullet}$ in the absence of antioxidant species upon reaction time was $<5\%$ after 120 min when the UV-transparent microplates (BD Falcon™, Ref. 353261)were used, whilst for the microplates used for visible region (Orange Scientific, Ref. 5530100)an absorbance decrease about 40% was obtained. This lower stability of the radical in the second type of microplate is due to the incompatibility of microplate material with high content of ethanol, because radical consumption was also observed for ABTS$^{\bullet+}$ when it was dissolved in ethanolic solution 50% (*v*/*v*) and it did not occur in aqueous medium. As stable reactants are required to measure the scavenging reaction for a time period long enough to reach endpoint conditions, the use of the former microplates for ethanolic media is recommended.

### 2.6. Microplate protocol for ABTS$^{\bullet+}$ assay

The modified ABTS$^{\bullet+}$ method [6] performed at pH 4.6 with sodium acetate buffer was adapted to microplate format. Hence, 150 μL of standard solutions or diluted red wine samples (between 1:1250 and 1:600) were placed in each well. Then, 150 μL of ABTS$^{\bullet+}$ solution in acetate buffer (50 mM, pH 4.6) was added and the reduction kinetics of colored ABTS$^{\bullet+}$ was monitored at 734 nm every minute during 300 min. To evaluate the absorbance of ABTS$^{\bullet+}$ in the absence of antioxidant species (control), 150 μL of water was added in place of antioxidant standards or samples. To evaluate the intrinsic absorption of red wines, 150 μL of acetate buffer was added to 150 μL of sample. The ABTS$^{\bullet+}$ assay was performed at pH 4.6 because the absorbance decrease obtained for the control after 60 min was $<2\%$, instead of the 10% decrease obtained at pH 7.4. The higher stability of ABTS$^{\bullet+}$ at acidic pH has also been previously described by other researchers [6,16]. This issue is important because it is necessary to guarantee the stability of the radical during the long-term monitoring of redox reactions required to reach endpoint conditions. Moreover, the selected low pH also provides conditions similar to those found in red wines.

### 2.7. Assessment of antioxidant capacity (AC) values and statistical analysis

The absorbance values at every minute during 120 and 60 min were measured for F–C and CUPRAC assays, respectively. For DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, the absorbance decrease was calculated by the difference between the absorbance of the radical in the absence and in the presence of antioxidants, for every minute of reaction, throughout 120 and 300 min, respectively. The absorbance and the absorbance decrease values obtained for diluted red wine samples were interpolated in the calibration curves of tested compounds assessed at the same reaction time and the antioxidant capacity (AC) values were expressed as equivalents (mM) of a given compound (kinetic matching approach).

For each tested compound, the calibration curves were determined with five different concentrations measured in quadruplicate and in three different days. The sensitivity for F–C and CUPRAC assays was determined by the slope of the calibration curve relating the absorbance and the concentration of compound, while for the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays the absorbance decrease was used.

For comparison purposes between the kinetic and the endpoint approaches, the antioxidant capacity of red wines expressed as equivalents of a given compound at a given reaction time ($AC_{(x\ min)}$) were converted to Trolox equivalents ($TE_{(x\ min)}$), taking into account the number of electrons transferred by the tested compound and by Trolox at endpoint conditions (reflected at the slope of calibration curves). For this, the following equation was applied:

$$TE_{(x\ min)} = AC_{(x\ min)} \times (\text{slope tested compound}_{(endpoint)} / \text{slope Trolox}_{(endpoint)}) \quad (1)$$

for instance, the tannic acid equivalents at 5 min for ABTS$^{\bullet+}$ assay were determined by the interpolation of absorbance decrease obtained for red wine samples in the calibration curve of this compound assessed at the same time span ($AC_{(5\ min)}$). Then, this value was converted to Trolox equivalents ($TE_{(x\ min)}$) by multiplying it by the ratio of sensitivities obtained for tannic acid and for Trolox at endpoint conditions (Eq. (1)). For F–C assay, gallic acid equivalents ($GAE_{(x\ min)}$) were determined by application of the same equation, using the slope of gallic acid at endpoint conditions (instead of the value for Trolox).

Moreover, the theoretical sensitivity achievable by the antioxidant mixture SPM, formed by caffeic acid, (+)-catechin, hesperetin, morin and EGCG, was determined by the weighted sum of sensitivities of individual constituents (Eq. (2)). For this, the sensitivity attained for each compound when analyzed separately was multiplied by the

molar fraction.

$$\text{Sensitivity of SPM} = \sum_i (\text{Slope compound}_i \times \chi_i) \quad (2)$$

Linear regression analysis and paired $t$ test were applied to compare the results of the endpoint procedure with those obtained by the proposed kinetic matching approach, after conversion to GAE (F–C assay) and to TE (other assays).

## 3. Results and discussion

The main shortcoming of electron transfer antioxidant assays as F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ is the extended reaction time required to provide the total antioxidant capacity of food samples. This issue arises because the kinetic behavior of the standard usually applied (Trolox) is different from that presented by food products. Fig. 1A describes the absorbance values obtained for different concentrations of Trolox and for a food sample (red wine). The slope of calibration curve of Trolox is similar throughout the reaction monitoring because this compound reacts fast with oxidizing species (ABTS$^{\bullet+}$, in this case). When the absorbance values determined for a sample after 5, 60 and 300 min are interpolated in Trolox calibration curves determined at the same reaction time, the antioxidant capacity values increase as the reaction time increases. For this reason, the oxidation kinetic of samples must be first examined and the measurements should be taken at a time when the reaction has reached the endpoint conditions (constant absorbance values) in order to provide the total antioxidant capacity of the sample [7]. For almost all food samples, this approach is time-consuming and is not suitable for routine analysis.

In this context, we have reasoned that a compound or mixture of compounds that have an oxidation kinetic profile similar to the sample will foster similar antioxidant capacity values, independently of the reaction time selected. As depicted in Fig. 1B, by selecting a compound (tannic acid, in this case) with a kinetic profile similar to the sample, the antioxidant values determined after 5, 60 and 300 min are similar, without achieving endpoint conditions and consequently reducing the time taken by each analysis. To prove the feasibility of this approach, the reaction kinetics for several compounds in F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays was evaluated and later compared to the kinetic profile shown by red wines. The compounds chosen were the standards conventionally used (Trolox and ascorbic acid) and phenolics pertaining to different chemical families namely gallic acid (hydroxybenzoic acid), caffeic acid (hydroxycinnamic acid), tannic acid (tannin), catechol (benzenediol), pyrogallol (benzenetriol), quercetin and rutin (flavonols), morin (flavone), hesperetin (flavanone), (+)-catechin and EGCG (flavan-3-ols). The standard phenolic mixture (SPM) formed by a mixture of caffeic acid, (+)-catechin, morin, hesperetin and EGCG, was also studied. The composition of this mixture was tailored based on the solubility, stability, cost and occurrence of these phenolics in

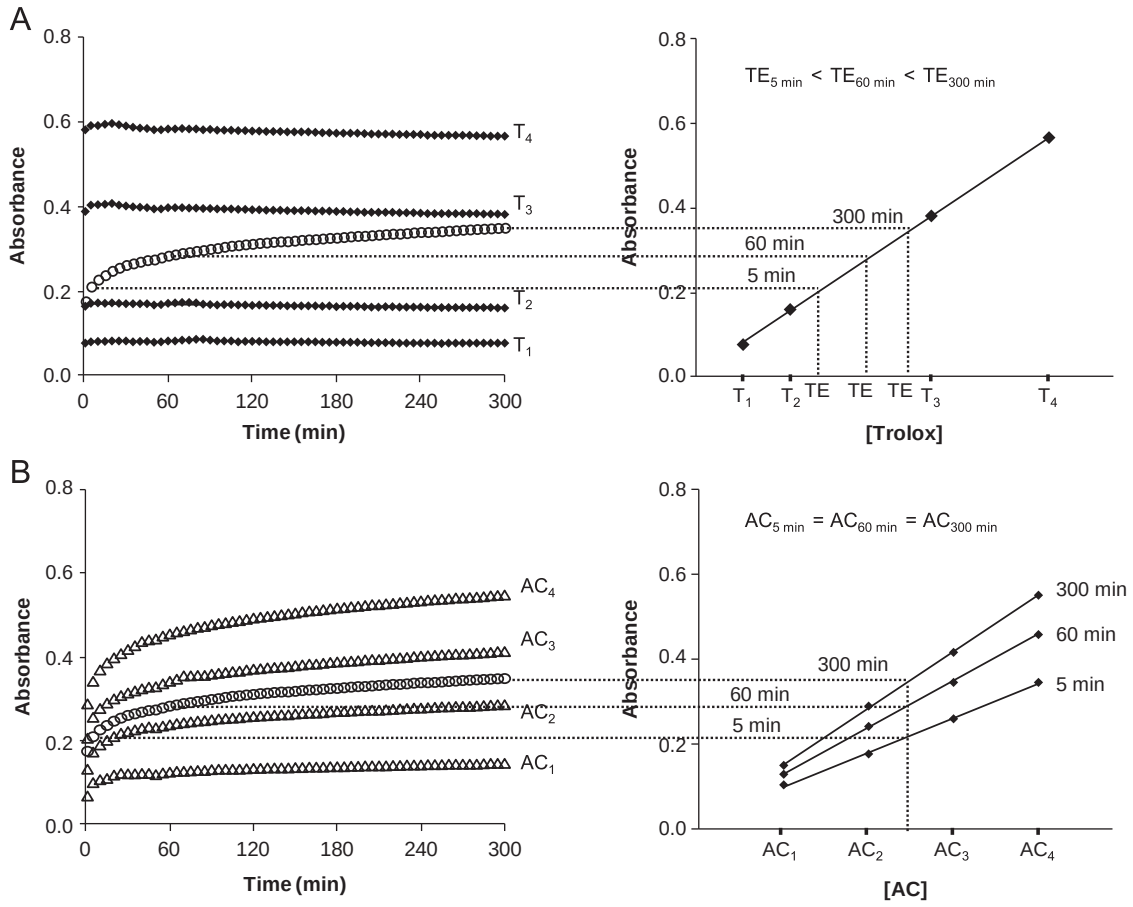


**Fig. 1.** Illustration of antioxidant capacity dependency on reaction time. **(A)** Experiment where the antioxidant capacity depends on the selected reaction time. Curves $T_1$ to $T_4$ represent the absorbance values obtained for increasing concentrations of Trolox (conventional standard); TE, Trolox equivalents, determined by the interpolation of absorbance at a given reaction time in the Trolox calibration curve assessed at the same reaction time. Note: for Trolox, in this assay, the calibration curve is the same along time. **(B)** Experiment where the antioxidant capacity is independent of the selected reaction time. Curves $AC_1$ to $AC_4$ represent the absorbance values obtained for increasing concentrations of a given tested compound with reaction kinetics similar to sample. This figure is based on the results obtained for ABTS$^{\bullet+}$ assay applied to sample $RW_{38}$ ($o$), with Trolox (♦) and tannic acid (△) as standard compounds.

common food products [11]. The chemical structures of all tested compounds are provided as supplementary data (Fig. S1).

### 3.1. Kinetic profile of red wines and antioxidant compounds

The oxidation profiles of red wines from different origins and vintages (Table S1, supplementary data) were also established and the reaction time that guaranteed the total oxidation of the sample (reaction endpoint) was assessed. In Fig. 2, the absorbance measurements upon reaction time for some red wines analyzed by F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays are shown. The absorbance decrease values upon reaction time for radical assays (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) are also presented.

For all antioxidant assays, red wines displayed a complex kinetic pattern involving fast and slow steps of oxidation, observed as a rapid variation in absorbance values in the first minutes of the reaction followed by a slower variation. This biphasic oxidation pattern may result from rapid oxidation of phenolic compounds forming semiquinone products (fast step), which further dimerize yielding new compounds that contribute to the antioxidant capacity [17]. Hence, the slow oxidation step is due to products formed in the first oxidation and to compounds that have slow reaction kinetics. Moreover, the time taken to

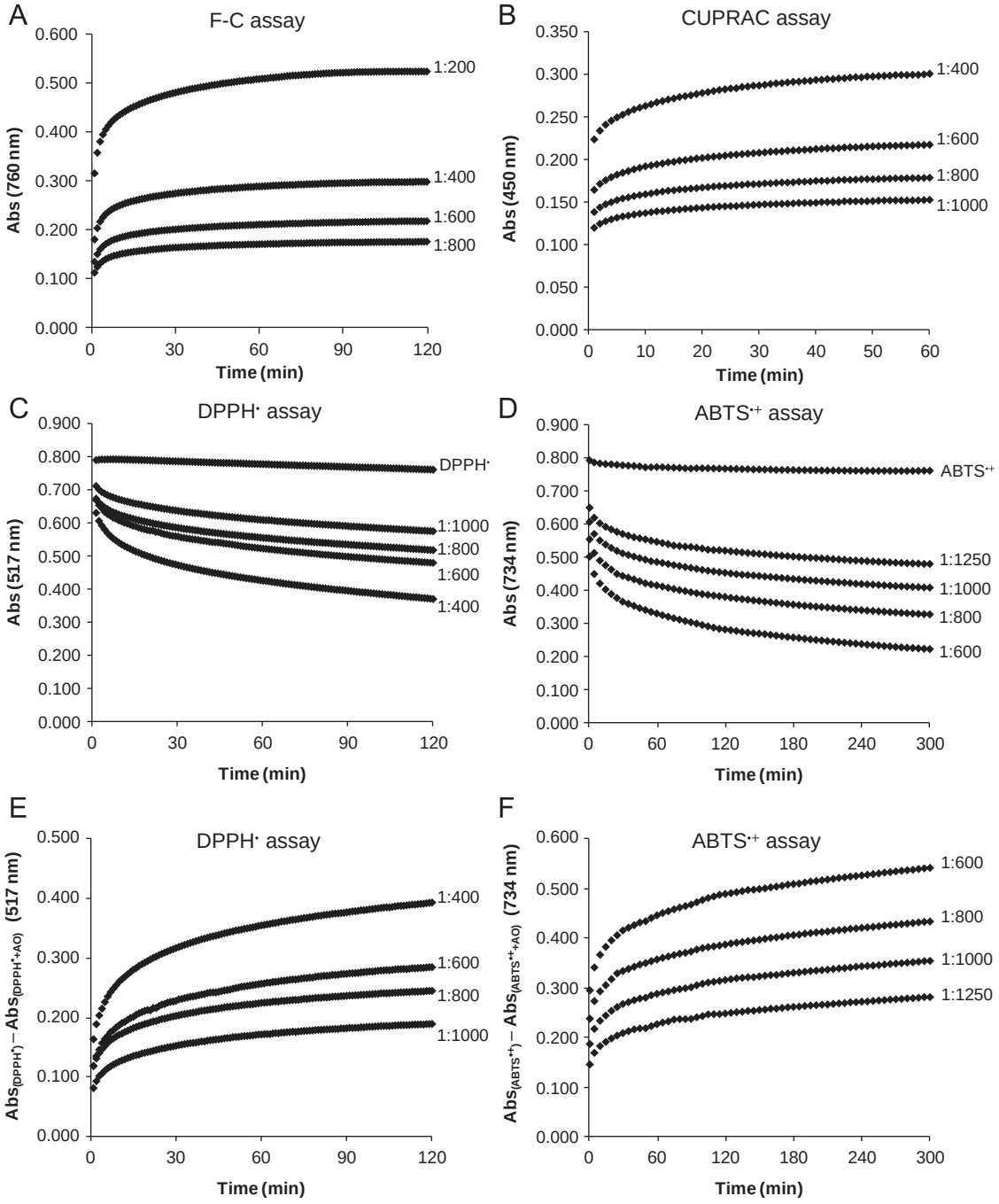


**Fig. 2.** Absorbance measurements along reaction time obtained for different dilutions (200 to 1250 times) of red wine samples (A)–(D). For DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, the absorbance decrease values were also represented (E) and (F), calculated as the difference of radical absorbance and its depletion in the presence of red wine samples. Analyzed samples: $RW_3$ (F–C assay); $RW_{13}$ (CUPRAC assay); $RW_{22}$ (DPPH$^{\bullet}$ assay); $RW_{38}$ (ABTS$^{\bullet+}$ assay).

attain constant values of absorbance (endpoint) increases as red wines are less diluted, i.e., as the concentration of antioxidant is higher the absorbance values stabilize later. Similar results have been previously described [8].

For F–C and CUPRAC assays, the endpoints were fixed at 120 and 60 min, respectively, since the absorbance increase in the last 10 min was negligible ( $<0.002$) (Fig. 2A and B). For DPPH$^{\bullet}$ assay, the reaction was monitored over 120 min but, for longer periods, a considerable decrease in the absorbance values of control (containing only DPPH$^{\bullet}$ solution) was observed. In fact, after 180 min the absorbance decrease of control was about 10% of the initial absorbance, while after 120 min it was $<5\%$. This took place because ethanol evaporates along the analysis and the DPPH$^{\bullet}$ radical precipitates in the reaction medium, as the percentage of ethanol decreases below a threshold value. For this reason, the reaction was monitored up to 120 min. Regarding ABTS$^{\bullet+}$ assay, the endpoint was fixed at 300 min because above this time an increase in absorbance values of control (about 5%) was observed due to solvent evaporation, which consequently concentrates the radical. The reaction times selected for DPPH$^{\bullet}$ (120 min) and ABTS$^{\bullet+}$ (300 min) assays can be considered near the endpoint conditions because the absorbance decrease attained in the last 30 min of the reaction correspond to c.a. 5 and 2% of absorbance decrease obtained after 90 and 270 min, respectively (Fig. 2C–F). These absorbance differences provide an increase in TE values that was lower than the standard deviation ($n=16$). Noteworthy, the time span to attain constant absorbance values were lower for electron transfer assays (F–C and CUPRAC) indicating that the reaction was faster than that obtained for radical scavenging assays (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) based on a mechanism of sequential proton loss electron transfer (SPLET), which the net result is the same as in hydrogen atom transfer to the free radicals [18,19]. Moreover, the higher pH values applied at F–C ($pH \approx 10$) and CUPRAC ($pH=7$) assays may also increases the rate of electron transfer to oxidizing species.

Using these reaction times, the kinetic profiles of 13 pure compounds and SPM were evaluated for each method. For this, the analytical signals obtained for each compound, tested at different concentrations ($n=5$), were measured and the sensitivity ($mM^{-1}$) was determined at every minute along the reaction monitoring. Some examples are given in Fig. 3. For all antioxidant assays, Trolox (generally accounted as a fast reacting compound) displayed a rapid oxidation kinetics reaching endpoint conditions in the first minutes of reaction, which is evidenced in Fig. 3 by the constant sensitivity values obtained throughout the analysis time (relative difference $<2\%$). Similar results were obtained for ascorbic acid after 10 min (data shown only for F–C assay). On the other hand, pure phenolic compounds and SPM provided a biphasic kinetic pattern with a relatively fast initial rate followed by a slow step.

The antioxidant capacity values of several compounds were determined at endpoint conditions, calculated as the ratio between the slope of a given compound and the slope of gallic acid for F–C assay or the slope of Trolox for the other methods

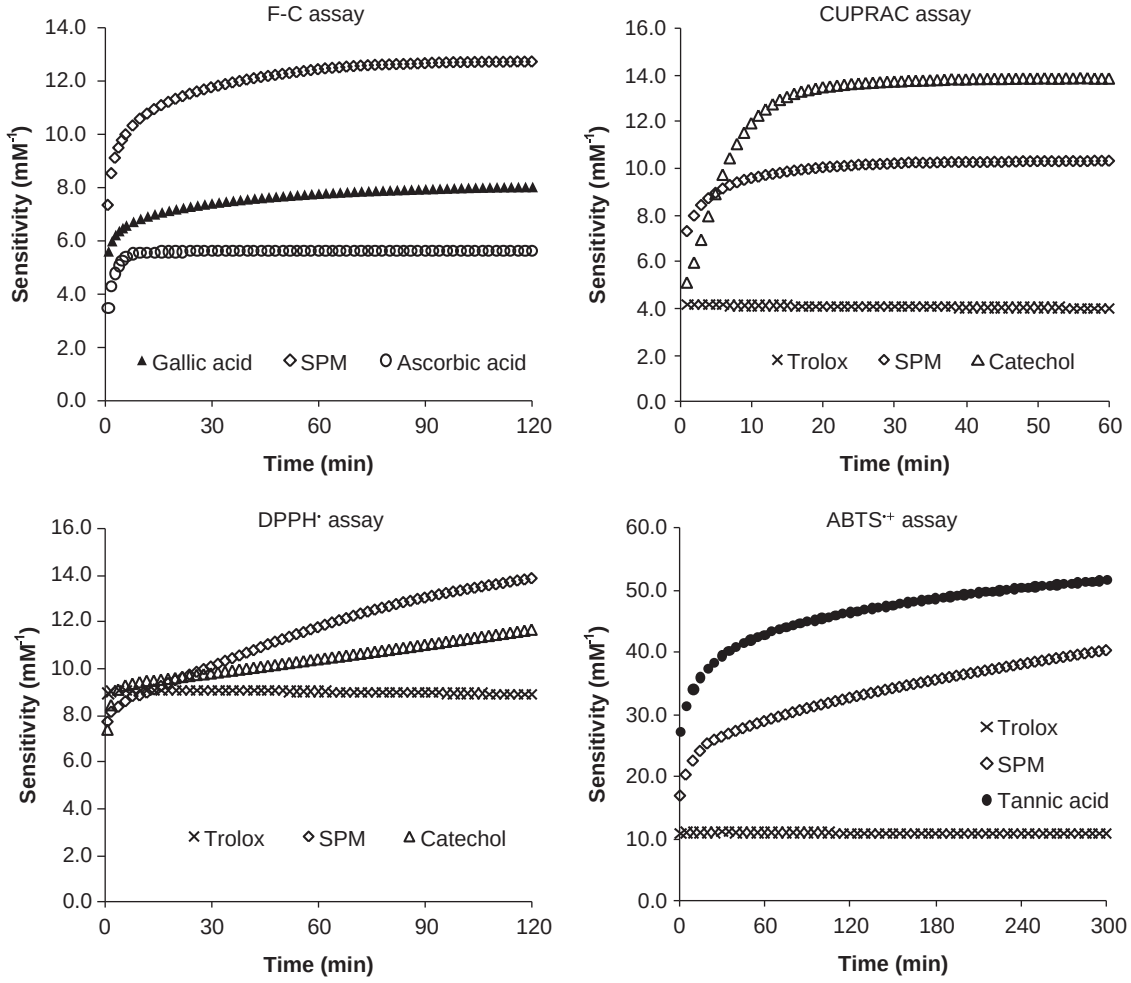


**Fig. 3.** Sensitivity values ($mM^{-1}$) upon reaction time obtained for some compounds tested for F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays ($R \geq 0.9995$, $n=5$). The values presented are the mean of three experiments performed in different days. Owing to scale reasons, the sensitivity values of tannic acid were divided by five.

**Table 1**
Antioxidant capacity values of several compounds expressed as GAE (F–C assay) and TE (other assays).

| Compound | Antioxidant assay | | | |
|---|---|---|---|---|
| | F–C (120 min)[a] | CUPRAC (60 min)[b] | DPPH$^{\bullet}$ (120 min)[b] | ABTS$^{\bullet+}$ (300 min)[a] |
| Trolox | 0.37 ± 0.01 | 1 | 1 | 1 |
| Ascorbic acid | 0.70 ± 0.01 | 1.13 ± 0.03 | 1.03 ± 0.05 | 1.04 ± 0.06 |
| Gallic acid | 1 | 3.2 ± 0.2 | 4.0 ± 0.2 | 5.7 ± 0.2 |
| Caffeic acid | 1.08 ± 0.03 | 3.5 ± 0.1 | 1.29 ± 0.05 | 3.3 ± 0.1 |
| Tannic acid | 8.3 ± 0.2 | 16.4 ± 0.4 | 22.4 ± 0.9 | 24 ± 1 |
| Catechol | 1.09 ± 0.03 | 3.5 ± 0.2 | 1.32 ± 0.05 | 3.5 ± 0.2 |
| Pyrogallol | 0.99 ± 0.02 | 3.19 ± 0.09 | 2.8 ± 0.1 | 4.6 ± 0.2 |
| Morin | 1.49 ± 0.03 | 2.07 ± 0.06 | 1.38 ± 0.07 | 4.8 ± 0.2 |
| Quercetin | 2.38 ± 0.09 | 3.8 ± 0.4 | 3.0 ± 0.1 | 7.0 ± 0.3 |
| Rutin | 1.82 ± 0.04 | 3.07 ± 0.08 | 2.4 ± 0.2 | 4.3 ± 0.2 |
| Hesperetin | 1.45 ± 0.06 | 0.58 ± 0.02 | 0.56 ± 0.02 | 1.56 ± 0.06 |
| (+)-Catechin | 1.62 ± 0.04 | 2.8 ± 0.2 | 2.8 ± 0.1 | 4.9 ± 0.2 |
| EGCG[c] | 2.5 ± 0.1 | 6.0 ± 0.2 | 6.3 ± 0.2 | 8.5 ± 0.3 |
| SPM[d] | 1.59 ± 0.03 | 2.59 ± 0.08 | 1.6 ± 0.1 | 3.7 ± 0.2 |

[a] For F–C assay, the slope of the calibration curve for gallic acid was 8.0 ± 0.2 mM$^{-1}$ ($n$=5, $R$ > 0.9998).
[b] For CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, the slope of calibration curves for Trolox were 4.0 ± 0.1 mM$^{-1}$ ($n$=5, $R$ > 0.9998), 8.9 ± 0.3 mM$^{-1}$ ($n$=5, $R$ > 0.9995) and 10.8 ± 0.4 mM$^{-1}$ ($n$=5, $R$ > 0.9994), respectively.
[c] EGCG, (−)-epigallocatechin gallate.
[d] SPM, standard phenolic mixture.

(Table 1). For F–C assay, the obtained gallic acid equivalents (GAE) are in agreement with other results previously reported because similar reaction times were applied [20,21]. Gallic acid is conventionally chosen as standard for F–C assay instead of Trolox, because the last compound presents a low reactivity towards F–C reagent [21]. Regarding the other three assays, for those compounds exhibiting complex oxidation kinetics, the antioxidant capacity values were usually higher than the values reported in the literature, because the reaction times selected here were higher than those reported in previous works [6,22,23]. For instance, the Trolox equivalents (TE) determined for caffeic acid in CUPRAC and ABTS$^{\bullet+}$ assays were about 3.5 ± 0.1 and 3.3 ± 0.1 (Table 1), while the values reported in other works [6,22] at 30 and 120 min of reaction were about 2.9 and 2.3, respectively. This dependence of antioxidant capacity values upon reaction time can be also observed for catechol in CUPRAC assay (Fig. 3), since the TE values determined after 1, 5, 10 and 20 min were about 37, 64, 86, and 97% of that obtained at 60 min. On the other hand, for fast reacting compounds such as ascorbic acid, the TE values were independent of the selected reaction time, since the determined endpoint values (1.1 and 1.0 for CUPRAC and ABTS$^{\bullet+}$ assays, Table 1) were similar to those determined at shorter reaction times [6,22]. These results clearly illustrate how the selected reaction time for ET based assays account for the large diversity of antioxidant values reported in literature, where measurements are seldom taken near the endpoint conditions.

Moreover, the data concerning the kinetic profile, the total antioxidant capacity and the potential interactions between phenolic compounds of SPM is provided here for the first time. The sensitivity obtained at endpoint reaction times for F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays was 12.7 ± 0.1, 10.3 ± 0.2, 13.9 ± 0.7 and 40 ± 1 mM$^{-1}$, respectively. Considering the amount of each phenolic compound present in the standard mixture and each respective sensitivity when analyzed separately, the theoretical sensitivity values for SPM (calculated as the weighted sum of each component, see Materials and Methods section) were 11.1, 10.0, 14.0, and 40 mM$^{-1}$ for F–C, CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, respectively. Hence, for F–C assay a synergistic effect between the phenolic compounds was obtained as the determined sensitivity was about 14% higher than the theoretical value, indicating that there are some interactions among the components of SPM in this assay. On the other hand, for CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays an additive effect was attained because the experimental sensitivities were similar to theoretical values. Similar results for ternary synthetic antioxidant solutions were attained for CUPRAC assay [13]. The total antioxidant capacity of polyphenolic mixtures measured by F–C assay may be overestimated because there are some interactions between phenolic compounds and/or oxidized products formed, while for the other ET assays there was no evidence for chemical interactions among the compounds because the total antioxidant capacity determined corresponds to the sum of the individual antioxidant capacity values of the SPM constituents.

### 3.2. Selection of standard compound and reaction time

One of the aims of this work was to validate that a compound that has an oxidation kinetic profile similar to the sample will provide endpoint antioxidant capacity values in a shorter period of time, as represented in Fig. 1B. For this, the absorbance values measured for samples at every minute of reaction were interpolated in calibration curves of each tested pure compound, determined at the same reaction time. These values were further divided by the antioxidant capacity determined at endpoint conditions and plotted against time, as represented in Fig. 4.

Results showed that when the kinetic profile of red wine samples is different from that exhibited by the tested pure compounds, the antioxidant capacity values determined were dependent of the reaction time. For instance, when the absorbance change for samples is larger than that obtained for compounds within the same time interval, antioxidant capacity values increase along experiment time, as shown for application of gallic and ascorbic acids as standards in the F–C assay or for application of Trolox in CUPRAC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ methods (Fig. 4). On the other hand, when the absorbance change for samples is lower than that obtained for pure compounds, the antioxidant capacity values (expressed as equivalents of pure compound) decrease along the reaction time (Fig. 4, application of catechol in CUPRAC assay and application of SPM in ABTS$^{\bullet+}$ assay).

When compounds have a kinetic profile similar to red wines, the antioxidant capacity values are constant and become independent of the time selected. As exemplified in Fig. 4, the SPM provided antioxidant capacity values independent of the analysis time after 3, 5 and 10 min of reaction for F–C, CUPRAC and DPPH$^{\bullet}$ assays, respectively, while tannic acid provided constant antioxidant capacity values for ABTS$^{\bullet+}$ assay after the first minute of the reaction. Therefore, the total antioxidant capacity can be determined at non-endpoint conditions using the proposed kinetic matching approach. For red wine samples, the lowest reaction times (min) required to attain constant antioxidant capacity values, similar to that attained at endpoint conditions (relative deviation < 5%) are presented in Table 2 for all tested compounds. The SPM, catechol and hesperetin are standards with suitable kinetic matching for F–C assay because the antioxidant values determined after 3 min of reaction were similar to those obtained at 120 min, while for most of the compounds the time needed was above 30 min (Table 2).

Regarding to CUPRAC assay, gallic acid provided similar antioxidant values after 3 min, but the intercept values of its calibration curves were lower than the reagent blank, causing an overestimation

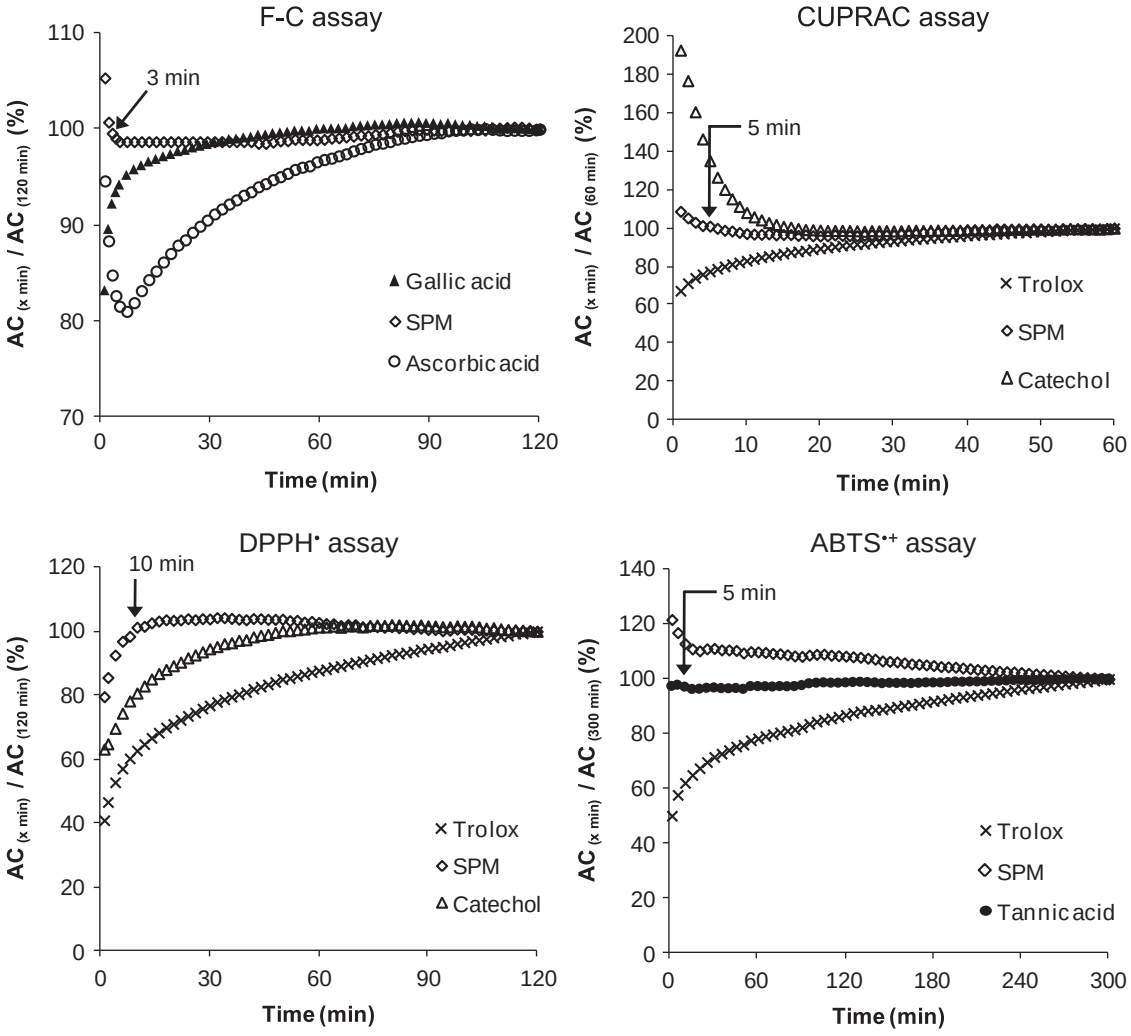


**Fig. 4.** Application of the kinetic matching approach to wine samples by representing relative antioxidant capacity (AC) values along reaction time for several compounds. Analyzed samples: $RW_3$ (F–C assay); $RW_{13}$ (CUPRAC assay); $RW_{22}$ (DPPH$^\bullet$ assay); $RW_{38}$ (ABTS$^{\bullet+}$ assay).

**Table 2**
Values of minimum reaction time [a] (min) necessary to attain constant antioxidant capacity values, independent of analysis time. Above these reaction times, the antioxidant values are similar ($\pm$5%) to those obtained at endpoint conditions.

| Compound | Antioxidant assay | | | |
|---|---|---|---|---|
| | F–C | CUPRAC | DPPH$^\bullet$ | ABTS$^{\bullet+}$ |
| Trolox | 30 | 45 | > 90 | 240 |
| Ascorbic acid | 90 | 10 | > 90 | 240 |
| Gallic acid | 30 | 3 | 90 | 240 |
| Caffeic acid | 30 | 30 | 90 | > 240 |
| Tannic acid | 30 | 45 | 90 | 5 |
| Catechol | 3 | 20 | 60 | > 240 |
| Pyrogallol | 30 | 30 | > 90 | 240 |
| Morin | 10 | 45 | 20 | 180 |
| Quercetin | 10 | 45 | > 90 | 30 |
| Rutin | 30 | 45 | 60 | 240 |
| Hesperetin | 3 | 20 | 60 | 30 |
| (+)-Catechin | 30 | 45 | 90 | 240 |
| EGCG[b] | 30 | 30 | 90 | 180 |
| SPM[c] | 3 | 5 | 10 | 240 |

[a] The values were determined considering, for each assay and compound, the results from ten red wine samples of different origins and vintages.
[b] EGCG, (−)-epigallocatechin gallate.
[c] SPM, standard phenolic mixture.

of the antioxidant capacity. Similar results were obtained for caffeic acid. This took place because both gallic and caffeic acids establish complexes with Cu(II) ions present in excess in the reaction media, decreasing the intrinsic absorption of blank [10]. Hence, the SPM was selected as standard because the antioxidant capacity values measured after 5 min were constant along the reaction time (Fig. 4) and the intercept values of calibration curves were near zero. The SPM was also chosen as standard for DPPH$^\bullet$ assay because the antioxidant capacity values were constant above 10 min (Fig. 4), which is far below the 90 min required for most of the compounds, including the classical standard Trolox. Although tannic acid provided similar antioxidant values for ABTS$^{\bullet+}$ assay after the first minute (Fig. 4), the reaction time of 5 min was selected because the repeatability of antioxidant capacity values (RSD $\approx$ 5%) was higher than that determined at the first minute of reaction (RSD $\approx$ 10%).

Another issue that limits the homogenization of antioxidant protocols is the absence of a common standard and/or expression of antioxidant results that allows the comparison of data between methods and laboratories [10]. Hence, in order to standardize the expression of antioxidant capacity results provided by different assays and to compare the results from the kinetic matching approach with the endpoint measurements, the antioxidant capacity of red wines expressed as equivalents of a given compound were converted to equivalents of generally used

standards, namely gallic acid equivalents (GAE) for F–C assay and Trolox equivalents (TE) for the other three methods. This was achieved after correction for the number of electrons transferred by each compound (see Eq. (1), described in Materials and Methods section). The antioxidant capacity values expressed as equivalents of a given compound and the GAE and TE values (mM) estimated for some red wine samples, at selected reaction times and also at endpoint conditions are given in Table 3. These results confirmed that the kinetic profile of the compound is not relevant when endpoint measurements are taken, as shown for GAE and TE values at endpoint conditions, which were similar independently of the compound used as standard after the correction for the number of transferred electrons. However, whenever measurements are taken before a stable absorbance is achieved (kinetic), GAE or TE values can be underestimated or overestimated. For instance, the TE values determined in the $ABTS^{\bullet+}$ assay after 5 min of reaction using Trolox and SPM as standards were 18.6 and 38.6 mM, which are different from that obtained at endpoint conditions (31.8 and 32.5 mM, respectively, Table 3). However, when tannic acid was evaluated as potential standard, the antioxidant value expressed as equivalents of tannic acid determined after 5 min of reaction ($1.32 \pm 0.06$ mM) and further converted to TE by multiplying it to the slope ratio of tannic acid and Trolox at endpoint conditions (258/10.8), provided a result (31.6 mM) similar to that attained at endpoint conditions (31.8 mM). This occurs because the reactivity of tannic acid was similar to that obtained for red wine samples (Fig. 4).

Therefore, for rapid assessment of endpoint antioxidant capacity of red wines, we recommend the use of SPM as standard for F–C, CUPRAC and $DPPH^{\bullet}$ assays and tannic acid for $ABTS^{\bullet+}$ assay, shortening the reaction time to 3, 5, 10 and 5 min, respectively, instead of the 120, 60, 120 and 300 min necessary with the classical standards. For other foodstuffs, a compound with an oxidation kinetic similar to target samples should be identified and similar calculations can be undertaken. Furthermore, a step towards homogenization should be given by converting the antioxidant capacity values to GAE and TE, using Eq. (1) and considering the number of electrons transferred by the kinetic matching compound and by the classical standard (gallic acid or Trolox) at endpoint conditions.

### 3.3. Comparison of the total antioxidant capacity of red wines assessed by the kinetic matching and by the endpoint approaches

The potential of the proposed kinetic matching approach for high-throughput assessment of total antioxidant capacity was shown by application to red wine samples ($n=10$, for each assay) from different origins and vintages (Table S1, supplementary data). The F–C and CUPRAC values obtained at endpoint conditions using gallic acid and Trolox as standard compounds, respectively, and the equivalents of SPM and their respective calculated GAE and TE at selected reaction time are presented in Table 4. The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacity results

**Table 3**
Antioxidant capacity values [a] of red wine samples expressed as equivalents of a given compound or as gallic acid equivalents (GAE) or Trolox equivalents (TE) estimated for the kinetic matching and the endpoint approaches.

| Antioxidant assay | Compound | Kinetic matching | | Endpoint | |
|---|---|---|---|---|---|
| – | – | **3 min** | **GAE (3 min)**[b] | **120 min** | **GAE (120 min)**[c] |
| F–C | Gallic acid | $10.9 \pm 0.3$ | 10.9 | $11.9 \pm 0.2$ | 11.9 |
| – | SPM | $7.4 \pm 0.2$ | 11.8 | $7.4 \pm 0.1$ | 11.8 |
| – | Ascorbic acid | $14.2 \pm 0.4$ | 10.0 | $16.7 \pm 0.3$ | 11.8 |
| – | – | **5 min** | **TE (5 min)**[d] | **60 min** | **TE (60 min)**[e] |
| CUPRAC | Trolox | $18.7 \pm 0.2$ | 18.7 | $24.2 \pm 0.3$ | 24.2 |
| – | SPM | $9.4 \pm 0.3$ | 24.3 | $9.37 \pm 0.08$ | 24.3 |
| – | Catechol | $9.6 \pm 0.3$ | 33.6 | $6.7 \pm 0.2$ | 23.5 |
| – | – | **10 min** | **TE (10 min)**[d] | **120 min** | **TE (120 min)**[e] |
| $DPPH^{\bullet}$ | Trolox | $13 \pm 2$ | 13.1 | $20 \pm 2$ | 20.4 |
| – | SPM | $13 \pm 1$ | 20.4 | $13 \pm 1$ | 20.5 |
| – | Catechol | $13 \pm 1$ | 16.7 | $15 \pm 1$ | 20.3 |
| – | – | **5 min** | **TE (5 min)**[d] | **300 min** | **TE (300 min)**[e] |
| $ABTS^{\bullet+}$ | Trolox | $18.6 \pm 0.8$ | 18.6 | $32 \pm 1$ | 31.8 |
| – | SPM | $10.3 \pm 0.4$ | 38.6 | $8.6 \pm 0.4$ | 32.5 |
| – | Tannic acid | $1.32 \pm 0.06$ | 31.6 | $1.33 \pm 0.05$ | 31.8 |

[a] Results are expressed as the mean ± standard deviation obtained for four red wine dilutions analyzed in quadruplicate ($n=16$); $RW_3$, $RW_{13}$, $RW_{22}$ and $RW_{38}$ analyzed for F–C, CUPRAC, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, respectively.

[b] Gallic acid equivalents (GAE) were estimated by multiplying the equivalent values obtained at 3 min by the slope ratio between the compound and gallic acid at endpoint conditions.

[c] Gallic acid equivalents (GAE) were estimated by multiplying the equivalent values obtained at 120 min by the slope ratio between the compound and gallic acid at endpoint conditions.

[d] Trolox equivalents (TE) were estimated by multiplying the equivalent values obtained at 5, 10 and 5 min and the slope ratio between the compound and Trolox at endpoint conditions.

[e] Trolox equivalents (TE) were estimated by multiplying the equivalent values obtained at 60, 120 and 300 min and the slope ratio between the compound and Trolox at endpoint conditions.

**Table 4**
F–C and CUPRAC values of red wine samples obtained by the kinetic matching and by the endpoint approaches. Antioxidant capacity values at endpoint conditions were assessed using gallic acid (F–C assay) and Trolox (CUPRAC assay) as standards, while the standard phenolic mixture (SPM) was used as standard for the kinetic matching approach.

| Antioxidant assay | Sample | Kinetic matching | | Endpoint | |
|---|---|---|---|---|---|
| | | **SPM (3 min)** | **GAE**[a] **(3 min)** | **GAE (120 min)** | **R.D. (%)** |
| **F–C** | $RW_1$ | $6.5 \pm 0.2$ | $10.4 \pm 0.3$ | $10.6 \pm 0.1$ | −1.9 |
| – | $RW_2$ | $8.7 \pm 0.4$ | $13.8 \pm 0.6$ | $13.9 \pm 0.3$ | −0.7 |
| – | $RW_3$ | $7.4 \pm 0.2$ | $11.7 \pm 0.2$ | $11.9 \pm 0.2$ | −1.7 |
| – | $RW_4$ | $9.8 \pm 0.4$ | $15.6 \pm 0.6$ | $15.6 \pm 0.5$ | 0.0 |
| – | $RW_5$ | $8.3 \pm 0.3$ | $13.2 \pm 0.4$ | $13.2 \pm 0.4$ | 0.0 |
| – | $RW_6$ | $8.2 \pm 0.3$ | $13.1 \pm 0.5$ | $13.7 \pm 0.3$ | −4.4 |
| – | $RW_7$ | $7.1 \pm 0.2$ | $11.2 \pm 0.2$ | $11.6 \pm 0.4$ | −3.4 |
| – | $RW_8$ | $7.3 \pm 0.3$ | $11.5 \pm 0.5$ | $11.8 \pm 0.2$ | −2.5 |
| – | $RW_9$ | $9.5 \pm 0.3$ | $15.1 \pm 0.6$ | $14.8 \pm 0.5$ | +2.0 |
| – | $RW_{10}$ | $8.5 \pm 0.4$ | $13.5 \pm 0.6$ | $13.1 \pm 0.5$ | +3.1 |
| – | – | **SPM (5 min)** | **TE**[b] **(5 min)** | **TE (60 min)** | |
| **CUPRAC** | $RW_{11}$ | $9.1 \pm 0.4$ | $24 \pm 1$ | $23.8 \pm 0.6$ | +0.8 |
| – | $RW_{12}$ | $7.0 \pm 0.4$ | $18 \pm 1$ | $17.8 \pm 0.5$ | +1.1 |
| – | $RW_{13}$ | $9.4 \pm 0.3$ | $24.3 \pm 0.7$ | $24.2 \pm 0.3$ | +0.4 |
| – | $RW_{14}$ | $9.4 \pm 0.8$ | $24 \pm 1$ | $24 \pm 1$ | 0.0 |
| – | $RW_{15}$ | $8.8 \pm 0.5$ | $23 \pm 1$ | $22.7 \pm 0.7$ | +1.3 |
| – | $RW_{16}$ | $8.4 \pm 0.5$ | $22 \pm 1$ | $21.9 \pm 0.5$ | +0.5 |
| – | $RW_{17}$ | $8.0 \pm 0.3$ | $20.7 \pm 0.8$ | $20.2 \pm 0.2$ | +2.5 |
| – | $RW_{18}$ | $8.6 \pm 0.6$ | $22 \pm 1$ | $22.3 \pm 0.6$ | −1.3 |
| – | $RW_{19}$ | $13 \pm 1$ | $35 \pm 1$ | $35 \pm 1$ | 0.0 |
| – | $RW_{20}$ | $8.4 \pm 0.4$ | $21.7 \pm 0.9$ | $21.9 \pm 0.4$ | −0.9 |

[a] GAE, gallic acid equivalents (mM) were calculated by multiplying the equivalents of SPM obtained after 3 min of reaction and the slope ratio between SPM and gallic acid at endpoint conditions (12.7/8.0).

[b] TE, Trolox equivalents (mM) were calculated by multiplying the equivalents of SPM obtained after 5 min of reaction and the slope ratio between SPM and Trolox at endpoint conditions (10.4/4.0). Each value corresponds to the mean ± standard deviation of four dilutions of red wine samples analyzed in quadruplicate ($n=16$). R.D. Relative deviation between the two approaches (GAE or TE values).

obtained at endpoint conditions using Trolox as standard and those attained by the kinetic matching approach using SPM ($DPPH^{\bullet}$ assay) and tannic acid ($ABTS^{\bullet+}$ assay) along with the respective calculated TE are presented in Table 5. For all tested samples, the antioxidant values determined by the kinetic matching approach and by the endpoint procedure were in agreement (relative deviation $<4.8\%$ for all assays). For comparison purposes, a linear relationship between these results was established as $C_{kinetic}=C_0+S\times C_{endpoint}$ for each assay ($n=10$). The values for intercept ($C_0$) and slope ($S$) were: for F–C assay, $-1.1$ ($\pm 1.9$), 1.08 ($\pm 0.15$); for CUPRAC assay, 0.4 ($\pm 1.0$), 0.99 ($\pm 0.04$); for $DPPH^{\bullet}$ assay, 1.4 ($\pm 2.9$), 0.92 ($\pm 0.14$); and for $ABTS^{\bullet+}$ assay, $-0.08$ ($\pm 4.47$), 0.99 ($\pm 0.15$). Considering the limits of the 95% confidence intervals presented (values in parentheses), the calculated intercept and slope values do not differ significantly from 0 and 1, respectively. Therefore, there is no evidence for systematic differences between the two sets of results obtained by the proposed kinetic matching approach and by the endpoint measurements for each assay [24]. Furthermore, when a paired $t$-test was performed on the data obtained for all red wines within the same method, calculated $|t|$ values of 1.15, 1.22, 1.64 and 1.28 were obtained for F–C, CUPRAC, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, respectively. The comparison between these values and the tabulated $t$ ($p=0.05$; $df=9$)$=2.26$ indicates no significant difference for the mean concentration values obtained by the two approaches [24]. Hence, the total antioxidant capacity of red wines can be rapidly assessed by selecting a compound with an oxidation kinetic behavior similar to samples without the necessity of achieving endpoint conditions.

**Table 5**
$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging capacity values of red wine samples obtained by the kinetic matching and by the endpoint approaches. Antioxidant capacity values at endpoint conditions were assessed using Trolox as standard, while the standard phenolic mixture (SPM) and tannic acid were used as standards for the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ kinetic matching approach.

| Antioxidant assay | Sample | Kinetic matching | | Endpoint | |
|---|---|---|---|---|---|
| | | SPM (10 min) | TE[a] (10 min) | TE (120 min) | R.D. (%) |
| **$DPPH^{\bullet}$** | $RW_{21}$ | $12\pm1$ | $19\pm2$ | $19\pm2$ | 0.0 |
| – | $RW_{22}$ | $13\pm1$ | $20\pm1$ | $20\pm2$ | 0.0 |
| – | $RW_{23}$ | $14.5\pm0.9$ | $23\pm1$ | $23\pm2$ | 0.0 |
| – | $RW_{24}$ | $13\pm1$ | $21\pm2$ | $21\pm2$ | 0.0 |
| – | $RW_{25}$ | $13.0\pm0.9$ | $20\pm1$ | $21\pm1$ | −4.8 |
| – | $RW_{26}$ | $13.8\pm0.9$ | $22\pm1$ | $22\pm2$ | 0.0 |
| – | $RW_{27}$ | $10.3\pm0.5$ | $16.1\pm0.8$ | $16\pm1$ | 0.6 |
| – | $RW_{28}$ | $12.6\pm0.5$ | $19.7\pm0.8$ | $20\pm1$ | −1.5 |
| – | $RW_{29}$ | $14\pm1$ | $23\pm2$ | $24\pm3$ | −4.2 |
| – | $RW_{30}$ | $12.4\pm0.6$ | $22\pm1$ | $22\pm3$ | 0.0 |
| – | – | **Tannic acid (5 min)** | **TE[b] (5 min)** | **TE (300 min)** | |
| **$ABTS^{\bullet+}$** | $RW_{31}$ | $1.40\pm0.04$ | $33\pm1$ | $34\pm2$ | −2.9 |
| – | $RW_{32}$ | $1.17\pm0.06$ | $28\pm2$ | $28\pm2$ | 0.0 |
| – | $RW_{33}$ | $1.09\pm0.04$ | $26\pm1$ | $26\pm1$ | 0.0 |
| – | $RW_{34}$ | $1.22\pm0.06$ | $29\pm1$ | $29.8\pm0.6$ | −2.7 |
| – | $RW_{35}$ | $1.28\pm0.06$ | $31\pm2$ | $30\pm1$ | +3.3 |
| – | $RW_{36}$ | $1.17\pm0.05$ | $28\pm1$ | $28.3\pm0.7$ | −1.1 |
| – | $RW_{37}$ | $0.97\pm0.05$ | $23\pm1$ | $23.6\pm0.9$ | −2.5 |
| – | $RW_{38}$ | $1.32\pm0.06$ | $32\pm1$ | $32\pm1$ | 0.0 |
| – | $RW_{39}$ | $1.33\pm0.07$ | $32\pm2$ | $32\pm1$ | 0.0 |
| – | $RW_{40}$ | $1.17\pm0.07$ | $28\pm2$ | $28.6\pm0.7$ | −2.1 |

[a] TE, Trolox equivalents (mM) were calculated by multiplying the equivalents of SPM obtained after 10 min of reaction and the slope ratio between SPM and Trolox at endpoint conditions (13.9/8.9).

[b] TE, Trolox equivalents (mM) were calculated by multiplying the equivalents of tannic acid obtained after 5 min of reaction and the slope ratio between tannic acid and Trolox at endpoint conditions (258/10.8). Each value corresponds to the mean ± standard deviation of four dilutions of red wine samples analyzed in quadruplicate ($n=16$). R.D. Relative deviation between the two approaches (TE values).

### 3.4. Figures of merit of kinetic matching approach

Considering that the SPM is proposed for the first time as standard for rapid assessment of antioxidant capacity of red wines instead of the endpoint approach using the classical standards, the figures of merit of the microplate protocols for the selected reaction times were determined. The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $S_{y/x}$, which estimates the standard deviation of $y$-residuals [24]. For four calibration curves performed in different days, the calculated detection limit was 1.8, 0.7 and 2.5 μM of SPM for F–C, CUPRAC and $DPPH^{\bullet}$ assays, respectively. The repeatability was estimated by calculating the relative standard deviation (RSD) from 10 consecutive determinations of SPM standard solutions (between 10.0 and 20.0 μM), providing values of 1.5, 1.3 and 4.5% for F–C, CUPRAC and $DPPH^{\bullet}$ assays, respectively. The reproducibility assessed by the RSD of calibration slopes performed in different days ($n=4$), was 2.5, 4.0 and 1.8% for F–C, CUPRAC and $DPPH^{\bullet}$ assays, respectively. For the $ABTS^{\bullet+}$ assay, where tannic acid is the proposed kinetic matching standard, the detection limit was 0.15 μM, the precision (RSD) was 3.9%, while the reproducibility was 2.3%, all calculated as described above.

Finally, sample throughput was estimated considering that for each 96-well plate, blank and five standards were analyzed in quadruplicate, leaving 72 wells available for red wine samples. Considering the analysis times proposed, sample throughputs (with four replica) were 360, 216, 108 and 216 $h^{-1}$ for F–C, CUPRAC, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, respectively, which are excellent figures when compared to 9, 18, 9 and 4 $h^{-1}$ achieved by endpoint measurements. In this regard, the kinetic matching approach proposed here under the microplate format represents a suitable tool for routine analysis in wine industries. Moreover, the SPM was shown here, through application as standard for real samples, to be an excellent candidate to fill the role of universal standard for antioxidant activity assessment, with pending acceptance attending to compromise and agreement among researchers working in the antioxidant field.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.05.002.

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