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Article

Determination of Total Antioxidant Capacity Using Thiamine as a Natural Fluorescent Probe

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This work proposes a spectrofluorometric method for the determination of total antioxidant capacity (C_{AO}) in beverage samples, based on inhibition of thiochrome formation (λ_{ex} = 370 nm, λ_{em} = 440 nm); a product of thiamine (vitamin B_1) oxidation from $K_3Fe(CN)_6$. In the development of the method, gallic acid (GA) was used as a reference, and inhibition of thiochrome formation (in percentage) was used as the analytical response. The selectivity of the method was evaluated using seven different compounds (gallic acid, ascorbic acid, quercetin, butylhydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2 (Trolox), cysteine, and glucose). As proof of concept, the proposed method was applied in the determination of C_{AO} in different samples, such as teas and infusions, red wines, and white wines. The results were compared with the Folin-Ciocalteu (FC), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$), and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) methods using the linear correlation between the methods (at 95% confidence). We observed excellent agreement between the proposed method and FC (correlation coefficient (r) = 0.9768) and $ABTS^{\bullet+}$ (r = 0.9842), compared with $DPPH^{\bullet}$ method (r = 0.8502). For the determination of total C_{AO} in beverages, the proposed method developed proved to be fast, sensitive, simple, and the results were in agreement with established assays.

Keywords: total antioxidant capacity, spectrofluorometric method, thiamine and thiochrome, selectivity, statistical comparison, proof of concept

Introduction

The consumption of foods rich in antioxidant compounds such as vitamins, carotenoids, and phenolic compounds has been associated with the prevention of the diseases related to oxidative stress. Due to these benefits, high interest has been associated, since, in general, these compounds help to eliminate reactive species of oxygen and nitrogen.[1,2] There is a distinct need to identify and classify foods and beverages that help provide adequate nutritional and yet also present antioxidant activity to help avoid the complications caused by oxidative damage. The composition of these foods is usually quite complex and isolating each antioxidant compound is expensive and can be imprecise. Thus, it is necessary to develop methods of quantifying total antioxidant capacity (C_{AO}). However, there is still no standardized and reliable method of measuring antioxidant capacity in food or biological samples.[3-6]

In view of this situation, Prior *et al.*[7] proposed guidelines for the standardization of *in vitro* methods of antioxidant capacity determination. The authors suggested that a method of determining antioxidant capacity should meet following requirements: (*i*) chemical measurements which can lead to potential applications; (*ii*) use of biologically relevant chemical species (radicals or not); (*iii*) simplicity; (*iv*) good reproducibility; (*v*) be adaptable to hydrophilic and lipophilic antioxidants; and (*vi*) it should present a high analytical frequency for routine analysis and quality control. Besides, aspects related to the sensitivity, matrix effect, repeatability, and recognition of possible interferences must also be considered.

In this sense, considering the aspects indicated, a species was sought that might act as a probe to monitor antioxidant species *in vitro*, that is biologically available in the organism and foods, and as well as which presents a well-established mechanism of performance and monitoring. In addition to being present in a wide variety of foods, thiamine (vitamin B_1), which is a crucial molecule for carbohydrate metabolism and maintenance of neural activity, was selected as a probe.[8,9]

During thiamine biosynthesis, the formation of oxidation products generated through enzyme activity and by reactive

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species occurs. Of the products made by thiamine oxidation, thiochrome has already been used as a probe to quantify vitamin B_1 (after oxidation) in samples of biological interest.[10,11] Thiochrome presents structural rigidity, planarity, and aromatic ring conjugation; thus, exhibits fluorescence ($\Phi = 0.28$) when excited in the UV region ($\lambda = 370$ nm).[12] Generally, for vitamin B_1 determination, various oxidizing agents have been used to oxidize thiamine to thiochrome in a basic medium, such as Hg^{II}, Cu^{II}, $K_3Fe(CN)_6$, and hydrogen peroxide in the presence of complexed Fe^{II}. In basic media, Hg^{II} and $K_3Fe(CN)_6$ are the most efficient oxidants for the formation of the fluorescent thiochrome.[13] Thiochrome generated by oxidation of thiamine has been used before in different analytical methodologies. Zhu *et al.*[14] developed a spectrofluorometric method to determine the concentration of ClO^- in water samples through catalytic oxidation of thiamine to thiochrome in the presence of $K_4Fe(CN)_6$. In this methodology, ClO^- oxidizes Fe^{II} to Fe^{III} within the complex, which then reacts with thiamine generating thiochrome. Metalloenzymes such as horseradish peroxidase (heme group) can catalyze the oxidation of thiamine to thiochrome in the presence of H_2O_2 and oleic acid. These have been used to develop methods of vitamin B_1 quantification in food, urine, and drug samples.[11]

Thus, the present work proposes to develop a simple, rapid, and sensitive analytical methodology for the determination of total antioxidant capacity, based on thiamine oxidation inhibition. This method is based on a reduction of thiochrome formation and a consequent decrease in the analytical signal, which is proportional to the concentration of the antioxidant. To validate the method, as proof of concept, we used well-established samples for studies of antioxidant determination. Finally, the method was applied in samples of wines (red and white), teas, and infusions. The results obtained by the proposed method were compared with previously established methodologies such as Folin-Ciocalteu (FC, total phenolic compounds), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$), and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radicals, which are mimetics of reactive nitrogen species.

Experimental

Reagents and samples

All reagents used in the assays are of analytical grade purity. Thiamine hydrochloride, ABTS, $DPPH^{\bullet}$, gallic acid, quercetin, butylhydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2 (Trolox®) were purchased from Sigma-Aldrich (St. Louis, USA). Potassium hexacyanoferrate(III), sodium carbonate, sodium bicarbonate, sodium monohydrogen phosphate, phosphoric acid, hydrochloric acid, boric acid, ascorbic acid, acetic acid, sodium acetate, sodium hydroxide, and potassium persulfate were obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu reagent and cysteine were purchased from Vetec (Rio de Janeiro, Brazil).

The samples of the wines (white and red), teas, and infusions analyzed were purchased from local commerce in the city of Maceió, Alagoas, Brazil. Teas and infusions after opened or prepared were conditioned in the refrigerator at 4 °C until 48 h.

All solutions were prepared with water (conductivity < 0.1 μS cm^{-1}) obtained from a Millipore Millipak Gamma Gold (Bedford, USA) purifier. In all methods described in this work, gallic acid (GA) was used as the reference standard. In this way, the results of the samples were expressed in GA equivalents (mg L^{-1}). The analytical reference signals were obtained from solutions analogous to those of the methods being applied by replacing the volume of the solution of the standard or the sample with deionized water.

Total phenolic compounds

The concentration of total phenolic compounds was determined by the Folin-Ciocalteu method.[15] For this assay, 500 μL of the previously diluted (1:10) Folin-Ciocalteu reagent, and the Na_2CO_3 solution (75 g L^{-1}) were used, respectively. Then, 2.0 mL of the reference solution or sample was added. The final volume was then adjusted to 5.0 mL with deionized water, and after 30 min, spectrophotometric measurements were performed at 770 nm.

$DPPH^{\bullet}$ radical scavenger assay

In order to evaluate the $DPPH^{\bullet}$ radical scavenger capacity, 0.2 mL of $DPPH^{\bullet}$ radical (600 μmol L^{-1}) in methanolic solution, 1.0 mL of the previously diluted sample or reference solution, and 2.80 mL of a 30% methanolic solution (v/v) were mixed, in this order. After 30 min of incubation removed from the light, the spectrophotometric measurement was performed at 527 nm.[15]

$ABTS^{\bullet+}$ radical scavenger assay

The $ABTS^{\bullet+}$ stock solution (1 mmol L^{-1}) radical was prepared by dissolving 26 mg ABTS in water, then 3.0 mL of 1 mmol L^{-1} $K_2S_2O_8$ was added, and the volume was completed to 10.0 mL with deionized water. After 16 h of incubation protected from light, the solution was diluted to 25.0 mL with 0.05 mol L^{-1} phosphate buffer (pH = 7.2).

To perform the $ABTS^{\bullet+}$ radical method, we proceeded as follows: 0.22 mL of the $ABTS^{\bullet+}$ radical solution was added to 1.0 mL of the standard solution (or the previously diluted sample), and 2.80 mL of deionized water. After 15 min, the spectrophotometric measurement was performed at 734 nm. As before, the reference signal was obtained from a similar solution, the sample being replaced with water.[16]

Proposed method (thiamine method)

To 1.50 mL of either antioxidant compound reference solution (or sample) is sequentially added 1.0 mL of the 50 μmol L^{-1} of $K_3Fe(CN)_6$ in 0.1 mol L^{-1} bicarbonate/carbonate buffer solution (pH = 8.0 ± 0.1), and 1.0 mL of 3.33 μmol L^{-1} thiamine hydrochloride solution. The final volume was then adjusted to 4.0 mL with water. Reaction was allowed to take place for 10 min and spectrofluorimetric measurements (λ_{ex} = 370 nm, λ_{em} = 440 nm) were performed. The analytical reference signal was obtained from a solution similar to the previous one, where the sample volume was replaced by deionized water. Inhibition of the analytical signal (I, in percentage), i.e., the presence of compounds with antioxidant capacity was calculated from equation 1:

$$I(\%) = \left(1 - \frac{F_{sample}}{F_{reference}}\right) \times 100 \quad (1)$$

where F_{sample} is the fluorescence after the addition of the sample or analytical standard to the solution containing the oxidizing agent ($K_3Fe(CN)_6$), and $F_{reference}$ is the analytical blank fluorescence.

Preparation of the analyzed samples

The wine samples analyzed were diluted in deionized water beforehand. The herb samples were prepared from the extraction of the compounds by infusion as follows: tea bags or commercial infusions were transferred to a beaker containing 100 mL of heated water (ca. 90 °C) for 10 min. After the extraction time, the tea bag was withdrawn, and the solution (tea or infusion) was allowed to cool to room temperature. The infusion sample preparations were carried out to reproduce the protocol suggested in each respective package methodically. Finally, the samples were diluted and then analyzed using different methodologies.

Statistical analysis

For optimization of specific analytical parameters, sensitivity was used as the criterion, based on the slope of the respective analytical curve in a given study condition. The analyzed analytical curve was constructed with at least five points according to the relation $I_F(\%) = aC_{AO} + b$, with I_F being the percentage inhibition of the fluorescent emission signal at 440 nm, C_{AO} being the concentration of the antioxidant compound, a the slope, and b the linear coefficient. The linear correlation coefficient (r) was calculated, aiming to evaluate the arrangement of points regarding adequacy towards linear behavior. A similar procedure was carried out for the proposed method, and the comparison methods aimed at quantifying the antioxidant capacity in different samples using interpolation of the data.

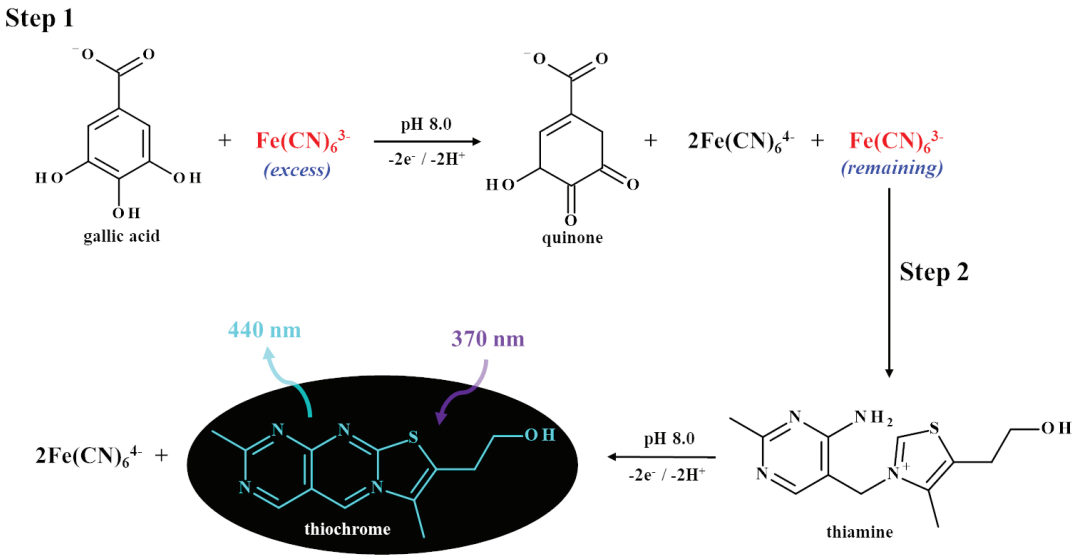
The calculations for the limits of detection (3σ, LOD) and quantification (10σ, LOQ) were performed according to the following equations: $LOD = 3s_b/a_c$ and $LOQ = 10s_b/a_c$, where s_b is the standard deviation of the analytical blank (n = 10), and a_c corresponds to the slope of the analytical curve employed. The relative standard deviation (RSD) was calculated according to the equation: $RSD = (s_p/x_p) \times 100$, where s_p is equivalent to the standard deviation of a given analytical standard within the linear range, and x_p is the average value found for this standard (n = 10).[17] The results of the different methods employed were evaluated using the paired Student's *t*-test, ANOVA (analysis of variance), and linear correlation procedures between the results obtained. For all procedures, a normal distribution of the data (random error) and a confidence interval of 95% were considered.

Results and Discussion

Thiamine in the basic medium in the presence of an oxidizing agent can be oxidized to thiochrome. Thus, the presence of antioxidant compounds in the reaction medium can inhibit fluorophore formation; we, therefore, used this strategy to develop an analytical methodology to determine the total antioxidant capacity of different systems (Scheme 1).

Oxidation of thiamine to thiochrome using different Fe^{III} complexes

The ability of different Fe^{III} complexes to oxidize thiamine to thiochrome was evaluated under physiological conditions. $K_3Fe(CN)_6$ (E^0 = 0.36 V) was used as the reference oxidant, and compared to the following systems: Fe^{III}-ethylenediamine tetraacetic acid (EDTA) ($[FeY]^-/E^0$ = 0.12 V), Fe^{III}-citrate (E^0 = 0.60 V), and Fe^{III}-glutamate (E^0 = 0.74 V).[18,19] Except for commercially purchased $K_3Fe(CN)_6$, all of the complexes were generated,



Scheme 1. The main steps of the proposed method. Step 1: oxidant consumption ($Fe(CN)_6^{3-}$) by the antioxidant compound; step 2: reaction of the remaining oxidant with thiamine, to form the thiochrome (fluorophore).

employing ligand excesses of 50 and 100 fold. The results of this evaluation are shown in Figure S1 (Supplementary Information (SI) section). Intensities of the fluorescence signals generated by thiamine oxidation were evaluated, which were from 94 to 76% lower than that of $K_3Fe(CN)_6$. Increasing the concentration of the EDTA, citrate, and glutamate in the medium decreased the thiamine oxidation efficiency.

Despite the high reduction potential of Fe^{III}-citrate and Fe^{III}-glutamate complexes, there was no appreciable formation of thiochrome, possibly due to the generation of non-fluorescent thiamine oxidation products.[20] Besides, the complexes volume may have had an influence, since FeY^-, $Fe(citrate)_3^{3-}$ and $Fe(glutamate)_2$ complexes are voluminous when compared to $Fe(CN)_6^{3-}$, possibly, making it difficult for the oxidant to reach the thiamine.

Although it does not participate in any biological process, $K_3Fe(CN)_6$ was selected due to several advantages, such as (*i*) low toxicity compared to other oxidants described in the literature (Hg^{II}, for example);[13] (*ii*) it is a Fe^{III} complex (which are widely present in the biochemical process); (*iii*) it has a large formation constant ($\log\beta_6 = 44$), and is stable over a wide pH range; (*iv*) it has a redox potential of 0.36 V; and is a selective oxidant, since most antioxidant compounds in foods present a redox potential range of 0.10 to 0.60 V; (*v*) it is commercially available with low cost and guaranteed purity; (*vi*) it does not exhibit intrinsic fluorescence and generally reacts rapidly with different reducing compounds, and (*vii*) is already regularly employed in other methods to evaluate the concentration of total phenolic compounds (Prussian Blue and 4-aminoantipyrine (4-AAP)).[5,21,22]

Preliminary evaluation of the system in the presence of an antioxidant compound

Preliminary tests were carried out aiming at an experimental condition where fluorescence reduction (oxidant consumption) would occur in the presence of an antioxidant compound, as compared to the reference system (absence of antioxidant compound). Following experimental protocols for thiamine determination, the oxidizing agent is always in high excess (20-fold, for example).[20,23] We, therefore, tried to evaluate how the variation of the $K_3Fe(CN)_6$ concentration might influence the analytical response of the proposed method. For this, the fluorescence signal variation was evaluated against two $K_3Fe(CN)_6$ concentration level, always in the presence and absence of an antioxidant compound (gallic acid, GA) which was used as an analytical standard for the optimization of all steps of the developed method (Figure 1).

From the comparison of the reference solutions signals (spectra a and c), an unexpected behavior was obtained; an increase in the $K_3Fe(CN)_6$ concentration led to a decrease in the fluorescence signal. The fluorescent intensity of the reference solution (blank) where $K_3Fe(CN)_6$ was in higher concentration (spectrum c) is less than the signal in the presence of the antioxidant compound, in this case, the GA (spectrum b). Contrasting behavior may be observed comparing spectra a and d where the concentration of $K_3Fe(CN)_6$ is 10 times lower in relation to the reactional conditions found for spectra b and c. This profile can be explained by oxidation/degradation of thiochrome (forming non-fluorescent species) due to the high excess (ca. 38 times) of oxidant concerning thiamine.[20] Thus, it

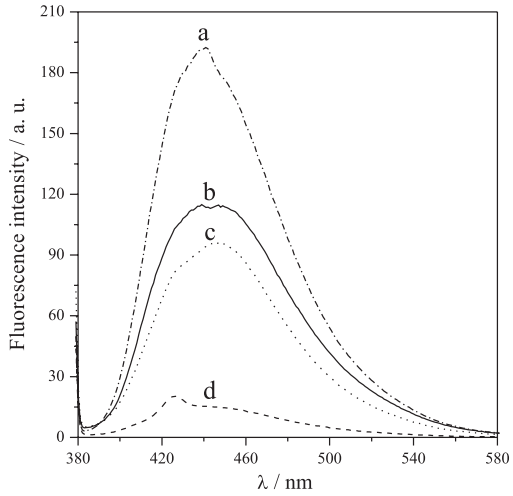


Figure 1. Influence of the oxidant ratio on the inhibition of thiamine oxidation by $K_3Fe(CN)_6$ in the intensity of fluorescence. (a) Reference solution with $K_3Fe(CN)_6$ (0.1 mM); (b) GA and $K_3Fe(CN)_6$ (1.0 mM); (c) reference solution with $K_3Fe(CN)_6$ (1.0 mM) and (d) GA and $K_3Fe(CN)_6$ (0.1 mM). Conditions: 1.0 mg L^{-1} GA (5.9 μM), 0.7 mg L^{-1} thiamine (2.6 μM) and 0.1 M bicarbonate/carbonate buffer (pH = 10).

became evident that the relationship of excess oxidant to thiamine cannot be too high, and is an essential parameter of the method to be optimized.

Evaluating the order of reagent addition and reactional kinetics

This evaluation was carried out to verify whether the order of addition of the reagents might influence the intensity of the analytical signal. Two distinct orders were evaluated: thiamine + $K_3Fe(CN)_6$ + GA (order 1), and GA + $K_3Fe(CN)_6$ + thiamine (order 2) (Figure S2, SI section). When the reagents were added in order 1, no decay of the fluorescence signal was observed; it remained practically constant, even with an increase in the concentration of the antioxidant compound. This result may be attributed to the reaction between thiamine and the oxidant that occurred before the addition of GA. Therefore, the reaction with the remaining $K_3Fe(CN)_6$ did not lead to a reduction of the analytical signal, since the formation of thiochrome had already occurred. However, when order 2 was evaluated, the fluorescence decay presented a linear trend, due to the previous reaction between GA and $K_3Fe(CN)_6$. Thus, the concentration of the oxidant was reduced in the reaction medium leading to lower thiochrome formation and the decrease in analytical signal. Thus, for future experiments, the addition of the reagents in order 2 was maintained.

The kinetics of thiochrome formation in the absence and presence of an antioxidant compound (0.5 mg L^{-1} GA) was evaluated. The reaction was quickly completed, and the GA did not alter the kinetic profile, only the magnitude of the analytical signal. Although 5 min is enough time to reach equilibrium, we chose to use 10 min, since when analyzing real samples where there is a mixture of compounds, longer reaction time is guaranteed.

Thus, a time minimum of 10 min after the addition of the reagents was selected (Figure 2).

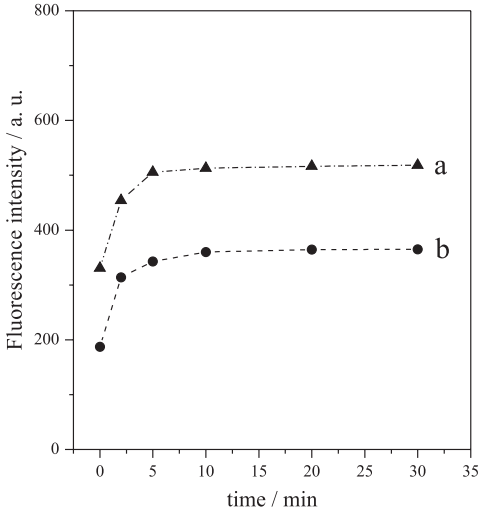


Figure 2. Reaction kinetic profile using gallic acid (GA) as a model antioxidant compound. (a) $K_3Fe(CN)_6$ + thiamine and (b) $K_3Fe(CN)_6$ + GA (0.5 mg L^{-1}) + thiamine. Conditions: thiamine (3.33 μM), $K_3Fe(CN)_6$ (50 μM) and bicarbonate/carbonate buffer (0.1 M, pH 10).

Finally, after established the parameters related to the reagent addition order and reaction time, an analytical curve was obtained to assess the performance of the proposed method (Figure 3), which showed a good linear fit up to 5 mg L^{-1} (gallic acid).

Evaluation of the pH and the buffer system

The reaction for thiochrome formation occurs preferentially in an alkaline medium. When the objective is the higher conversion of thiamine to thiochrome, it is usually done with strong bases (NaOH or KOH) to promote the necessary conditions for the reaction. At this stage of the optimization, the pH of the medium was evaluated, as well as the buffer system. Three buffer systems were assessed in different pH ranges: bicarbonate/carbonate buffer (pH 8 to 11), borate (pH 8 to 10), and Britton-Robinson (pH 9 to 12). The formation of the thiochrome (reference signal) under different pH conditions was evaluated, and the fluorescent probe production was favored in the pH range from 9 to 10, using bicarbonate/carbonate and Britton-Robinson buffer solutions (Figure 4).

The pH values above 10 led to a reduction in fluorescent signal (except for the borate buffer) which is associated with

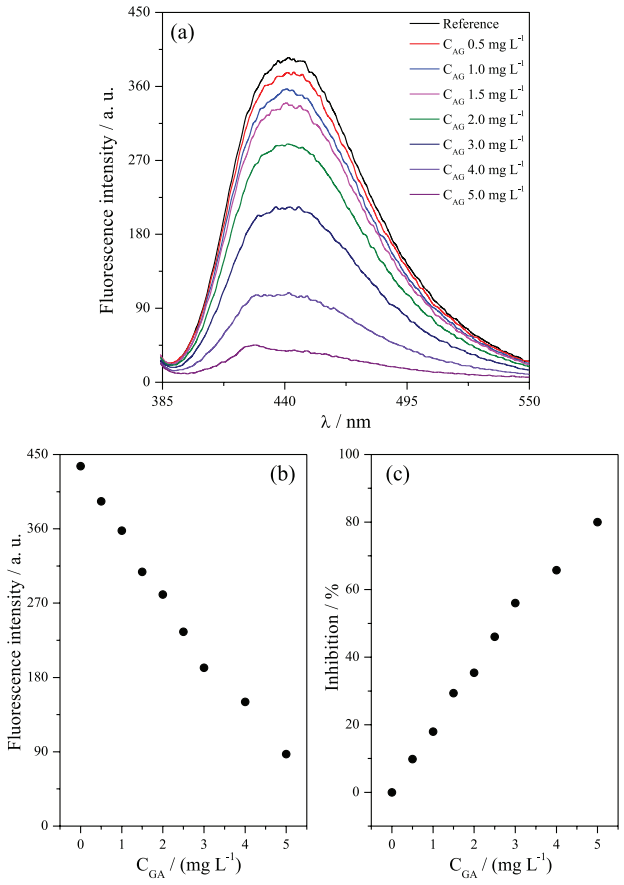


Figure 3. (a) Spectra of the generated thiochrome, in the absence (reference), and with different concentrations of gallic acid (0.5-5.0 mg L^{-1}); (b) linear decreasing of the analytical signal at 440 nm proportional to GA concentration; (c) inhibition of analytical signal proportional to the increase of GA concentration. Conditions: thiamine (3.33 μM), $K_3Fe(CN)_6$ (50 μM), bicarbonate/carbonate buffer (0.1 M, pH 10). Measurements were performed after 10 min at least.

less fluorescent probe (thiochrome) formation (Figure 4a); this is possibly due to the generation of intermediate (non-fluorescent) species.[24] Additionally, the differentiated behavior of the borate buffer in relation to the others may be associated with the formation of an ionic pair through interaction between the thiamine (positive charge) and the borate ion, or by the anion generated from the reaction between H_3BO_3 and chloride ions in solution.[25]

The inhibition of thiochrome formation in the presence of the antioxidant compound was also evaluated (Figure 4b). The pH of the medium is determinant for the degree of antioxidant compound dissociation, altering potential, selectivity, and chemical behavior. For phenolic compounds, an increase in pH facilitates proton abstraction and, consequently, oxidation of the species.[26] In this study, it was observed that an increase in pH resulted in analytical sensitivity reduction, which may be associated with an increased concentration of the dissociated base fraction, and thus more significant electrostatic interaction with thiamine, preventing the activity of the oxidant. Therefore, the bicarbonate/carbonate buffer at pH 8.0 was selected, for the following criteria: (*i*) it presented the highest sensitivity; (*ii*) bicarbonate/carbonate buffer is a base already used in a method for determination of total phenolic compounds (Folin-Ciocalteu); and (*iii*) the pH value selected and the bicarbonate/carbonate buffer constituents are common, and close to physiological pH conditions. Thus, for our subsequent studies, the pH and buffer solution were maintained.

Evaluation of the buffer solution concentration

After the selection of the buffer system and its respective pH value, the effect of the concentration of this solution from 0.025 to 0.2 mol L^{-1} (HCO_3^-/CO_3^{2-}) was evaluated in relation to thiochrome inhibition and GA. Following the

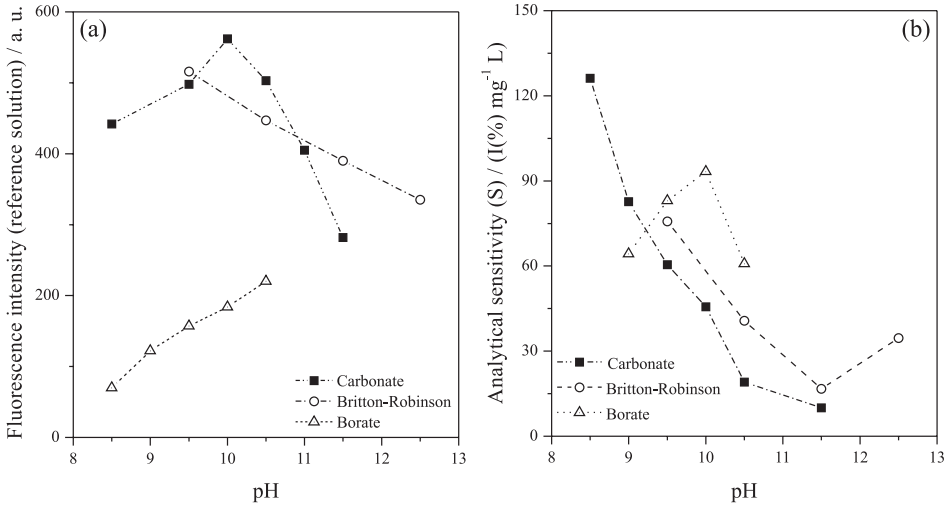


Figure 4. Evaluation of pH and buffer system influence on (a) fluorescence of the reference solution and (b) analytical sensitivity of the method. Conditions: thiamine (3.33 μM), $K_3Fe(CN)_6$ (50 μM), reaction time of 10 min. All buffer solutions in this study were fixed at 0.1 M.

results presented in Table 1, it was observed that the buffer concentration leading to the highest sensitivity would be equal to 0.05 mol L^{-1}. However, aiming to ensure greater buffer system efficiency, we selected the 0.1 mol L^{-1} concentration even with a 20% reduction in analytical sensitivity. For higher buffer solution concentration values, an increase in the linear range was noticed, despite the sensitivity being similar to the selected condition. Besides, at 0.1 mol L^{-1}, better linearity was obtained (r = 0.9993), when compared with the other conditions. Thus, for subsequent studies, the final concentration of the buffer solution was fixed at 0.1 mol L^{-1}.

Table 1. Analytical curve parameters associated with bicarbonate/carbonate buffer concentration. Conditions: thiamine (3.33 μM), $K_3Fe(CN)_6$ (50 μM), pH = 8.0 and reaction time of 10 min

Concentration / M	Linear range (GA) / (mg L^{-1})	$I = aC_{GA} + b$		
		a	b	r
0.025	0.025-0.3	151.3	20.6	0.9935
0.05	0.025-0.3	158.5	18.6	0.9867
0.10	0.025-0.3	126.2	12.5	0.9993
0.15	0.05-0.4	127.7	12.2	0.9946
0.20	0.05-0.4	137.3	−2.72	0.9989

GA: gallic acid; I: inhibition percentage; a: slope; C_{GA}: gallic acid concentration; b: linear coefficient; r: correlation coefficient.

Evaluation of the $K_3Fe(CN)_6$/thiamine proportion

Since excess of oxidant can promote oxidation of thiochrome to non-fluorescent products, we initially chose to evaluate the ratio of $K_3Fe(CN)_6$ to thiamine and not the concentration of each compound separately. In this experiment, the proportions ranged from 5 to 30 times $K_3Fe(CN)_6$ to thiamine. As observed in Figure 5a, fluorescent compound formation reaction increased with the $K_3Fe(CN)_6$/thiamine ratio until reaching an excess of 15-fold. For the fixed thiamine concentration (3.33 μmol L^{-1}), an oxidant excess of 15-fold led to maximum thiochrome production.

In another approach, the influence of $K_3Fe(CN)_6$/thiamine ratio on the analytical sensitivity of the method

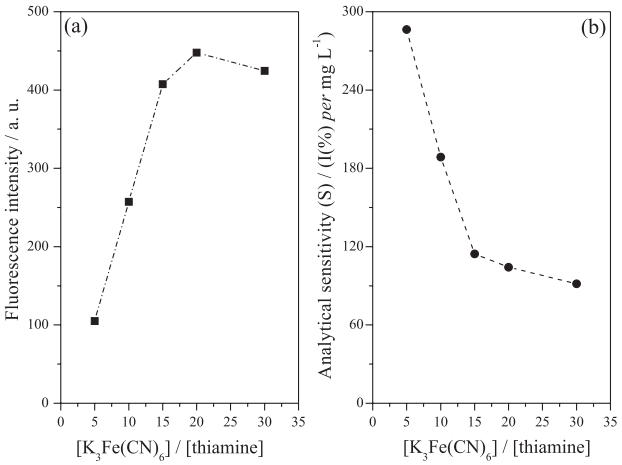


Figure 5. Evaluation of the $K_3Fe(CN)_6$/thiamine molar ratio. (a) Fluorescence signal from the reference solution (λ_{em} = 440 nm) and (b) analytical sensitivity. Conditions: $K_3Fe(CN)_6$ (50 μM), bicarbonate/carbonate buffer at 0.1 M (pH = 8) and reaction time equal to 10 min.

was observed in the presence of GA (Figure 5b). The lower the $K_3Fe(CN)_6$/thiamine ratio, the higher the sensitivity (S) of the proposed method. This result is related to the lower concentration of $K_3Fe(CN)_6$ in the reaction medium (presenting a lower excess) after being consumed by reaction with GA and resulting in more significant inhibition of the analytical signal. Above an excess of 15 times, smaller variation in sensitivity was observed. Thus, the ratio selected for $K_3Fe(CN)_6$/thiamine ratio was 15, since above this ratio, the thiamine to thiochrome conversion was higher, and the method was more robust due to its lower sensitivity variation (9%) with an increase in excess $K_3Fe(CN)_6$.

Evaluation of reagent concentrations

After the selection of the molar $K_3Fe(CN)_6$/thiamine ratio, the influence of the concentration of the species was evaluated, maintaining the molar ratio constant at 15:1. The results obtained for this evaluation are presented in Table 2. With higher reactant concentrations, the thiochrome formation was favored (reference signal), and consequently, analytical sensitivity decreased. In accordance with the results, it was established that the

Table 2. Evaluation of $K_3Fe(CN)_6$/thiamine ratio multiples (1, 2 and 4). Conditions: bicarbonate/carbonate buffer at 0.1 M (pH = 8) and reaction time of 10 min

[Thiamine] / μM	$[K_3Fe(CN)_6]$ / μM	Signal reference	Linear range / (mg L^{-1})	$I = aC_{AG} + b$		
				a	b	r
1.67	25	146	0.025-0.3	185.2	25.5	0.9899
3.33	50	360	0.025-0.3	144.7	17.3	0.9986
6.66	100	772	0.125-1.0	56.1	20.6	0.9872

I: inhibition percentage; a: slope; C_{GA}: gallic acid concentration; b: linear coefficient; r: correlation coefficient.

concentrations of the solutions would be maintained at $[K_3Fe(CN)_6] = 50\ \mu mol\ L^{-1}$ and $[thiamine] = 3.33\ \mu mol\ L^{-1}$.

Finally, the concentration of thiamine used was close to the saturation concentration of this vitamin in the human body (ca. 0.7 μmol L^{-1}),[27] which meets the requirement of using species that act as probes and that are naturally present in biological systems.[7]

Evaluation of the methodology using different types of thiamine

Thiamine (combined with phosphate groups) acts in the pyruvate dehydrogenase enzymatic complex as a coenzyme for oxidative decarboxylation of pyruvate to form active acetate (acetyl-CoA), which is the main component in the metabolic pathway of the citric acid cycle.[28] Thus, we tried to evaluate the methodology against other thiamines containing a phosphate group in their structure and that participate in important metabolic events. The analytical performances of thiamine, thiamine monophosphate (TMP), and thiamine pyrophosphate (TPP) were compared as part of the evaluation of the methodology. The influence on thiamine structural alterations was evaluated using analytical curves employing I_F (in percentage) as an inhibition parameter for the fluorescence signal (uF) in the function of gallic acid concentration in the medium. The obtained curves are related to the following equations:

$$I_{thiamine} = (218 \pm 6)C_{GA} + (2.1 \pm 1.0),\ r = 0.9984 \quad (2)$$
$$I_{TMP} = (607 \pm 75)C_{GA} + (6.3 \pm 4.6),\ r = 0.9777 \quad (3)$$
$$I_{TPP} = (248 \pm 20)C_{GA} + (2.4 \pm 2.1),\ r = 0.9847 \quad (4)$$

In this step, it was verified that all of the thiamines could be used for the analysis of total antioxidant capacity. The higher sensitivity obtained from the phosphorylated thiamines led to a decrease in the linear range. However, considering the cost of phosphorylated thiamines, the use of thiamine despite the lower sensitivity would still be advantageous for this type of application allowing analysis cost savings.

Figures of merit for the proposed method

After the optimization of the spectrofluorimetric method variables as intended for the determination of total antioxidant capacity, the figures of merit were established. The proposed method presented a linear concentration range expressed in GA of 0.025 to 0.3 mg L^{-1} corresponding to the analytical curve (Figure S3, SI section), described by the equation $I_F = (218.4 \pm 6.2)C_{GA} + (2.06 \pm 1.0)$ ($r = 0.9984$), with a limit of detection (3σ) of 0.008 mg L^{-1}. The RSD was calculated for concentration values at the extremes of the analytical curve since they are the regions with the most significant instrumental error. Thus, for the concentrations of 0.05 and 0.3 mg L^{-1} of GA, RSD values of 3.4 and 1.1% were respectively obtained, indicating that the proposed methodology presented good precision.

Evaluation of proposed method with other antioxidant and reducing compounds

To evaluate the proposed method, the following antioxidant and reducing capable compounds were selected: gallic acid, ascorbic acid, quercetin, BHT, Trolox®, cysteine, and glucose. The results of this assay are presented in Table 3. The comparison parameter for the different compounds in this study was EC_{50}; that is, the concentration of the compound capable of inhibiting 50% of the reference signal.[15] Thus, the lower the concentration, the more efficiently the compound consumes the oxidizing agent. As observed (Table 3), it was found that gallic acid more effectively inhibited the formation of thiochrome, followed by quercetin, ascorbic acid, Trolox, cysteine, BHT, and glucose.

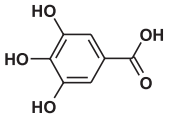
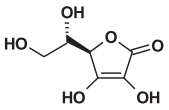
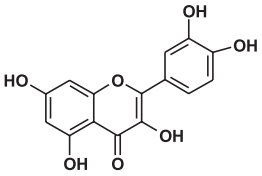
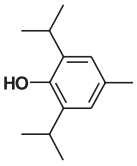
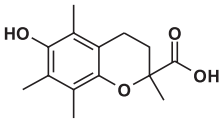
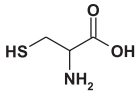
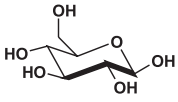
Glucose did not efficiently inhibit the formation of the fluorescent compound. When a sample with 100 mg L^{-1} in glucose was evaluated, I ca. 10% was observed. Thus, the concentration of glucose in a given sample should not interfere with the results obtained by the proposed method in the function of dilution. Besides, the oxidation reaction of reducing sugars is more effective at higher pH values, which allows dislocation of the sugar molecule equilibrium from cyclic to acyclic, facilitating oxidation.[29]

From the results, it was possible to infer that for reducing and antioxidant properties of these compounds, gallic acid and quercetin were the most effective antioxidants.[15] Although ascorbic acid and Trolox are substances of different classes, they have similar EC_{50}. Cysteine and BHT presented the same EC_{50} value, which indicates that the results may be influenced by aspects related to lipo- or hydrophobicity as well as by the redox potential of each compound since the method is based on electron transfer.

Determination of antioxidant capacity in beverages

The assay results for antioxidant capacity in beverages were compared to the results achieved using $DPPH^{\bullet}$ radicals (antioxidant), $ABTS^{\bullet+}$ (antioxidant), and Folin-Ciocalteu (total phenolics) methods. Thus, to validate the proposed method, as proof of concept, we used well-established samples, in this case, wines (white and red), tea, and infusions for studies of antioxidant determination (Table 4).

Table 3. Analytical parameters of different antioxidants and reducing compounds against the proposed method. Conditions: thiamine (3.33 μM), $K_3Fe(CN)_6$ (50 μM), bicarbonate/carbonate buffer at 0.1 M (pH = 8) and reaction time equal 10 min

Compound	Structure	Linear range / (mg L^{-1})	Analytical curve $I = aC_{AO} + b$	r	EC_{50} / (mg L^{-1})	EC_{50} / μM
Gallic acid		0.025-0.3	$I = (218.2 \pm 6.2)C_{AO} + (2.1 \pm 1.0)$	0.9984	0.2	1.0
Ascorbic acid		0.5-2.0	$I = (35.8 \pm 2.6)C_{AO} + (3.4 \pm 2.6)$	0.9953	1.2	7.1
Quercetin		0.1-0.75	$I = (97.5 \pm 7.8)C_{AO} + (4.9 \pm 3.3)$	0.9936	0.4	1.3
BHT		0.5-8.0	$I = (10.2 \pm 0.3)C_{AO} + (0.5 \pm 1.2)$	0.9985	5.0	22.7
Trolox		0.5-4.0	$I = (21.5 \pm 0.5)C_{AO} - (3.2 \pm 1.0)$	0.9988	2.4	9.7
Cysteine		0.5-8.0	$I = (10.2 \pm 1.3)C_{AO} + (0.5 \pm 1.4)$	0.9985	5.0	22.7
D-Glucose		10-100	$I = (0.09 \pm 0.1)C_{AO} + (0.07 \pm 0.1)$	0.9986	500[a]	1315[a]

[a]Value extrapolated from the analytical curve. I: inhibition percentage; a: slope; C_{AO}: antioxidant capacity; b: linear coefficient; r: correlation coefficient; EC_{50}: concentration of the compound capable of inhibiting 50% of the reference signal; BHT: butylhydroxytoluene.

In accordance with the results, the samples of red wine and tea (white and green) presented the highest antioxidant capacities. This result was expected since the concentrations of phenolic compounds are high in these types of samples.[16,30] The results are consistent with other studies[31] considering the total concentration of phenolic compounds. The results obtained by the proposed method were compared statistically with the methods of Folin-Ciocalteu, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$.

By means of the paired Student's *t*-test at a 95% confidence interval, the following experimental *t*-values were obtained: $t = 1.58$ (proposed *vs*. FC); $t = 3.49$ (proposed *vs*. DPPH$^{\bullet}$), and $t = 2.64$ (proposed *vs*. ABTS$^{\bullet+}$), with $t_{critical} = 2.18$ (degree of freedom (ν) = 12, two-tailed). From this evaluation, the proposed method would be statistically similar to the method of Folin-Ciocalteu alone ($t_{experimental} < t_{critical}$), but this statistical evaluation of the results is not adequate, due to the different principles of each method, and which parameter is measured.[32]

For a second statistical evaluation, ANOVA (one-way) was applied to the data at a 95% ($\alpha = 0.05$) confidence interval. In this test, it was verified that there was no significant difference between the groups ($F_{calculated} = 2.12$ *vs*. $F_{critical} = 2.79$; $p = 0.1092$) for the same factor (antioxidant capacity). Except for the DPPH$^{\bullet}$ method, the other methods presented good alignment values. This statistical method uses the mean and total group variance for comparison. When the values of all samples are used as the basis for the box plot graph (Figure 6), it is verified that even the results of the DPPH$^{\bullet}$ method are systematically

Table 4. Determination of total antioxidant capacity in beverages using the proposed method, Folin-Ciocalteu (FC), DPPH•, and ABTS•+ (n = 3)

Sample	Beverage	Type	Dilution				Equivalents of GA / (mg L^{-1})			
			I	II	III	IV	Folin-Ciocalteu (I)	DPPH• (II)	ABTS•+ (III)	Proposed method (IV)
1	wine	white	15	80	250	800	295 ± 1	70 ± 6	232 ± 3	265 ± 1
2	wine	white	20	80	250	800	330 ± 1	43 ± 2	244 ± 3	228 ± 2
3	wine	white	20	80	250	800	387 ± 1	63 ± 1	273 ± 3	322 ± 1
4	wine	red	100	600	1000	3000	1227 ± 1	435 ± 20	976 ± 25	801 ± 1
5	wine	red	100	600	1000	3000	1082 ± 6	264 ± 1	777 ± 12	605 ± 2
6	wine	red	100	600	1000	3000	1263 ± 6	181 ± 1	893 ± 12	816 ± 1
7	tea	green	80	1500	900	2000	1117 ± 1	694 ± 16	1213 ± 13	872 ± 1
8	tea	white	80	1500	900	2000	1067 ± 3	625 ± 16	1140 ± 2	836 ± 1
9	tea	black	25	500	300	1000	432 ± 2	231 ± 16	324 ± 6	190 ± 15
10	infusion	fennel	5	25	40	100	73 ± 1	15.0 ± 0.5	46 ± 1	25 ± 1
11	infusion	balm	2	5	10	20	19.0 ± 0.1	4.0 ± 0.1	8.0 ± 0.1	5.0 ± 0.2
12	infusion	gorse	15	80	150	400	139 ± 1	26.0 ± 0.1	99 ± 2	85 ± 1
13	infusion	chamomile	5	30	40	100	87 ± 1	18.0 ± 0.1	33 ± 1	13 ± 1

GA: gallic acid; DPPH•: 2,2-diphenyl-1-picrylhydrazyl; ABTS•+: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate).

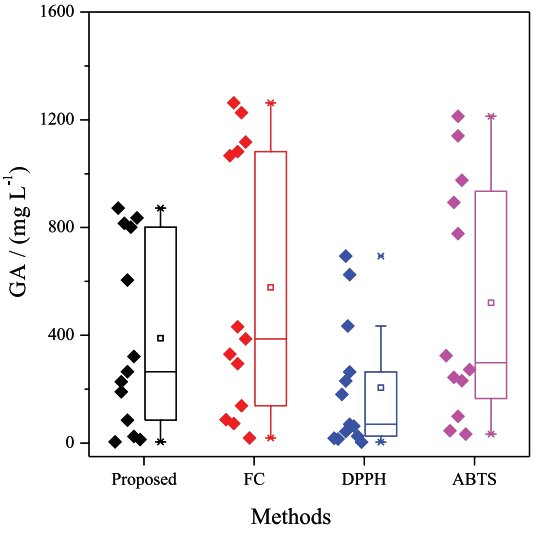


Figure 6. Box plot for the results obtained by the different methods (n = 13).

smaller relative to the overall mean (without outlier values considered).

However, the comparisons carried out above have serious limitations since although all of the methods evaluated were calibrated with GA, it cannot be guaranteed that within the universe of complex samples (such as wine) that each probe used would react in the same (or similar) way with the different compounds of the samples. Besides, between the methods, there are different mechanisms of action and competition between the type(s) of mechanism(s) in the same method (as in the case of DPPH•, for example), which may still occur. Therefore, the results obtained from the applied statistical tests are subject to variations depending on the type of sample and the methods being compared.

To confirm any relation between results obtained by the different methods, we chose to perform correlation analysis (95% confidence interval). For these relations, the following equations were obtained (Figure S4, SI section):

$$C_{FC} = (1.38 \pm 0.09)C_{proposed} + (39 \pm 47),\ r = 0.9768 \quad (5)$$

$$C_{DPPH} = (0.59 \pm 0.11)C_{proposed} + (23 \pm 56),\ r = 0.8502 \quad (6)$$

$$C_{ABTS} = (1.28 \pm 0.07)C_{proposed} + (17 \pm 35),\ r = 0.9842 \quad (7)$$

When the slope is close to unity, and the linear is near a null value (considering the range of values employed), the correlation is considered to be perfect.[17] Based on the equations obtained above and Table 4, it can be seen that the results of the proposed method are systematically inferior to the Folin-Ciocalteu and the ABTS•+ radical methods. Therefore, these methods show a similar trend in the distribution of results, indicating that there is a good correlation, which may be associated with the electron transfer mechanism that is dominant in both methods. However, both have a higher redox potential than the proposed method, being FC (0.70 V) and ABTS•+ (0.68 V).[33,34] Thus, they can oxidize species that are outside the range potential of $K_3Fe(CN)_6$ (0.36 V),[35] and this way, presenting a concentration level higher than proposed method.

For the DPPH• radical, the results obtained were systematically inferior in relation to the proposed method, which may be related to the fact that the DPPH•

radical preferred mechanism of action is the transfer of hydrogen atoms, and not the transfer of electrons. In addition, this radical reacts preferentially with lipophilic compounds, which in the samples analyzed, must be in low concentration. Thus, little correlation with the results of the proposed method was observed (Figure S4, SI section).

Spectrophotometric methods are widely used to determine antioxidant capacity due to their simplicity, speed, and low cost. However, colored samples may present spectral interference and compromise the accuracy of the result.[7,36] Therefore, fluorimetric methods prove to be an alternative because they are simple, sensitive, and present high selectivity.[37,38] In this context, there are few methods in the literature for determining the total antioxidant capacity based on fluorimetric detection. Among the methods already established can be mentioned: oxygen radical absorbance capacity (ORAC),[39,40] cerium(IV) ions reducing antioxidant capacity (CERAC),[37] and the method based on the oxidation of lucigenin to *N*-methylacridone (NMA) with H_2O_2[38] (Table S1, SI section). Considering the comparison, although the proposed method shows a promising alternative for the determination of total antioxidant capacity using a probe of biological relevance (vitamin B1), the analysis of hydrophobic samples and compounds can be compromised due to the low solubility in an aqueous medium of the thiamine and $K_3Fe(CN)_6$.

Conclusions

The proposed method allows spectrofluorometric determination of the total antioxidant capacity of beverage samples using thiochrome inhibition, as derived from the oxidation of thiamine (vitamin B_1) through $K_3Fe(CN)_6$. The method was shown to be fast and simple, using easily available instrumentation, and biological molecules. The endpoint of the reaction was well established and performed at close to physiological conditions. The application of the proposed method to determine the antioxidant capacity of wine samples (red and white), teas, and infusions, presented excellent correlation with the results obtained by both the Folin-Ciocalteu and $ABTS^{\bullet+}$ methods. However, the method still shows limited application to samples and compounds of a hydrophobic nature. Finally, the proposed method proved to be versatile, allowing the use of antioxidant compounds that present different properties as standards, as well as potential application with phosphorylated thiamines.

Supplementary Information

The supplementary information associated with this work can be found at http://jbcs.sbq.org.br as PDF file.

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