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Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity

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ABSTRACT

In the present work, a rapid and high-throughput Folin-Ciocalteu (F-C) reducing capacity assay adapted to routine/screening analysis was developed. In order to attain a fast F-C reducing kinetic reaction, the reaction conditions of the classical time-consuming F-C assay were modified and the influence of alkali and F-C reagent concentration was evaluated using gallic acid as standard. The proposed method was performed in a 96-well microplate format and it was applied to several phenolic compounds and food products (wines, beers, infusions and juices) providing F-C reducing capacity results after 3 min of reaction similar to those obtained by the time-consuming (120 min) conventional method. The additive and synergistic effect of reducing nonphenolic compounds usually found in food samples was also investigated. Ascorbic acid and ferrous sulfate provided an additive effect, while for fructose, glucose and sodium sulfite a synergistic effect was obtained. The detection limit was 0.25 mg L^{-1} (as gallic acid) and the repeatability was <1.6% (n = 12).

1. Introduction

The health effects of dietary antioxidants depend on their respective intake levels, bioavailability, synergism and reducing capacity to protect against the harmful action of reactive oxygen and nitrogen species [1,2]. In this regard, several epidemiological studies have shown an inverse association between the development of a wide range of degenerative and nondegenerative diseases and the consumption of food rich in antioxidant compounds as wines, fruits and vegetables [2,3]. For this reason, in the last decade a huge interest has been dedicated to develop and/or improve reliable analytical methods to determine the antioxidant capacity of foodstuffs.

Among the battery of antioxidant methods available [4,5], the Folin-Ciocalteu (F-C) assay is a widespread method in agrochemical area and food industry owing to its simplicity, availability of commercial reagent and fairly standardized procedure. This method has been used for many years to measure the total phenolic content in natural products, but the reaction mechanism is an oxidation/reduction reaction not specific to phenolic compounds. In fact, the F-C assay measures the ability of both phenolic and nonphenolic compounds in alkaline medium to reduce the phosphomolybdic/phosphotungstic acid reagent to blue complexes that are detected spectrophotometrically [6]. For this reason, the parameter assessed was stated as "F-C reducing capacity" and the results have been correlated to antioxidant activity determined by other widely used antioxidant methods [7].

In the original F-C assay [6], the carbonate buffer is used for pH adjustment and the end-point of the reaction was attained after 120 min at room temperature, which makes its implementation for routine analysis difficult. Considering that the reaction kinetics increases with F-C reagent concentration, alkalinity and temperature of the medium, different combinations of carbonate concentration and reaction temperature have been used to reduce the time necessary to attain the maximum color. Procedures using higher temperatures of 40 and 50 °C have reduced the end-point times to 20 and 15 min, respectively [8,9]. However, under these conditions the thermolabile antioxidants may be subject to destruction/decomposition and thereby an underestimate F-C reducing value is obtained. Recently, an automatic flow procedure for the assessment of F-C reducing capacity at room temperature of food products was developed [10]. In this method, the carbonate buffer solution used as alkaline reagent in the classical F-C assay [6] was replaced by sodium hydroxide solution as the rate of reduction of F-C reagent is substantially increased. The reaction time was reduced from 2 h to 4 min with similar results for all samples analysed. Despite of the strict control of reaction conditions and high level of automation attained, the implementation of automatic flow methods in common research/industry laboratories is more complex when compared to batch methods. Thus, a

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microliter and rapid F-C assay performed at room temperature and adequate to routine/screening determinations in a large number of food samples and dietary supplements is of upmost importance [4,7,11].

Therefore, the main purpose of the present work is the improvement of the time-consuming F-C assay in order to attain a fast and high-throughput methodology providing F-C reducing capacity results similar to those obtained by the classical procedure. To achieve this objective, the carbonate buffer was replaced by sodium hydroxide and the assay was adapted to a 96-well microplate format. The concentration of the reagents and their influence in the reaction kinetics were thoroughly studied, using gallic acid as standard compound. The proposed methodology was applied to several phenolic compounds and food products and the results were compared with the classical method. Moreover, the additive and/or synergistic effect of some reducing nonphenolic compounds usually found in food samples such as organic acids (ascorbic, citric and tartaric), sugars (fructose, glucose and sucrose), ferrous ion and sulfites were also investigated.

2. Experimental

2.1. Reagents and solutions

All chemicals used were of analytical-reagent grade with no further purification. Ferrous sulfate, Folin-Ciocalteu reagent (FCR), gallic acid and resorcinol were purchased from Sigma (St. Louis, MO). Ascorbic acid, caffeic acid, catechol, cinnamic acid, ferulic acid and propyl gallate were obtained from Aldrich (Milwaukee, WI). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ellagic acid, (±)-catechin and sodium carbonate were obtained from Fluka (Buchs, Switzerland). Pyrogallol and tannic acid were purchased from Riedel-de-Haën (Seelze, Germany). Acetone, citric acid, ethanol, methanol, tartaric acid, sodium sulfite, D-(+)-glucose, fructose, sodium hydroxide and sucrose were obtained from Merck (Darmstadt, Germany). Water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol absolute pro-analysis were used for the preparation of all solutions. Methanol and acetone pro-analysis was also used.

The stock solutions of ascorbic acid (3.0 mM), citric acid (25 mM), ferrous sulfate (3.0 mM), fructose (555 mM), gallic acid (3.0 mM), D-(+)-glucose (555 mM), sodium sulfite (80 mM), sucrose (300 mM) and tartaric acid (33.3 mM) were prepared by dissolving the respective compound in water. Stock solutions of caffeic acid (3.0 mM), catechol (4.5 mM), cinnamic acid (5.0 mM), ferulic acid (3.0 mM), propyl gallate (3.0 mM), pyrogallol (3.0 mM), resorcinol (5.0 mM), tannic acid (0.3 mM) and trolox (3.0 mM) were prepared in ethanol solution 50% (v/v). Stock solutions of (±)-catechin (2.0 mM) and ellagic acid (1.0 mM) were prepared in ethanol. The working solutions were prepared by rigorous dilution of the respective stock solutions in water.

For assessment of F-C reducing capacity of compounds and food products, sodium hydroxide solution (0.35 M) was daily prepared from a stock solution of approximately 0.85 M, whose rigorous concentration was determined by titration with an acid standard solution (Tritisol, Merck). FCR was diluted 1:5 (v/v) with water. Working standard solutions containing gallic acid (2.5–37.5 mg L^{-1}) were daily prepared by rigorous dilution with water from a stock solution of 500 mg L^{-1}. For the classical F-C assay, sodium carbonate 6% (w/v) was prepared.

All food products (n = 20) were purchased at local markets. Herbal and tea infusions were prepared by pouring 200 mL of deionized water at 90 °C into a glass with herbal or tea bag and by brewing for 5 min. Carbon dioxide from white wines and beers were completely removed by magnetic stirring. All beverages were diluted with water just before measurement. The dilutions performed varied from 1:10 to 1:400.

2.2. Analytical procedure for the rapid microplate Folin-Ciocalteu assay

The high-throughput assay proposed for the assessment of F-C reducing capacity was performed on a microplate reader (Synergy HT, BIO-TEK) using spectrophotometric detection and microtiter 96-well plates. Hence, 50 μL of gallic acid standard solution or food sample and 50 μL of FCR (1:5, v/v) were placed in each well. After that, 100 μL of sodium hydroxide solution (0.35 M) was added. The absorbance at 760 nm of the blue complex formed was monitored with 1 min intervals until it reaches a maximum value (for optimized conditions it was 3 min). To evaluate the intrinsic absorption of sample, 50 μL of 0.4 M of acid solution was added instead of the FCR. The reagent blank was evaluated by the addition of 50 μL of water instead of standard compound or sample. All experiments were performed in quadruplicate at room temperature (25 ± 1 °C).

For comparison purposes, the classical F-C assay [6] was adapted to a microplate format. In this case, the alkali added was the carbonate buffer 6% (w/v), providing a concentration of 3% (w/v) in each well, and the absorbance was monitored during 120 min.

2.3. Assessment of F-C reducing capacity for pure compounds and for samples

For phenolic and nonphenolic compounds studied, the F-C reducing capacity was estimated by establishing a linear calibration curve from the plot of absorbance as a function of concentration of testing compound (mM). Then, the slope of this calibration curve was compared to the slope of the calibration curve for the standard compound (gallic acid). This ratio (%) reflected the F-C reducing capacity of the testing compound.

For food samples, a calibration curve relating the absorbance and the concentration of gallic acid in the range from 2.5 to 37.5 mg L^{-1} was established ($R \geq 0.9997$). The absorbance values obtained for samples were related to that of gallic acid standard curve and the F-C reducing capacity was expressed as gallic acid equivalents (mg L^{-1}). This result was multiplied by the respective dilution factor.

For the classical (carbonate medium) and for the proposed F-C assay (hydroxide medium), the absorbance values were determined after 120 and 3 min of reaction, respectively.

3. Results and discussion

3.1. Study of chemical aspects

In the F-C chemistry, the oxidation of phenols is faster when the alkalinity of the medium increases and also when phenolic moieties are not protonated. Moreover, during the oxidative reaction, the phenolate ion may be regenerated by the polymerization of oxidized phenols to dimerized products; thus, the oxidation may then not only be repeated, but also the regenerated phenol is often oxidized more easily than the original one. Therefore, the reaction must occur at alkaline pH and must reach the end-point quickly to minimize such regenerative polymerization [6]. Using the classical F-C assay, the alkalinity is assured by the carbonate buffer (pH ≈ 10) and the end-point of the reaction was fixed at 120 min. This time-consuming procedure may promote the phenolate ion regeneration as also is not suitable for routine analysis. Moreover, it is important to assure that the buffering capacity of the interconversion of carbonate and bicarbonate is not exceeded, since the production of free CO_2 bubbles may interfere in the colorimetric determination.

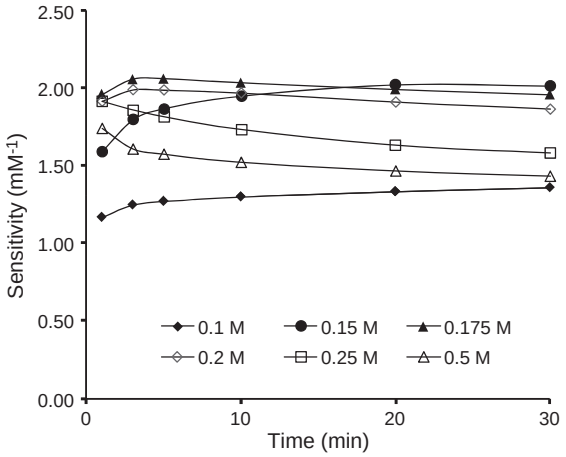

Fig. 1. Influence of NaOH concentrations (M) on sensitivity (mM^{-1}) of the methodology upon reaction time of 1, 3, 5, 10, 20 and 30 min. FCR concentration, 1:20 (v/v); $R \geq 0.9994$ ($n = 5$).

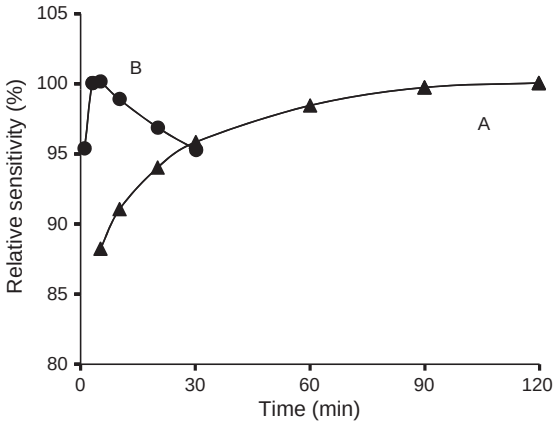

Fig. 2. Relative sensitivity (%) for gallic acid standard curves determined in different reaction conditions: (A) 3% (w/v) sodium carbonate; (B) 0.175 M sodium hydroxide. The relative sensitivity (%) was estimated by the ratio of the sensitivity at a fixed time to the sensitivity obtained at 120 min (experiment A) and 3 min (experiment B). For all calibration curves $R \geq 0.9994$ ($n = 5$).

To overcome these issues, in the proposed F-C assay the carbonate buffer was replaced by sodium hydroxide (pH > 12), since FCR reduction kinetics increases for higher alkali levels. For this reason, the influence of alkali (NaOH) and the FCR concentrations in the kinetic and sensitivity of the new methodology was evaluated. The concentrations presented in the following paragraphs are the values present in the reaction medium (after dilution).

To evaluate the influence of alkali concentration, the slope of calibration curves of gallic acid in different NaOH concentrations at several reaction times (1, 3, 5, 10, 20 and 30 min) were estimated (Fig. 1). The increase of alkali concentration decreases the reaction time necessary to attain the maximum of sensitivity. In fact, for concentrations of 0.1 and 0.15 M, the maximum of sensitivity was obtained after >30 min and 20 min, respectively. For higher concentrations as 0.175 and 0.2 M, the maximum value was obtained at 3 min of reaction time, while for 0.25 and 0.5 M it was obtained <1 min, respectively. Moreover, for concentration of 0.175 M and higher, the sensitivity of the methodology slightly decreased along the reaction time after reaching the maximum value. These results show that, the blue complex is developed more quickly as the alkali levels increase, but also a rapid destruction of the complex formed takes place, yielding to a decrease of the sensitivity during the time [10]. Considering the maximum sensitivity obtained, when the NaOH concentration varied from 0.1 to 0.175 M, the sensitivity increased about 30%. For higher concentrations as 0.2, 0.25 and 0.5 M, the sensitivity decreased approximately 3, 7 and 15%, respectively. Noteworthy, pyrogallol showed similar results to that provided by gallic acid. Therefore, the NaOH concentration of 0.175 M was selected for further studies since it provided the highest sensitivity in a short reaction time.

Adequate reagent concentration is important to assure that all phenols and other oxidizing substrates react with FCR before the reagent itself is destroyed, since it is not stable under alkaline conditions. Therefore, different FCR concentrations (expressed as dilution ratio from the commercial reagent) were evaluated: 1:40, 1:20, 1:13.3, and 1:10 (v/v), corresponding to 0.05, 0.1, 0.15 and 0.2 M of acidic solution in the final mixture. The NaOH concentration was modified in each condition in order to attain the same excess of alkali (which was 0.075 M). For concentrations of 0.15 and 0.2 M, an intense absorbance increase along the reaction time was obtained due to slight suspension formed in the reaction medium and therefore ruling out these two concentrations. To clarify that this behaviour was not specific to gallic acid, other phenolic compound (pyrogallol) and a red wine sample were tested and similar results were attained. Hence, the sensitivity of gallic acid standard curves for 0.05 and 0.1 M was estimated at 1, 3, 5, 10, 20 and 30 min. For both concentrations, the maximum of sensitivity was achieved after 3 min and a slightly decrease of 10 and 5% was attained after 30 min, respectively. Thus, the concentration of FCR chosen was 0.1 M (corresponding to 1:20, v/v) since the sensitivity attained was about 17% higher than that provided with 0.05 M of FCR.

As the majority of protocols for plant and food extracts are performed with organic solvents, especially methanol and acetone, it is important to evaluate their interference in the colorimetric reaction and establish the limit concentration (%, v/v) in the final mixture compatible with the assay. In this regard, using the optimized reaction conditions stated above (0.175 M NaOH, FCR 1:20, v/v and 3 min), the absorbance value of gallic acid standard solution (10 mg L^{-1}) with different methanol or acetone concentrations was related to that attained without organic solvent. The concentrations present in the final mixture varied from 0.25 to 10% (v/v). For methanol, the absorbance increase was <2.3% for concentrations up to 2.5% (v/v), whereas 5, 7.5 and 10% (v/v) originated 3.2, 6.7 and 7% of absorbance decrease, respectively. In the case of acetone, 0.25% (v/v) gave an absorbance decrease of 1.2%, while 1.25, 2.5 and 5% (v/v) originated an absorbance decrease of 3.6, 11 and 25%, respectively. Thus, the maximum concentration of methanol and acetone in food samples allowed for the determination was 20 and 5% (corresponding to 5 and 1.25% of organic solvent in the microplate well), respectively, which makes the proposed methodology suitable to assay small volumes of plant extracts with low phenolic content.

3.2. Application to phenolic and other reducing compounds present in food products

The F-C reducing capacity of several phenolic compounds (caffeic acid, catechin, catechol, ellagic acid, ferulic acid, gallic acid, propyl gallate, pyrogallol, resorcinol and tannic acid) commonly found in food samples were evaluated by the classical and by the proposed F-C assay. Trolox, a water soluble analogue of vitamin E, was also studied since it is the standard compound of other widely applied antioxidant assays [4]. Cinnamic acid was chosen as negative control.

The absorbance values obtained for each phenolic compound at different concentrations were monitored during 120 and 30 min using the classical and the proposed method, respectively. Fig. 2 describes the relative sensitivity (%) for gallic acid estimated as the ratio between the slope of the calibration curve at a fixed time

Table 1
F-C reducing capacity obtained for phenolic compounds determined by the classical ($C_{120\,min}$) and by the proposed method ($C_{3\,min}$).[a]

Phenolic compounds	$C_{120\,min}$	$C_{3\,min}$
Caffeic acid	105 ± 2	99 ± 1
(±)-Catechin	139 ± 4	138 ± 4
Catechol	103 ± 2	96 ± 3
Ellagic acid	196 ± 6	198 ± 4
Ferulic acid	80 ± 2	82 ± 3
Propyl gallate	92 ± 2	98 ± 2
Pyrogallol	108 ± 2	107 ± 2
Resorcinol	83 ± 2	73 ± 3
Tannic acid	802 ± 17	806 ± 16
Trolox	39 ± 1	40 ± 1

[a] Results are expressed as the ratio (%) ± standard deviation between the slope of the calibration curves obtained for the testing compound and for gallic acid (for all calibration curves $R > 0.9995$, $n \geq 4$).

Table 2
Linear range (g L^{-1}) and F-C reducing capacity[a] obtained for some nonphenolic compounds determined by the classical ($C_{120\,min}$) and by the proposed method ($C_{3\,min}$).

Nonphenolic compounds	$C_{120\,min}$		$C_{3\,min}$	
Ascorbic acid	0.005–0.050	64	0.005–0.050	59
Citric acid	–	NA[b]	–	NA[b]
Tartaric acid	–	NA[b]	–	NA[b]
Fructose	12.5–200	0.009	1.0–50	0.03
D-Glucose	25–200	0.0005	5.0–25	0.008
Sucrose	–	NA[c]	–	NA[c]
Ferrous sulfate	0.023–0.230	17.2	0.023–0.230	19.2
Sodium sulfite	0.40–2.0	2.0	1.0–10.0	0.2

[a] Results are expressed as the ratio (%) between the sensitivity (mM^{-1}) of tested compound and the sensitivity of gallic acid (mM^{-1}).
[b] No absorbance change up to 5.0 g L^{-1}.
[c] No absorbance change up to 50.0 g L^{-1}.

and the maximum sensitivity attained. In the carbonate medium, the sensitivity increase along the reaction time reaches the maximum value after 90–120 min, while using the hydroxide solution the maximum value was obtained after 3 min of reaction time. The sensitivity obtained after 30 min was about 95% of that attained at 3 min. For all the other phenolic compounds tested, a similar profile was obtained. Cinnamic acid which presents a similar structure but without phenolic moiety did not react in both methods.

Therefore, the F-C reducing capacity of phenolics was determined at 120 and 3 min of reaction time for the carbonate and hydroxide medium, respectively. The ratio (%) between the slope of the calibration curve determined for each phenolic compound and for gallic acid is given in Table 1. The results obtained by the proposed assay (3 min) were in agreement with those obtained by the time-consuming classical protocol (120 min); they were also similar to those described in the literature [6,12].

Caffeic acid, catechol, propyl gallate and pyrogallol with 2, 2, 3 and 3 free hydroxyl groups, respectively, in *ortho*-position gave similar reactivity to gallic acid due to the possibility to form *ortho*-quinone derivatives. In contrast, resorcinol which is a *meta*-phenol showed lower reactivity. Methoxyl substitution of phenolic group in ferulic acid partially removed the reactivity of that phenolic group (82%, Table 1). Tannic acid, as a pentadigalloylglucose, gave the highest reactivity due to the high quantity of free phenolic groups available to reduce FCR. On the other hand, the monophenol trolox gave less than half the reactivity of gallic acid.

Actually, the chemistry of F-C assay is non-specific to measure the total phenolic content as other oxidation substrates can interfere in an inhibitory, additive or synergistic manner. Inhibitory effects can occur as a result of oxidants competing with FCR or air oxidation after sample is made alkaline. The first is difficult to occur because such oxidative reaction in samples should have been completed in advance and the second is surpassed by the addition of FCR before the alkali. The additive effect is the ability of nonphenolic compounds to reduce directly the FCR, while the synergistic effect is produced by the regeneration of oxidized phenols to allow further oxidation by FCR. Among the interfering compounds described [4], organic acids (ascorbic, citric and tartaric), sugars (fructose, glucose and sucrose), ferrous sulfate and sodium sulfite are the major nonphenolic compounds present in food samples that may reduce the FCR. Moreover, as some of them are not considered as antioxidants an overestimation of antioxidant/reducing capacity can also be attained.

Therefore, considering the levels present in food products, their potential additive and/or synergistic effect in the improved method was studied. Additive effect was evaluated by the ability of nonphenolic compound to reduce FCR and a calibration curve was established between the absorbance increase and the concentrations tested. The linear range and the F-C reducing capacity obtained by the classical ($C_{120\,min}$) and by the proposed method ($C_{3\,min}$) are presented in Table 2. To evaluate the synergistic effect, the absorbance signal obtained by gallic acid standard solution (10 mg L^{-1}) containing different concentrations of nonphenolic compounds was compared with the sum of the absorbance values of their individual solutions (Table 3).

Organic acids as citric and tartaric are particularly abundant in fruit/vegetable juices and wines. The content of citric acid in orange juices ranges from 1.5 to 12.5 g L^{-1}, while for white wine samples levels of tartaric acid at 2.38–5.09 g L^{-1} have been found [13]. Red wine samples have low values of citric acid while the concentration of tartaric acid can vary from 2.4 to 2.9 g L^{-1} [14,15]. For both methods, no reactivity with FCR at concentrations up to 5 g L^{-1} was observed (Table 2). Moreover, for this concentration with concomitant presence of 10 mg L^{-1} of gallic acid, an absorbance increase of 1.1 and 3.4% was obtained for citric and tartaric acid, respectively.

Table 3
Evaluation of additive or synergistic effect of nonphenolic compounds in the F-C methodology proposed.

Compound	Measured absorbance[b]	Calculated absorbance[c]	Interference effect
Gallic acid (10 mg L^{-1})[a]	0.149 ± 0.002		
Ascorbic acid (10 mg L^{-1})[a]	0.067 ± 0.001		
Ascorbic acid + gallic acid	0.212 ± 0.002	0.216 ± 0.002	Additive
Fructose (5 g L^{-1})[a]	0.034 ± 0.005		
Fructose + gallic acid	0.236 ± 0.002	0.183 ± 0.005	Synergistic
Glucose (10 g L^{-1})[a]	0.010 ± 0.001		
Glucose + gallic acid	0.194 ± 0.001	0.159 ± 0.002	Synergistic
Ferrous sulfate (20 mg L^{-1})[a]	0.075 ± 0.002		
Ferrous sulfate + gallic acid	0.221 ± 0.001	0.224 ± 0.003	Additive
Sodium sulfite (0.4 g L^{-1})[a]	0.014 ± 0.001		
Sodium sulfite + gallic acid	0.274 ± 0.003	0.163 ± 0.002	Synergistic

[a] Concentration of standards.
[b] Absorbance values corrected for blank signal, each value corresponds to the mean ± standard deviation ($n = 4$).
[c] Sum of measured absorbance for gallic acid and for nonphenolic compound.

Table 4
F-C reducing capacity values (mg L^{-1}) obtained by the classical method (Na_2CO_3) and by the proposed method (NaOH) after 1, 3 and 5 min of reaction applying different dilution factors.[a]

Food sample	Dilution factor	Na_2CO_3	NaOH		
		(120 min)	(1 min)	(3 min)	(5 min)
Red wine C	400	2324 ± 69	2242 ± 58[b]	2341 ± 17	2362 ± 63
	200	2386 ± 31	2277 ± 71	2361 ± 70	2342 ± 65
White wine B	25	237 ± 3	217 ± 5[b]	239 ± 2	242 ± 5
	10	238 ± 2	230 ± 3[b]	242 ± 3	243 ± 4
Orange juice	50	263 ± 10	245 ± 13	261 ± 18	266 ± 13
	16.6	259 ± 7	238 ± 18[b]	260 ± 8	260 ± 6

[a] Each value corresponds to the mean ± standard deviation ($n = 4$).
[b] Value significantly different from the other means ($p < 0.05$) considering the effect of time for the same sample and dilution.

Thus, in view of the dilution factor applied (see Section 3.3) and the levels usually found in food products, their interference in F-C assay is negligible.

In contrast, ascorbic acid readily reacts with FCR independently of the alkaline reagent used (Table 2). In fact, the coefficient ratios to gallic acid for both reaction media are similar and a linear range of reactivity was obtained from 5.0 to 50 mg L^{-1}. The absorbance value obtained for a solution containing both ascorbic and gallic acid (either at 10 mg L^{-1}) was equal to the sum of their individual absorbance values; thus, ascorbic acid has only an additive effect in the F-C chemistry (Table 3). This took place because the reaction of ascorbic acid with FCR is sufficiently fast, not promoting the regeneration of oxidized phenols [6]. For concentrations of ascorbic acid of 0.5, 1.0 and 2.5 mg L^{-1} with concomitant presence of 10 mg L^{-1} of gallic acid, an absorbance increase of 0.9, 3.8 and 7.4% was obtained, respectively. Hence, the interference of ascorbic acid was considerable for concentrations higher than 1.0 mg L^{-1}. Recently, de Quiros et al. [16] analysed 17 samples including fruit juices, soft drinks and isotonic beverages and the contents of ascorbic acid ranged from 6.6 to 840 mg L^{-1}. In this regard, the F-C results obtained for fruit/vegetable juices with low phenol levels takes into consideration the contribution of ascorbic acid, which should not be interpreted as interference since it contributes to the overall antioxidant/reducing capacity and it is suitable for eventual comparison of the values determined by other electron transfer based assays (for instance TEAC, DPPH and FRAP assays). However, a correction on ascorbic acid content should be applied for the determination of total phenolic content [12,17].

To assess the contribution of ascorbic acid to total phenolic values, a procedure based on the quantification of the absorbance increase before adding the alkali was proposed [6], considering that ascorbic reacts with FCR under acidic conditions. This idea was evaluated in the present method, although we observed that appreciable blue formation before the addition of alkali was also attained for gallic acid. Therefore, the absorbance value obtained may be due to the presence of ascorbic acid or other easily oxidized substance (for instance ferrous ion) as well as in less extent to phenolic compounds. For this reason, the content of ascorbic acid should be evaluated by more specific assays [12,18].

The results obtained for sugars in the proposed medium (Table 2) are in accordance with those reported by other authors which stated that, sugars alone do not reduce FCR appreciably [6,12,17]. Sucrose concentrations up to 50 g L^{-1} did not react. For both monosaccharides, a synergistic effect was observed since the absorbance value of the mixture of gallic acid and sugar was superior to the sum of absorbance of their individual solutions (Table 3). In fact, fructose (0.25 g L^{-1}) and glucose (1.25 g L^{-1}) provided an absorbance increase of 3.9 and 3.4%, respectively, while 9.5 and 6.2% was obtained for concentrations of 0.5 and 2.5 g L^{-1}, respectively. Thus, the concentration of sugar that can interfere in the methodology decreased to 0.25 and 1.25 g L^{-1} for fructose and glucose, when compared to the additive effect, for which the linear range was between 1.0–50 and 5.0–25 g L^{-1}, respectively. Considering the concentration range between 20 and 90 g L^{-1} of glucose and fructose found in some fruit juices [19,20], sugars can induce overestimation of antioxidant/reducing capacity, especially in sweet samples with low phenol levels. Standard corrections at room temperature and at 55 °C have been applied to express the theoretical quantitative influence of sugars depending on quantity of phenolic content and sugars [6,17].

Ferrous sulfate displays an F-C reducing capacity about five times lower than gallic acid in both methods (Table 2). Similar to ascorbic acid, ferrous sulfate gave an additive but no synergistic effect (Table 3). For concentrations higher than 2.5 mg L^{-1}, the interference on the absorbance value of gallic acid solution was superior to 2.3%. Nevertheless, the levels found in red wines (1.61–9.3 mg L^{-1}), white wines (0.96–6.11 mg L^{-1}) and juices (1.24–5.51 mg L^{-1}) [21,22], do not represent an interference owing to the dilution factor applied before analysis (see Section 3.3).

The reactivity of sodium sulfite was also investigated and it was 10 times lower in proposed method when compared to classical assay; coefficient ratio to gallic acid was 0.2 and 2.0%, respectively (Table 2). However, a synergistic effect was observed since the absorbance value of solution containing 10 mg L^{-1} of gallic acid and 0.4 g L^{-1} of sodium sulfite was 68% higher than the sum of absorbance values of the individual compounds analysed separately (Table 3). Thus, even though the additive effect was observed for concentrations between 1.0 and 10 g L^{-1} (Table 2), the synergistic effect owing to the rapid regeneration of oxidized phenols makes that the concentration of 2.5 mg L^{-1} of sodium sulfite + 10 mg L^{-1} of gallic acid originated an absorbance increase of 4.5% when compared to the standard gallic acid solution. Sulfites are particularly found in wine samples due to their antioxidative and antimicrobial properties and the concentration can vary from 60.8 to 235 mg L^{-1} for white wines and from <10.0 to 130.4 mg L^{-1} in red wines [23]. Therefore, according to the dilution factor applied (50 and 200 for white and red wines, Section 3.3), the interference from sulfites should be considered for white wines with sulfite concentrations higher than 125 mg L^{-1}.

As the classical assay, the present method is not suitable for determination of total phenolic content as other reducing nonphenolic compounds as ascorbic acid, fructose and sulfites may reduce FCR. For this purpose, a cleanup procedure using solid phase extraction (SPE) could be applied to separate phenolic compounds and eliminate interfering water-soluble compounds [9,24,25]. Nevertheless, all these methods applied the classical time-consuming F-C assay after SPE procedure. Despite of these issues, this assay is suitable to assess the antioxidant/reducing capacity since it measures mono- and polyphenolic compounds, ascorbic acid and sulfites which have recognised antioxidant properties [4]. Therefore, the microliter assay developed here can be a suitable alternative as a fast and high-throughput method for

Table 5
Results (mg L^{-1}) obtained for analysis of different food products by the conventional ($C_{120\,min}$) and by the proposed methodology ($C_{3\,min}$) for the determination of F-C reducing capacity.[a]

Food product	$C_{120\,min}$		$C_{3\,min}$		R.D.(%)[b]
Red wine A	2108 ± 18	(1:100)	2087 ± 58	(1:200)	−1.0
Red wine B	2036 ± 35	(1:50)	2032 ± 37	(1:200)	−0.2
Red wine C	2327 ± 35	(1:50)	2361 ± 70	(1:200)	+1.5
Red wine D	2268 ± 34	(1:50)	2287 ± 38	(1:200)	+0.9
White wine A	242 ± 10	(1:25)	260 ± 8	(1:50)	+7.4
White wine B	237 ± 3	(1:25)	243 ± 11	(1:50)	+2.6
White wine C	252 ± 2	(1:25)	254 ± 8	(1:50)	+0.8
White wine D	276 ± 2	(1:25)	257 ± 11	(1:50)	−6.7
Blond beer A	306 ± 5	(1:50)	308 ± 8	(1:50)	+0.7
Blond beer B	314 ± 7	(1:50)	318 ± 9	(1:50)	+1.4
Blond beer C	313 ± 9	(1:50)	322 ± 4	(1:50)	+2.9
Dark beer	497 ± 17	(1:100)	515 ± 10	(1:100)	+3.5
Green tea A	327 ± 8	(1:25)	324 ± 16	(1:25)	−0.9
Green tea B	644 ± 2	(1:25)	627 ± 6	(1:25)	−2.6
Herbal infusion[c]	81 ± 2	(1:25)	76 ± 2	(1:25)	−5.0
Herbal infusion[d]	918 ± 9	(1:25)	880 ± 11	(1:100)	−4.2
Orange juice	263 ± 10	(1:50)	261 ± 18	(1:50)	−0.8
Fruit juice A	499 ± 12	(1:25)	510 ± 19	(1:50)	+2.2
Fruit juice B	622 ± 7	(1:25)	623 ± 8	(1:50)	+0.2
Fruit juice C	264 ± 2	(1:25)	244 ± 12	(1:50)	−7.6

[a] Each value corresponds to the mean ± standard deviation ($n = 4$). The values in parentheses correspond to the dilution performed prior to analysis.
[b] R.D. = relative deviation between the two methods.
[c] Infusion of Camomile.
[d] Infusion of green tea with mulberry and strawberry flavour.

the determination of total phenolic content after the cleanup SPE procedure as well as to screening analysis of antioxidant/reducing capacity in whole sample.

3.3. Application of the rapid microplate F-C assay to food products

The rapid microplate method was applied to determine the F-C reducing capacity of 20 beverages including red and white wines, blond and dark beers, herbal and tea infusions and juices. To establish the lowest reaction time at which the maximum of absorbance was achieved, the reducing reaction was monitored during 30 min. For all samples tested, the maximum of absorbance was obtained after 3–5 min of reaction and after that a slightly decrease of absorbance was attained, similar to that described for gallic acid. Therefore, the F-C reduction capacity expressed as equivalents of gallic acid (mg L^{-1}) after 1, 3 and 5 min were determined and the results were compared to those provided by the classical assay (120 min). Table 4 illustrates the results obtained for some samples applying different dilution factors. One-way analysis of variance (ANOVA) was performed to determine the effect of reaction time on the F-C reducing results obtained. For the same sample and dilution applied, the results obtained after 3 min of reaction using the proposed method showed no significant difference ($p > 0.05$) from those obtained after 120 min applying the classical assay. Moreover, the F-C reducing capacity determined was not affected by the dilution factor applied.

Hence, the F-C reducing capacity of food products were estimated by interpolation of absorbance values after 3 min of reaction in the following calibration curve: $A = 0.0131 \pm (0.0002) \times C + 0.080$ (± 0.004) and $R \geq 0.9997$, where A is the absorbance and C is the concentration of gallic acid (mg L^{-1}); values between parenthesis are the standard deviation of the parameters corresponding to six calibration curves performed on different days. These values were multiplied by the respective dilution factor. The sample blank for the lowest dilution applied was measured for all samples tested. Absorbance values obtained were similar to those provided by the blank signal (<2.0%); thus their contribution to the absorbance measurements was not significant.

The results obtained by the proposed methodology ($C_{3\,min}$) and by the classical assay ($C_{120\,min}$) for the analysis of the samples are presented in Table 5. For comparison purposes, a linear relationship ($C_{3\,min} = C_0 + S \times C_{120\,min}$) was established ($n = 20$), and the values for intercept (C_0), slope (S), and correlation coefficient were −2.9 (±11.4), 1.004 (±0.011), and 0.9998, respectively. Considering the limits of the 95% confidence intervals presented (values in parentheses), the calculated slope and intercept do not differ significantly from the values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results obtained by the improved method and by the classical approach [26]. Furthermore, when a paired t-test was performed on the data obtained for all samples, a t value of 0.049 was calculated. The comparison between this value and the tabulated t ($p = 0.05$; df = 19) = 2.093 indicates no significant difference for the mean concentrations obtained by the two methods [26].

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ [26]. For six different calibration curves, the calculated detection limit in the microplate was about 0.25 mg L^{-1} of gallic acid. The repeatability of the developed method was assessed by calculating the relative standard deviation (R.S.D.) from 12 consecutive determinations of three gallic acid standard solutions (2.5, 10.0, and 37.5 mg L^{-1}) providing values of 1.6, 1.3, and 0.8%, respectively. The reproducibility of the method assessed by the R.S.D. of calibration slopes performed in different days ($n = 6$) was 1.3%.

4. Conclusions

A microliter and simple procedure of F-C assay for the determination of F-C reducing capacity of food products was developed. Using the sodium hydroxide as alkaline support instead of the carbonate buffer, the reaction kinetics of FCR reduction increased dramatically. In fact, it was possible to decrease the reaction time from 120 to 3 min, providing similar results for pure compounds and for a large variety of food samples. Moreover, the microplate format allowed the reduction of reaction volume about 100 times compared to the classical F-C assay [6]. Low reagent/sample consumption and effluent produced also contributed here to the implementation of greener analytical procedures.

As the classical assay, the present method is not suitable for determination of the total phenolic content, especially in samples

with low phenol levels, unless interfering substances (ascorbic acid, fructose and sulfite) are considered or removed. The proposed assay would be a suitable rapid and high-throughput procedure for routine assessment of antioxidant/reducing capacity of food products and also to measure the total phenolic content after a SPE cleanup procedure.

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