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Development and validation of a sequential-injection method with chemiluminescence detection for the high throughput assay of the total antioxidant capacity of wines

Evdoxia Fassoula, Anastasios Economou *, Antony Calokerinos

Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, 157 71, Greece

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ABSTRACT

This work reports a sequential-injection analysis (SIA) method with chemiluminescence (CL) detection for the rapid assay of the total antioxidant capacity (TAC) in wines. The method exploited the Co(II)-catalysed CL reaction of luminol with hydrogen peroxide in alkaline medium. Zones of sample, hydrogen peroxide, catalyst (Co(II) solution) and alkaline luminol were sequentially aspirated into the holding coil of the SIA manifold. Then, the flow was reversed and the stacked zones were directed to the CL detector. As the zones overlapped, antioxidants in the samples scavenged a portion of hydrogen peroxide and the decrease in the CL intensity was monitored and related to the TAC. The chemical and geometric conditions were studied and the method was validated in terms of linearity, accuracy (trueness and precision), matrix effects, signal additivity and robustness. The reproducibility of the method (expressed as the between-days % relative standard deviation) was between 2.5 and 3.4% and the trueness (expressed as the % recovery in wines spiked with gallic acid) was in the range 96.7–97.3%. The sampling frequency was 60 samples h^{-1}. The proposed SIA-CL method was compared with the DPPH method and the Folin–Ciocalteau (FC) method for the analysis of 25 wine samples.

1. Introduction

Over the last years, antioxidant compounds contained in foods have received special attention. Antioxidants play a major role in scavenging the reactive oxygen species (ROS) generated during aerobic metabolism. ROS are implicated in several health problems including diabetes, infertility, rheumatoid arthritis and cardiovascular diseases [1–3]. ROS have been shown to be potentially damaging to cells by oxidative mechanisms such as lipid peroxidation in the cellular membranes, enzyme inactivation and oxidative DNA damage [4–6].

The ingestion of foods with high antioxidant activity can be important in the prevention of the oxidative stress due to ROS and, consequently, in the prevention of health disorders [7,8]. In wines, the major natural antioxidants are polyphenols which exhibit strong antioxidant activity via a free radical-scavenging mechanism and metal ion chelation [9,10]. A wide range of studies has shown the effective antioxidative properties of these compounds in the protection against arteriosclerosis, coronary heart disease and tumour growth [11–14].

The array of the different analytical methodologies to determine the total antioxidant capacity (TAC) in foods *in vitro* has been reviewed previously and the relative strengths and weakness of the various approaches have been discussed in detail [15–21]. Considering that these methods are routinely used for screening purposes, their scope for automation is highly relevant. Flow-based methods, including flow-injection analysis (FIA), sequential-injection analysis (SIA), multisyringe FIA (MS-FIA) and multicommutation, are ideally suited to fulfil the need for automated analysis and have been applied for the purposes of fast screening of TAC in foods (including wines) [22,23]. In addition to the higher sample throughput and the minimisation of reagent consumption, these on-line approaches contribute to increase in precision, accuracy and cost-effectiveness.

The majority of the reported flow-based methods for TAC assessment utilise spectrophotometric detection (mainly based on the scavenging of the coloured $ABTS^{\bullet+}$ or $DDPH^{\bullet}$ radicals by antioxidants in the sample), electrochemical detection (based on the direct electro-reduction of the antioxidants in the sample on a suitable working electrode) and chemiluminescence (CL) detection (mainly based on enhancement or suppression of a CL reaction due to the presence of antioxidants in the sample) [22,23]. CL

* Corresponding author. Tel.: +30 210 7274298; fax: +30 210 7274750.
E-mail address: aeconomo@chem.uoa.gr (A. Economou).

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detection requires simpler instrumentation and is more sensitive than spectrophotometry while it is preferable to electrochemical detectors that are often subject to electrode fouling. Although various on-line CL approaches have been reported for the study of scavenging capacity of specific ROS in different matrices [23], a very limited number of such methods exists dealing with the determination of TAC in food samples [24–29]. In the particular case of wine analysis, there are only three applications of FIA-CL which exploit the CL reaction of acidic potassium permanganate or acidic soluble manganese(IV) with antioxidant compounds in the sample in order to determine the TAC and total phenolic levels [27–29]. However, according to our knowledge, there are no reports of SIA coupled to CL detection for the assay of TAC in food samples, including wines. SIA has evolved as the latest-generation version of flow analysis techniques, possessing some distinct advantages over FIA, namely manifold simplicity, lower consumption of reagents and wider scope for sample pre-treatment and manipulation [30].

Therefore, in this work, the first SIA-CL method has been developed for the rapid assay of TAC in wine samples based on scavenging of hydrogen peroxide by antioxidants in the sample and determination of the residual hydrogen peroxide by its CL reaction with alkaline luminol; the decrease in the CL intensity was monitored and related to the TAC of the sample. The method was thoroughly validated (in terms of linearity, accuracy, matrix effects and robustness) and was applied to the analysis of wine samples.

2. Experimental

2.1. Reagents

Gallic acid, caffeic acid, epicathechin, p-coumaric acid, 2,2 diphenyl-1-picryl-hydrazyl (DPPH), tartaric acid were purchased from Sigma (St. Louis, MO), catechin, luminol (97%), hydrated cobalt(II) nitrate ($Co(NO_3)_2{\cdot}6H_2O$) from Fluka (Buchs, Switzerland), sodium hydroxide, hydrogen peroxide (30%) and ethanol from Panreac (Barcelona, Spain) and methanol from Fischer Scientific (Hampton, NH).

A 1.0×10^{-2} mol L^{-1} stock solution of H_2O_2 was prepared daily in doubly distilled water and more dilute solutions were prepared by serial dilution. A 0.1 mol L^{-1} stock solution of NaOH was prepared by dissolving the solid compound in doubly distilled water. A 1.0×10^{-1} mol L^{-1} stock solution of luminol was prepared in 0.01 mol L^{-1} of NaOH and more dilute solutions were prepared by appropriate dilution with the 0.1 mol L^{-1} stock solution of NaOH in order to achieve the desired NaOH concentration; all the luminol solutions were stored in the dark at 4 °C. A 1000 mg L^{-1} stock solution of Co(II) was prepared by dissolving the solid compound in doubly-distilled water and more dilute solutions were prepared by appropriate dilution with doubly distilled water.

Solutions of the antioxidants were prepared daily. Stock solutions of 1.0×10^{-2} mol L^{-1} of gallic acid and 1.0×10^{-3} mol L^{-1} of catechin and epicatechin were prepared by dissolving the solid compound in doubly distilled water. Stock solutions of 1.0×10^{-3} mol L^{-1} of caffeic acid and p-coumaric acid were prepared by dissolving the solid compound in 1 ml of ethanol and further dilution with doubly distilled water. More dilute solutions of the antioxidants were prepared in doubly distilled water.

The 1.0×10^{-3} mol L^{-1} solution of DPPH was prepared daily by dissolving the solid compound in methanol and storing in the dark.

A wine matrix simulator was prepared by mixing 7.3 g of tartaric acid, 12.0 mL of ethanol and 8.0 g of glucose with 800 mL of doubly distilled water, adjusting the pH to 3.5 with NaOH and dilution to a final volume of 1 L.

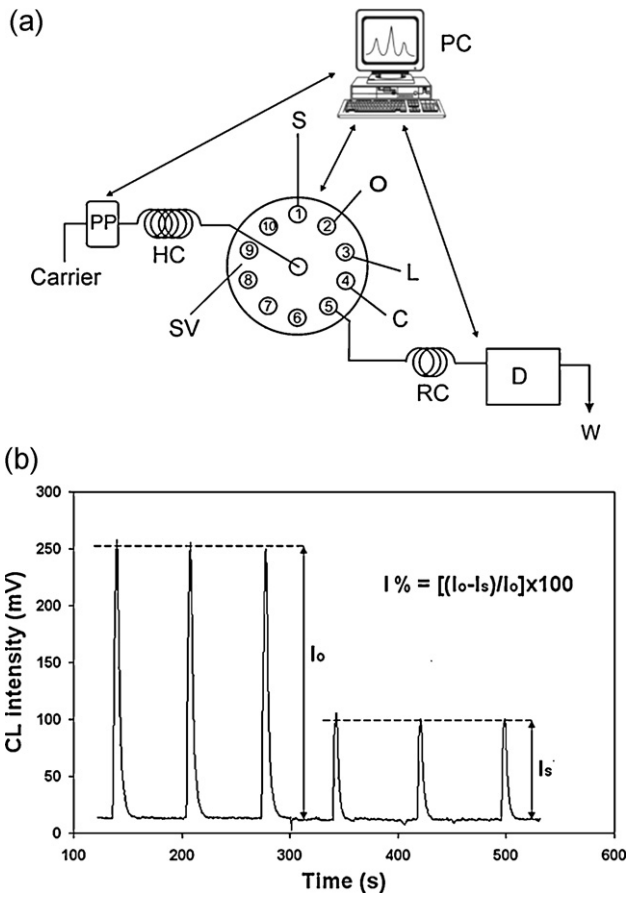

Fig. 1. (a) Schematic diagram of the SIA flow system. Symbols: PP, pump; RC, reaction coil; SV, selection valve; PC, personal computer; HC, holding coil; D, detector; W, waste; S, sample; C, catalyst (Co(II) solution); O, oxidant (hydrogen peroxide); L, luminol; C, carrier (distilled water). For selected conditions see Table 2. (b) SIA peaks recorded for a blank solution, I_0, and antioxidant solution, I_s (in this case gallic acid with $c_{\text{gallic acid}} = 1.0 \times 10^{-5}$ mol L^{-1}) obtained under the conditions shown in Table 2. The method of the calculation of the *I*% value is also illustrated.

The samples were 15 white and 10 red wines purchased from local supermarkets or donated from the Wine Institute, National Agricultural Research Foundation (NAGREF, Athens, Greece).

2.2. Instrumentation

The 10-port selection valve with microelectric actuator was from Valco (Vici Valco, Schenton, Switzerland). The peristaltic pump was a Gilson Minipuls 3 (Villiers-le-Bel, France) featuring 0.5 mm i.d. Tygon tubing. The CL detector was a miniature Hamamatsu H6780 photomultiplier tube operating at 600 V (Hamamatsu Photonics, Japan). The optical flow-cell was a spiral home-made glass unit and was placed in front of the detector in a light-tight box. The tubing was PTFE 0.75 mm i.d. (Jour Research, Sweden). A 6025 E PCI multi-function interface card (National Instruments, Austin, TX) was used to interface the valve, pump, and detector to a personal computer. The experimental set-up is illustrated in Fig. 1a. The control and data acquisition programme was developed in LabVIEW 6.1 (National Instruments, Austin, TX) and allowed complete automation of the experimental sequence. The CL signal was displayed on the screen of the PC in real time and saved as an ASCII text file at the end of each experimental sequence. A single-beam spectrophotometer (Helios, Thermo Scientific) equipped with 1 cm flow-path plastic cuvettes was used for the DPPH and Folin–Ciocalteau (FC) measurements.

Table 1
SIA sequence for the determination of TAC.

Step	Valve position	Action	Time (s)	Flow rate ($mL\,min^{-1}$)
1	1	Sample aspiration	15	0.30
2	2	Aspiration of H_2O_2 solution	5	0.30
3	3	Aspiration of luminol solution	5	0.30
4	4	Aspiration of Co(II) solution	5	0.30
5	5	Delivery to the flow-cell	30	3.50

2.3. Experimental procedure

Before the analysis, the wines were purged for 5 min with nitrogen to expel sulphur dioxide. Then, the samples were analysed directly after dilution 1:200 (v/v) (white wines) and 1:1000 (v/v) (red wines) with doubly distilled water.

The experimental sequence for the SIA-CL method for assessment of the TAC is illustrated in Table 1. A zone of sample or standard was aspirated in the holding coil (Table 1, step 1) followed by zones of hydrogen peroxide (Table 1, step 2), luminol (Table 1, step 3) and Co(II) solution (Table 1, step 4). The stacked zones were delivered to the flow-cell and the CL intensity was monitored by the PMT (Table 1, step 5). Initially, the mean blank signal, I_0, was calculated from 3 consecutive measurements by aspirating doubly distilled water in step 1 of the sequence shown in Table 1. Then, the sample or standard was aspirated in triplicate and the mean analytical signal, I_s, was calculated. The % signal suppression, $I\%$, due to the scavenging activity of hydrogen peroxide by the antioxidant(s) was derived according to the formula:

$$I\% = \frac{I_0 - I_s}{I_0} \times 100 \quad (1)$$

The graphical method to calculate $I\%$ is illustrated in Fig. 1b. Calibration plots for individual antioxidants were constructed as $I\%$ *vs* the logarithm of the antioxidant concentration. The TAC of the sample or standard was ultimately expressed as gallic acid equivalents (GAE). The GAE value of a standard of sample represents the concentration of gallic acid that would cause an identical $I\%$ signal suppression to the standard or sample analysed. Gallic acid was selected to express TAC both because it is a main phenolic constituent of wines and because it is widely used in the literature to express TAC of food samples [24,29,31].

The experimental procedures for the analysis using the DPPH and the Folin–Ciocalteau (FC) methods were similar to those described earlier [32,33].

3. Results and discussion

3.1. Study of the chemical and geometrical variables

So far, no SIA-CL methods have been reported for the TAC assessment of food or wine samples. However, the CL reaction between luminol and various oxidation agents has been exploited for the assessment of selected standard antioxidants and for the analysis of herbal medicines by FIA [34–37] and for the assay of antioxidant capacity of multivitamin supplements by SIA [38]. Regarding applications of on-line methods with luminol CL detection for TAC assessment in food, a MS-FIA method has been described for the analysis of tea [26] and two FIA approaches for the analysis of oil [24,39].

Initially, the oxidation reaction of luminol by hydrogen peroxide (oxidant) in the absence of a catalyst was studied in the SIA mode. However, the blank CL signal obtained in the absence of antioxidants was weak and both the sensitivity and linear range were poor. As an alternative, the addition of Co(II) (serving as a catalyst) was tested. Metal cations can dramatically increase the CL intensity of the luminol–hydrogen peroxide system since they catalyse the formation of highly reactive and transient free radicals via the superoxide-driven FENTON reaction; this might potentially complicate the interpretation of the measurement results due to the presence of more than one antioxidant-scavenging species [34,35]. However, in practice the analytical characteristics of the method were significantly improved in the presence of Co(II) and, therefore, the luminol–hydrogen peroxide-Co(II) CL system was adopted. These results are in agreement with those observed in the CL detection of standard antioxidants in the FIA mode in the presence of Co(II) [35].

Table 2
Range studied and selected conditions for the determination of gallic acid by SIA–CL (S, sample; O, oxidant; L, luminol; C, catalyst).[a]

Parameter	Units	Range studied	Selected value
Stacking order	–	S–O–L–C, O–S–L–C, S–O–C–L, O–S–C–L	S–O–L–C
$c_{Co(II)}$	$mg\,L^{-1}$	0 to 10	2
c_{NaOH}	$mol\,L^{-1}$	2.0×10^{-3} to 2.0×10^{-1}	2.0×10^{-2}
$c_{luminol}$	$mol\,L^{-1}$	1.0×10^{-4} to 1.0×10^{-2}	5.0×10^{-4}
$c_{H_2O_2}$	$mol\,L^{-1}$	1.0×10^{-4} to 1.0×10^{-2}	5.0×10^{-4}
$V_{H_2O_2}$	μL	10 to 100	25
V_{sample}	μL	10 to 100	75
$V_{luminol}$	μL	10 to 100	25
$V_{Co(II)}$	μL	10 to 100	25
$F_{flow\,rate}$	$mL\,min^{-1}$	1.5 to 4.0	3.5
$l_{reaction\,coil}$	cm	36 to 102	36

[a] Initial conditions for the selection of the chemical and instrumental variables were: $c_{gallic\,acid} = 5.0\times10^{-5}\,mol\,L^{-1}$; $c_{H_2O_2} = 1.0\times10^{-4}\,mol\,L^{-1}$; $c_{luminol} = 1.0\times10^{-3}\,mol\,L^{-1}$; $c_{NaOH} = 1.0\times10^{-2}\,mol\,L^{-1}$; $c_{Co(II)} = 2\,mg\,L^{-1}$; $V_{luminol} = V_{H_2O_2} = V_{gallic\,acid} = V_{Co(II)} = 25\,\mu L$; $F_{flow\,rate} = 3.5\,mL\,min^{-1}$; $l_{reaction\,coil} = 36\,cm$.

While in FIA mixing can be controlled very efficiently by multichannel manifolds, mixing in SIA depends exclusively on zone dispersion and overlap. In the context of this work, the order of aspiration of the sample and reagents, the concentration of the reagents, the volumes of the different zones, the delivery flow rate and the length of the mixing coil were critical since they affected the zone overlap profile and the temporal and spatial chemical conditions prevailing within the overlapping zones. The values of the chemical and geometrical parameters were selected using a univariate approach using gallic acid ($c_{gallic\,acid} = 5.0\times10^{-5}\,mol\,L^{-1}$) as the analyte. The different variables were studied sequentially by varying the values of each variable in the ranges shown in Table 2 while keeping the other variables constant. Judicious selection of the working values was based on a compromise between the following requirements: (i) high value of the $I\%$ in order to increase the sensitivity; (ii) satisfactory precision; (iii) low residence time in order to increase the sample throughput; (iv) low consumption of reagents; (v) good buffering capacity in the reaction mixture (since the wine samples are moderately acidic while the CL reaction is favoured at alkaline pH). The selected values of the chemical and instrumental parameters are shown in Table 2. The concentration of hydrogen peroxide was critical since it affected both the dynamic range and the limit of detection and was further investigated in Section 3.2.

3.2. Calibration features of selected wine antioxidants

In the present method, calibration curves were plotted for four main typical antioxidants found in wines (gallic acid, catechin, epicatechin and coumaric acid) as well as of Trolox (which is commonly used as a reference antioxidant compound). Calibration plots ($I\%$ *vs* the logarithm of the antioxidant concentration) exhibited typically a sigmoidal shape. The lower part of the plot corresponded to low antioxidant concentrations which scavenged only a small percentage of the total hydrogen peroxide and, there-

Table 3
Calibration features of selected antioxidants (the calibration curves for all antioxidants were logarithmic of the type: $I\% = (a \pm s_a)\ln c_{\text{antioxidant}} + (b \pm s_b)$). Conditions as in Table 2.

Antioxidant	Linear range (mol L^{-1})	b ($\pm s_b$)	a ($\pm s_a$)	LOD (mol L^{-1})	R^2
Gallic acid	1.0×10^{-6} to 2.0×10^{-4}	223.7 (±6.7)	15.8 (±0.6)	1.0×10^{-6}	0.992
Caffeic acid	1.0×10^{-7} to 2.0×10^{-6}	323.3 (±12.7)	18.8 (±0.9)	1.0×10^{-7}	0.994
Cathechin	1.0×10^{-7} to 1.0×10^{-5}	255.2 (±6.7)	15.2 (±0.5)	1.0×10^{-7}	0.995
Epicatechin	1.0×10^{-7} to 5.0×10^{-6}	263.6 (±5.4)	15.7 (±0.4)	1.0×10^{-7}	0.998
p-Coumaric acid	5.0×10^{-6} to 1.0×10^{-3}	203.2 (±4.2)	15.6 (±0.4)	5.0×10^{-6}	0.995
Trolox	1.0×10^{-6} to 1.0×10^{-4}	245.8 (±6.8)	16.7 (±0.6)	1.0×10^{-6}	0.994

fore, induced only weak change between the blank signal and the sample signal. The upper horizontal part of the plot corresponded to high antioxidant concentrations that caused almost exhaustive consumption of the hydrogen peroxide and led to high $I\%$ values. In the middle part of the plot, corresponding to intermediate antioxidant concentrations, the $I\%$ value was a linear function of the logarithm of the antioxidant concentration obeying the equation: $I\% = (a \pm s_a)\ln c_{\text{antioxidant}} + (b \pm s_b)$ (where a and b are the slope and intercept of the calibration plot and s_a and s_b are the standard deviations of the slope and intercept of the calibration plot) and could be used for analytical purposes. Such logarithmic calibration plots are typically obtained using the luminol–hydrogen peroxide CL system in the presence [35] or absence [26,38] of Co(II). The calibration features corresponding to the linear range of the selected antioxidants are shown in Table 3. This simple qualitative model of the shape of the calibration plots accounted for the profound effect of the hydrogen peroxide concentration on the linear range and the limit of detection of the method. At low hydrogen peroxide concentrations, the presence of even moderate concentration of antioxidant caused a measurable decrease in the CL intensity (leading to low limits of detection) but the presence of modest antioxidant concentrations consumed almost quantitatively the hydrogen peroxide present (which limited the linearity of the calibration plot at higher antioxidant concentration). In contrast, at high hydrogen peroxide concentrations, the presence of low concentration of antioxidant did not cause a measurable decrease in the CL intensity (leading to higher limits of detection) but the linearity of the calibration plots extended to higher concentration of the antioxidant until exhaustive scavenging of hydrogen peroxide occurred. This effect was reflected in the calibration features of the plots of gallic acid obtained with different concentrations of hydrogen peroxide shown in Table 4. Although using lower hydrogen peroxide concentrations resulted in lower limits of detection, in practice a nominal concentration of hydrogen peroxide equal to $c_{H_2O_2} = 5.0 \times 10^{-4}$ mol L^{-1} was selected for further work in order to prevent changes in the effective concentration of hydrogen peroxide in the flow system due to the instability of low concentrations of this compound in aqueous solutions. This hydrogen peroxide concentration provided sufficiently low limits of detection and a suitable linear range for the analysis of wine samples.

In view of the detection principle of the present method, the limit of detection (LOD) was defined as the concentration of the antioxidant that would induce a CL intensity equal to $I_{\text{LOD}} = I_0 - 3 \times s_0$ (where I_0 is the blank signal in the absence of antioxidant and s_0 is the standard deviation of I_0). In other words, the LOD was considered as the concentration of the antioxidant that could induce a statistically significant change (decrease) with respect to the blank signal. Considering that the calculated value was always lower than the lower concentration of the linear part of the calibration plot for all the antioxidants, the LOD coincided with the lowest concentration of the linear range. Similarly, the limit of quantification (LOQ) was calculated as the concentration of the antioxidant that could induce a CL intensity equal to $I_{\text{LOQ}} = I_0 - 10 \times s_0$. Again, the calculated values were always lower than the lower concentrations of the linear parts of the calibration plots for all the antioxidants. Therefore, detection and quantification was practically possible for any concentration within the linear parts of the calibration plots. The LOD values for the selected antioxidants are summarised in Table 3.

3.3. Additivity of selected antioxidants

In the case of antioxidants, occasional interaction (either synergy or antagonism) between the individual antioxidants has been postulated, even *in vitro* [40]. In this work, additivity between gallic acid and the selected other antioxidants was assessed by preparing binary solutions containing a fixed total concentration, $c_{\text{total antioxidant}}$, of gallic acid and each antioxidant ($c_{\text{total antioxidant}} = 2.0 \times 10^{-6}$ mol L^{-1} of total antioxidants except in the case of the mixture of gallic acid-coumaric acid with $c_{\text{total antioxidant}} = 2.0 \times 10^{-5}$ mol L^{-1} of total antioxidants). In these mixtures, the content of gallic acid (expressed as $c_{\text{gallic acid}}/c_{\text{total antioxidant}} \times 100$) varied between 0% and 100% (0, 30, 50, 70% and 100%).

For each mixture, experimental plots were constructed in which the x axis represented the % content of the solutions in gallic acid and the y axis the GAE values of the solutions determined by means of the experimental $I\%$ values. Theoretical plots were constructed in the same way but with the y axis representing the GAE values of the solutions determined mathematically by means of the calibration plots of antioxidants. Additivity was ascertained when the respective theoretical plots statistically coincided with experimental plots; the respective plots are illustrated in Fig. 2. The theoretical and experimental values of the slopes and intercepts were compared using the t-test. In all cases $t_{\text{experimental}} < t_{\text{theoretical}}$ (where $t_{\text{experimental}}$ and $t_{\text{theoretical}}$ are the experimental and theoretical t-test values) suggesting that the antioxidants in each binary mixture behaved in an additive way.

Additivity was also ascertained via the study of the trueness during method validation where wine samples containing a variety of antioxidants were spiked with gallic acid and satisfactory recoveries were obtained (see Section 3.5).

Table 4
Features of the calibration plots for gallic acid with different concentrations of hydrogen peroxide (the calibration curves at all hydrogen peroxide concentrations were logarithmic of the type: $I\% = (a \pm s_a)\ln c_{\text{antioxidant}} + (b \pm s_b)$). Other conditions as in Table 2.

$c_{H_2O_2}$ (mol L^{-1})	Linear range (mol L^{-1})	b ($\pm s_b$)	a ($\pm s_a$)	LOD (mol L^{-1})	R^2
1.0×10^{-3}	1.0×10^{-5} to 5.0×10^{-4}	248.3 (±10.1)	19.8 (±1.0)	1.0×10^{-5}	0.995
5.0×10^{-4}	1.0×10^{-6} to 2.0×10^{-4}	223.7 (±6.7)	15.8 (±0.6)	1.0×10^{-6}	0.992
1.0×10^{-4}	5.0×10^{-7} to 1.0×10^{-4}	234.5 (±7.5)	14.8 (±0.6)	5.0×10^{-7}	0.993
4.0×10^{-5}	1.0×10^{-7} to 5.0×10^{-5}	178.4 (±1.3)	10.0 (±0.1)	1.0×10^{-7}	0.999

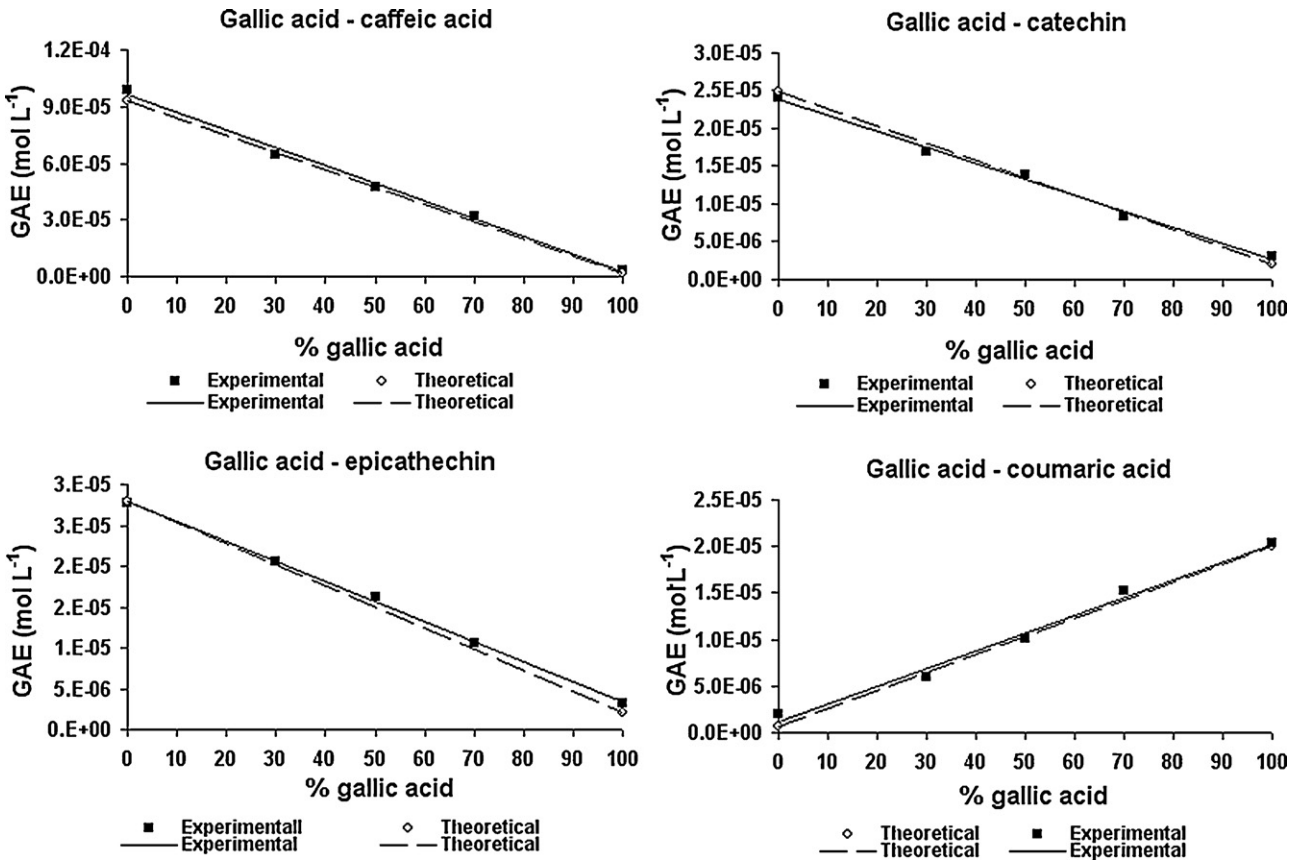

Fig. 2. Theoretical and experimental additivity curves for binary solutions of gallic acid and selected wine antioxidants. In all cases the total antioxidant concentration in the solution was $c_{\text{total antioxidant}} = 2.0 \times 10^{-6}$ mol L^{-1} except in the mixture of gallic acid with coumaric acid which had a total antioxidant concentration equal to $c_{\text{total antioxidant}} = 2.0 \times 10^{-5}$ mol L^{-1}. The content of gallic acid in the *x* axis was expressed as $(c_{\text{gallic acid}}/c_{\text{total antioxidant}}) \times 100$. Conditions as in Table 2.

3.4. Matrix effects

Experiments were conducted to study the effect of the main wine constituents, namely tartaric acid, ethanol and glucose, on the determination of gallic acid by SIA-CL. Standards containing 5.0×10^{-5} and 5.0×10^{-6} mol L^{-1} of gallic acid were prepared in doubly distilled water and in solutions containing different contents of wine matrix simulator (prepared as described in the Section 2) and the *I*% values were determined with the present method; the results are illustrated in Fig. 3. For low contents of the wine matrix simulator (i.e. <2% (v/v)), the matrix effects were statistically insignificant and increased with increasing content of wine matrix becoming statistically important at wine matrix >2% (v/v). However, the high sensitivity of CL detection allowed high dilution factors to be used resulting in effective dilution of the interfering matrix components. Considering that the samples were typically diluted at least 1:200 (v/v) with water (corresponding to a maximum of 0.5% (v/v) content of wine matrix), the effect of the major wine constituents was considered negligible.

3.5. Method validation

3.5.1. Precision

To determine the within-day instrumental repeatability, 6 consecutive measurements were acquired at 6 concentration levels of gallic acid spanning the whole linear range of the calibration plot (1.0×10^{-6} to 2.0×10^{-4} mol L^{-1} of gallic acid): the within-day % relative standard deviation ranged from 0.4 to 1.8%. To determine the between-days instrumental reproducibility, 5 consecutive measurements were acquired at 6 concentration levels of gallic acid spanning the whole linear range of the calibration plot (1.0×10^{-6} to 2.0×10^{-4} mol L^{-1} of gallic acid) at 3 different days: the between-days % relative standard deviation ranged from 1.8 to 5.3%. From the between-days instrumental reproducibility experiments, 3 calibration plots were constructed for the 3 different working days. To determine whether the plots differed statistically, a *t*-test was conducted comparing the slopes and intercepts. In all cases $t_{\text{experimental}} < t_{\text{theoretical}}$, meaning that the plots did not differ statistically. For the assessment of the between-days reproducibil-

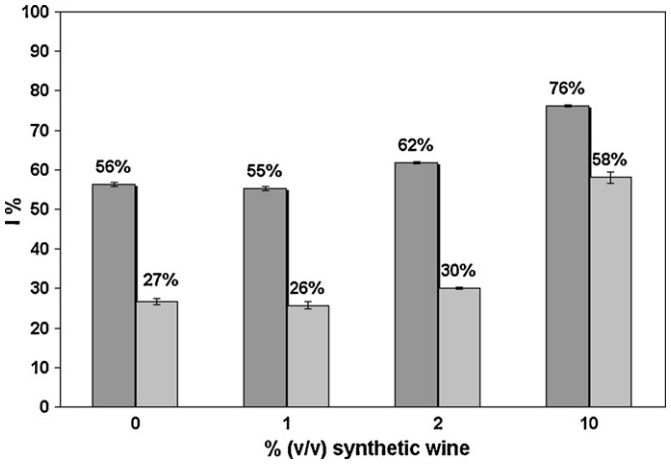

Fig. 3. Effect of the content of wine matrix simulator on the *I*% values of 5.0×10^{-5} mol L^{-1} (dark grey) and 5.0×10^{-6} mol L^{-1} (light grey) of gallic acid. Conditions as in Table 2.

Table 5
Between-days reproducibility of the method (3 samples analysed on 3 different days with 6 repeats per day, total 3 × 6 = 18 measurements per sample).

Day	GAE (mol L^{-1})		
	Sample 1	Sample 2	Sample 3
1	1.26×10^{-5}	4.44×10^{-5}	5.62×10^{-5}
2	1.20×10^{-5}	4.41×10^{-5}	5.45×10^{-5}
3	1.24×10^{-5}	4.17×10^{-5}	5.76×10^{-5}
Mean	1.23×10^{-5}	4.34×10^{-5}	5.61×10^{-5}
s_r	3.0×10^{-7}	1.5×10^{-6}	1.5×10^{-6}
% RSD	2.5	3.4	2.7

ity of the method (including not only the detection variability but also random errors introduced by the sample pretreatment, i.e. the sampling, purging and dilution steps), 3 wines were analysed at 3 different days (6 replicates per day) according to the procedure described in Section 2.3. The results (expressed as GAE values) are shown in Table 5: the between-days % relative standard deviation was in the range 2.5–3.4% suggesting that the method exhibited satisfactory between-days reproducibility.

3.5.2. Trueness

The trueness was determined by recovery experiments. Two wine samples were analysed according to the procedure described in Section 2.3 before and after spiking with 1.0×10^{-3} and 2.0×10^{-3} mol L^{-1} gallic acid. The % recovery, *R*%, was calculated according to the formula:

$$R\% = \frac{\mathrm{GAE_s}}{\mathrm{GAE_{in}} + C_s} \times 100 \quad (2)$$

where $\mathrm{GAE_s}$ is the GAE valued of the sample after spiking; $\mathrm{GAE_{in}}$ is the GAE value before spiking; C_s is the added concentration of gallic acid (1.0×10^{-3} or 2.0×10^{-3} mol L^{-1}).

The recoveries in the two wines analysed were satisfactory and were in the range 96.7–97.3%.

3.5.3. Robustness

For the robustness experiments, a two-level Plackett–Burman design was employed following the procedure described earlier [41]. The number of factors examined was $n = 11$, thus the number of required experiments was $N = n + 1 = 12$. The factors and the values at the two testing levels (upper (+) and lower (−)) together with the selected value are shown in Table 6. The parameters *A* and *K* (valve position and % wine matrix simulator) were "dummy factors" (known *ad hoc* that they did not have an effect on the experiments) but were introduced to complete the set of 11 factors required in the selected design. The 12 experiments were conducted using the Plackett–Burman design and the effect, E_X, of each factor, *X*, on the response was calculated according to the equation [41] (Table 6):

$$E_X = \frac{\sum Y(+)}{N/2} - \frac{\sum Y(-)}{N/2} \quad (3)$$

where $\sum Y(+)$ and $\sum Y(-)$ are the sum of the responses in which the factor *X* was at the upper (+) and lower (−) levels, respectively.

The effects of the factors B to J (excluding the "dummy" factors A and K), calculated with Eq. (3) are shown in Table 6 (last column). From the results of the experimental design, the interval of each factor within which the method is robust could be calculated as [41]:

$$\left\langle X_{(0)} - \frac{\left|X_{(1)} - X_{(-1)}\right| E_{\mathrm{critical}}}{2\left|E_X\right|}, X_{(0)} + \frac{\left|X_{(1)} - X_{(-1)}\right| E_{\mathrm{critical}}}{2\left|E_X\right|} \right\rangle \quad (4)$$

where $X_{(0)}$ is the selected value of the factor.

$X_{(1)}, X_{(-1)}$ are the values of the factor *X* at the upper (+) and lower (−) levels, respectively.

$$E_{\mathrm{critical}} = t_{\mathrm{critical}}\,(\mathrm{SE})_e \quad (5)$$

The value of $(\mathrm{SE})_e$ could be calculated as:

$$(\mathrm{SE})_e = \sqrt{\frac{S_a^2}{N_a} + \frac{S_b^2}{N_b}} \quad (6)$$

where S_a, S_b are the standard deviations in two different days at a concentration of gallic acid equal to $c_{\mathrm{gallic\,acid}} = 1.0 \times 10^{-5}$ mol L^{-1}.

N_a and N_b are the number of repeats at each day ($N_a = N_b = 5$).

From the within-day reproducibility experiments and using Eq. (6), $(\mathrm{SE})_e = 0.48$. In addition, for a confidence level of 95% and for 8 degrees of freedom ($n = N_a + N_b - 2$), $t_{\mathrm{critical}} = 2.31$ and therefore, using Eq. (5), yields $E_{\mathrm{critical}} = 1.11$.

When the value of E_{critical} and the data in Table 6 were substituted in Eq. (4), the interval of each factor within which the method was robust were calculated as shown in Table 6.

3.6. Application of the SIA-CL method to wines

The SIA-CL method developed and validated in this work has been applied to the determination of the TAC of 15 white and 10 red wine samples according to the procedure described in Section 2.3 and the results were compared to those obtained with the DPPH and Folin–Ciocalteau (FC) approaches. The comparison was carried out using the GAE values calculated from the samples by means of the respective calibration curves for gallic acid.

Table 6
Factors and testing levels used in the experimental design for assessment of the robustness and limits within which the method is robust ($c_{\mathrm{gallic\,acid}} = 1.0 \times 10^{-5}$ mol L^{-1}).

	Factor	Selected value	Testing levels		Interval in which the method is robust[a]	$\lvert E_X \rvert$[b]
			Upper level (+)	Lower level (−)		
A[c]	Valve position connected to the detector	5	6	7	-	-
B	c_{NaOH} (mol L^{-1})	2.0×10^{-2}	2.2×10^{-2}	1.8×10^{-2}	1.9×10^{-2} to 2.1×10^{-2}	2.63
C	c_{luminol} (mol L^{-1})	5.0×10^{-4}	5.5×10^{-4}	4.5×10^{-4}	2.4×10^{-4} to 7.6×10^{-4}	0.07
D	$c_{\mathrm{H_2O_2}}$ (mol L^{-1})	5.0×10^{-4}	5.5×10^{-4}	4.5×10^{-4}	4.4×10^{-4} to 5.6×10^{-4}	0.89
E	$c_{\mathrm{Co(II)}}$ (mg L^{-1})	2.0	2.2	1.8	1.7 to 2.3	0.69
F	$V_{\mathrm{Co(II)}}$ (μL)	25	27	23	24 to 26	1.90
G	V_{sample} (μL)	75	70	80	45 to 105	0.18
H	$V_{\mathrm{H_2O_2}}$ (μL)	25	27	23	24 to 26	1.97
I	V_{luminol} (μL)	25	27	23	22 to 28	0.85
J	$F_{\mathrm{flow\ rate}}$ (mL min^{-1})	3.5	3.7	3.3	3.3 to 3.7	0.92
K[c]	% wine matrix simulator content (v/v)	0.5	0.4	0.6	-	-

[a] Calculated according to Eq. (4).
[b] Effect of each factor on the response calculated according to Eq. (3).
[c] The factors A and D are "dummy".

The correlation plot between the SIA-CL method and the DPPH method was of the form:

$$GAE_{DPPH} = (0.23 \pm 0.01)GAE_{SIA\text{-}CL} + (7.0 \pm 1.3) \times 10^{-5} \quad (R^2 = 0.975) \tag{7}$$

where GAE_{DPPH}: the GAE value (mol L^{-1}) using the DPPH method; $GAE_{SIA\text{-}CL}$: the GAE value (mol L^{-1}) using the SIA-CL method.

On the other hand, the correlation plot between SIA-CL method and the DPPH method was of the form:

$$GAE_{FC} = (0.83 \pm 0.09)GAE_{SIA\text{-}CL} + (1.1 \pm 0.8) \times 10^{-3} \quad (R^2 = 0.905) \tag{8}$$

where GAE_{FC}: the GAE value (mol L^{-1}) using the FC method; $GAE_{SIA\text{-}CL}$: the GAE value (mol L^{-1}) using the SIA-CL method.

It is well known that different *in vitro* methods for the determination of antioxidant activity produce significantly different TAC values due to the different scavenging mechanism of the reactive reagent by the antioxidants in the sample [17]. In this sense, the determination of the absolute TAC values is meaningless without referring to the method used. Therefore, what can be practically expected from a TAC assay is to provide a means to rank samples according to their relative TAC. Depending on the sample matrix and the CL reaction utilised, the DPPH and different CL assays have been shown to correlate at different degrees; for instance, no clear correlation between the two methods was observed in oil samples [24,39] but a good correlation was observed for fruit juices, plant extracts and wine [27–29,37]. In this work, the correlation between the SIA-CL method and the DPPH method was satisfactory (R^2 = 0.975) despite the fact that the slope of the correlation slope differed significantly from unity (i.e. the two methods did not yield comparable absolute values of the TAC of the samples) suggesting that the present method could used for ranking samples according to their TAC.

In addition, several studies exist discussing the possible correlation between the polyphenol content (as measured mainly by the FC method) and the TAC (measured by various methods) in wines revealing various degrees of correlation [31–33,42,43]. In the present work, the results for the wine samples using the SIA-CL method exhibited a poorer correlation with the FC method (R^2 = 0.905) than with the DPPH method. This conclusion is consistent with previous reports [27,29] and indicates that the present method would be less satisfactory in ranking wines according to their phenolic content with respect to the standard FC method.

4. Conclusions

The present work describes the development and validation of a SIA-CL method for the assay of the TAC of wines. The method is rapid (with a sampling frequency of 60 samples h^{-1} as opposed to a reaction time of 30–150 min normally required for the batch DPPH method), simple, fully automated, sensitive, accurate, economical in terms of sample and reagent consumption and makes use of inexpensive instrumentation. Comparison of the results of the present SIA-CL assay with the DPPH method for the analysis of several wine samples revealed that the two approaches exhibit a positive correlation (R^2 = 0.975) suggesting that the present method could be applied for the relative ranking of wines with respect to their TAC.

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