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Evaluation of the total antioxidant capacity by using a multipumping flow system with chemiluminescent detection

Silvia R.P. Meneses [a], Karine L. Marques [b], Cherrine K. Pires [a], João L.M. Santos [b,*], Eduarda Fernandes [b], José L.F.C. Lima [b], Elias A.G. Zagatto [a]

[a] *Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, P.O. Box 96, Piracicaba 13400-970, Brazil*
[b] *REQUIMTE, Departamento de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal*

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Abstract

An automated flow-based procedure for assessment of total antioxidant capacity was developed. It involved a multipumping flow system, a recent approach to flow analysis, and exploited the ability of selected compounds to inhibit the chemiluminescence reactions of luminol or lucigenin with hydrogen peroxide. The system included several discretely actuated solenoid micropumps as the only active components of the flow manifold. This enabled the reproducible insertion and efficient mixing of very low volumes of sample and reagents as well as the transportation of the sample zone toward a flow-through luminometer, where the chemiluminometric response was monitored. With luminol as the chemiluminogenic reagent, linearity of the analytical curves was noted up to 3.2×10^{-4}, 1.1×10^{-3}, and 8.8×10^{-8} mol L^{-1} for Trolox, ascorbic acid, and resveratrol, respectively. With lucigenin, linear calibration plots up to 2×10^{-5} mol L^{-1} of Trolox and 5.7×10^{-5} mol L^{-1} of ascorbic acid were obtained. As favorable analytical figures of merit, the measurement precision (RSD typically between 0.2 and 2.0%, $n = 10$), low operational costs, low reagent consumption, sampling rate (160 and 70 h^{-1}), and versatility should be highlighted. The proposed system can be used in distinct analytical circumstances without requiring physical reconfiguration.

Keywords: Total antioxidant capacity; Chemiluminescence; Flow analysis; Multipumping; Hydrogen peroxide; Luminol; Lucigenin

Biological structures are permanently exposed to potentially damaging oxidative stresses [1], usually associated with reactive oxygen species (ROS)[1] that may include nonradical species such as hydrogen peroxide. These are often implicated in the pathology of several human disorders such as cardiovascular diseases, cancer, and degenerescence of the central nervous system (e.g., Alzheimer's disease) [2]. Cellular damages arising from ROS generation and oxidative scavenging systems can be prevented by means of the intake of antioxidant agents able to effectively inhibit these harmful reactions [3].

During recent years, an increasing interest in the therapeutical application of antioxidants and in the consumption of dietary supplements containing mixtures of antioxidant molecules has been observed. To this end, knowledge of the total antioxidant capacity (TAC) of a specific chemical, either natural or artificial, is essential for understanding its susceptibility to oxidative stress and for evaluating its therapeutical potential. In this context, there is a need for identification of more economic and effective natural antioxidants with potential to be incorporated into foodstuffs. Several assays for measuring the scavenging capacity of different molecules

* Corresponding author. Fax: +351 22 2004427.
E-mail address: joaolms@ff.up.pt (J.L.M. Santos).
[1] *Abbreviations used:* ROS, reactive oxygen species; TAC, total antioxidant capacity; MPFS, multipumping flow system; FIA, flow injection analysis.

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have been developed, most involving the generation of free radicals and the assessment of the ability of the antioxidant molecule to inhibit this generation [4]. These assays are usually characterized by a low automation level and based on manual discrete methods involving the use of a single reaction cell or a multiwell microplate detector whereby the solutions are added successively. The analytical signal is usually obtained after a preselected delay time from the instant of mixing as the integral of the signal over a given time period or as the entire integral. Measurements are time-consuming, laborious, and costly and also are susceptible to operational errors, such as inadequate sample/reagent mixing, that could affect precision and accuracy.

The distinct artificially generated oxidative systems used for TAC assessment differ in the selection of the oxidation source and target as well as in the strategy for evaluating the oxidation products. The latter generally involves spectrophotometric or fluorimetric methods. In this context, different assays, such as the total radical trapping parameter [5], the Trolox equivalent antioxidant capacity [6], the oxygen radical absorbance capacity [7], the total oxyradical scavenging capacity [8], and the ferric reducing/antioxidant power [9], should be highlighted. A commonly used method for the determination of the antioxidant capacity is based on the evaluation of the inhibition of the luminol/hydrogen peroxide chemiluminescent reaction [10]. Implementation of fast chemiluminescence reactions for traditional procedures involving a single cuvette or multiwell microtiter plate luminometers suffers from an additional limitation in that fast mixing and a short time prior to measurement are required.

The highly advantageous analytical characteristics associated with chemiluminescent measurements [11]—operational simplicity, use of inexpensive equipments, wide linear dynamic range, sensitivity, and selectivity—become more evident when the procedure is implemented in an automated analyzer, and flow analysis represents an advanced alternative in this regard. Improved measurement reproducibility and reliability of results are attained at higher sampling rates [12,13]. The multipumping flow system (MPFS) was recently proposed as an approach to automated handling of sample and reagent solutions and, thus, to implementation of fast, low-cost, reliable, and versatile analytical procedures [14]. The MPFS is a computer-controlled system using several solenoid actuated micropumps as the only active components in the manifold. The micropumps act as sample/reagent insertion, fluid propelling, mixing, and commutation units. In contrast to the typical laminar flow conditions of flow injection analysis (FIA) systems, the MPFS generates pulsed flowing streams with high mixing potential, a valuable feature because this provides improved sample/reagent intermixing that facilitates reaction development. These are particularly attractive circumstances when the mixing of sample and reagents occurs within the detector flow cell and when carrying out the measurement of short-lived chemiluminescence emissions.

The main purpose of this work was to take advantage of the mixing potential, versatility, and operational simplicity of the MPFS to develop an automated analytical flow methodology for the chemiluminometric determination of antioxidant capacity of Trolox (a water-soluble vitamin E analog), ascorbic acid, and resveratrol (an antioxidant found in grapes and present in red wine) based on the inhibitory effect of these compounds on the chemiluminescent reaction of luminol or lucigenin with hydrogen peroxide. The developed flow system was also applied to the determination of the antioxidant capacity of several pharmaceutical formulations and tea extracts.

Materials and methods

Reagents and solutions

All chemicals were of analytical grade, and purified water obtained with a Milli-Q system was used throughout (conductivity $<0.1\ \mu s\ cm^{-1}$).

The 1.0×10^{-2} mol L^{-1} luminol (3-aminophthalhydrazide, Sigma, St. Louis, MO, USA) solution was prepared by dissolving 17.7 mg in 10.0 ml of 0.1 mol L^{-1} KOH. The oxidizing reagent was prepared by diluting 0.1 ml of 9.7 mol L^{-1} H_2O_2 in 100 ml water. The 5.7×10^{-5} to 1.1×10^{-3} mol L^{-1} ascorbic acid (Sigma), 8.0×10^{-5} to 3.2×10^{-4} mol L^{-1} Trolox (6-methoxy-2,5,7,8–tetramethylchromane-2-carboxylic acid, Sigma), and 8.8×10^{-9} to 8.8×10^{-8} mol L^{-1} resveratrol (3,4′,5-trihydroxy-*trans*-stilbene, Sigma) solutions were prepared daily in water, 0.1 mol L^{-1} KOH, and 10% (v/v) methanol, respectively.

The 1.0×10^{-2} mol L^{-1} lucigenin (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate, Sigma) solution was prepared by dissolving 17.8 mg in 10 ml of a buffer solution (pH 7.4) that was prepared by dissolving 0.60 g Tris in 100 ml of 0.04 mol L^{-1} HCl. The oxidizing reagent was prepared by diluting 28 ml of 9.7 mol L^{-1} hydrogen peroxide in 100 ml water. The 1.2×10^{-5} to 6.0×10^{-5} mol L^{-1} ascorbic acid and 1.6×10^{-5} to 4×10^{-5} mol L^{-1} Trolox solutions were prepared daily in water and buffer solutions, respectively.

Pharmaceutical preparations (Redoxon, Cecrisina, and Vit C Alter) and tea extract (Gincoben) were analyzed after solubilization and without any prior sample treatment.

The multipumping flow system

The detector was a model CL2 luminometer (Camspec, Cambridge, UK) furnished with a 60-μl inner

volume flow cell. The solenoid micropumps (Bio-Chem Valve, Boonton, NJ, USA) were of the fixed displacement diaphragm type, operated through a CoolDrive (NResearch, West Caldwell, NJ, USA) power drive circuit. The pumps were filled up when switched on, and solution delivering was accomplished when the pumps were switched off. For each solution, the pulse frequency determined its flow rate, whereas the number of pulses and the pump stroke defined the volume inserted in the analytical path. The pumps dispensed 8-μl strokes and could be actuated at pulse frequencies as high as 250 min^{-1} [14]. A Pentium III microcomputer equipped with a PCL 711 Advantech interface card (San Jose, CA, USA) was used for the control of the micropumps and the real-time data acquisition and processing. Software was developed in Microsoft QuickBasic 4.5. The flow setup also included reaction coils and transmission lines of 0.8 mm internal diameter, PTFE tubing, homemade connectors, and accessories.

The flow diagram (Fig. 1) was designed with three micropumps (P_1, P_2, and P_3) for insertion and propulsion of the involved solutions. Antioxidant solution (sample), carrier, and reagent solutions were carried out toward the detector by repeated actuation of the corresponding micropumps. The antioxidant (Trolox, ascorbic acid, or resveratrol), oxidant (hydrogen peroxide, also acting as carrier stream), and chemiluminogenic reagent (luminol or lucigenin) solutions were inserted through actuation of P_1, P_2, and P_3 pumps, respectively, and no specific insertion valve was required. Depending on the chemiluminescent reagent under evaluation, sample insertion was accomplished in a different mode. When luminol was used, several sample/reagent pulses were introduced by fast repetitive simultaneous on–off switching of P_1 and P_3 micropumps (Fig. 2), whereas

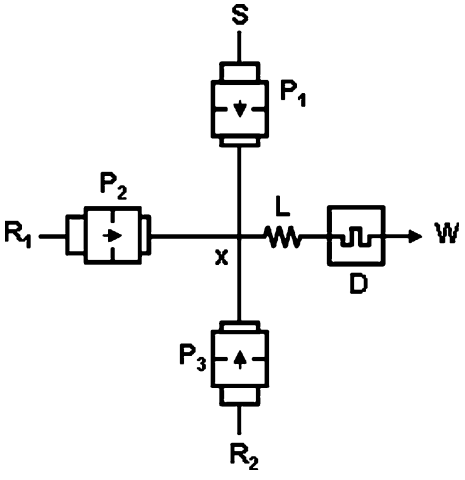

Fig. 1. The multipumping flow system. P_1, P_2, and P_3, solenoid micropumps 1, 2, and 3, respectively; S, sample; R_1, oxidant reagent; R_2, chemiluminogenic reagent (luminol or lucigenin); x, confluence point; L, transmission line (reaction coil); D, detector; and W, waste.

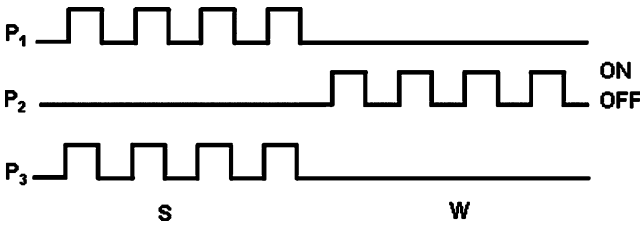

Fig. 2. Micropump status during analytical cycle. Figure refers to luminol. Upper and lower lines, pump on (filling up) and off (solution delivering), respectively; S stage, sample introduction and mixing with oxidant; W stage, transport of the reaction zone (mixing with luminol) and system washing.

P_2 was maintained off to avoid dilution at the merging point x. Because these two micropumps were operated simultaneously, the sample was mixed with luminol inside the reaction coil. Subsequently, by actuation of P_2 (P_1 and P_3 switched off), the sample/luminol initial reaction zone was transported toward the detector, reacting with the hydrogen peroxide carrier solution and producing a chemiluminescence response. Alternatively, when lucigenin was used as chemiluminogenic reagent, all pumps were operated simultaneously and a sample/lucigenin/hydrogen peroxide reaction zone was formed immediately inside the analytical path. This reaction zone was subsequently carried toward detection by repetitive actuation of P_2, as in the previous situation. Passage of the sample zone through the detector caused a transient signal recorded as a peak. Lessening in peak heights due to the different concentrations of the investigated antioxidant constituted the measurement basis.

In both situations of sample/reagent mixing, reaction zone establishment and development was accomplished by means of two complementary processes: the simultaneous insertion of the solutions in a configuration analogous to the merging zones approach [15] and the pulsed nature of the flowing stream. In fact, the sudden diaphragm displacement of the micropump generated a burst of solution that was suddenly introduced into the flow system, followed by a short resting period for filling up the micropump. These alternating flow conditions originated a pulsed flowing stream characterized by a chaotic movement of the solutions in all directions, contributing to an efficient homogenization of the sample zone and, thus, favoring reaction development [16–18].

After system design, the main analytical figures of merit were evaluated. The antioxidant capacity of the assayed compounds (Trolox, ascorbic acid, and resveratrol) was evaluated in terms of their ability to inhibit the oxidation of luminol (or lucigenin) by hydrogen peroxide. The inhibition percentage ($I\%$) was calculated as

$$I\% = (h_{\text{blank}} - h_{\text{sample}}) \times 100/h_{\text{blank}}, \quad (1)$$

where h_{blank} and h_{sample} represent recorded peak heights related to water and to solutions of the tested antioxi-

dant compound. Another evaluated parameter indicates the antioxidant concentration providing 50% inhibition (IC_{50}) and was estimated from the I% versus log C (antioxidant concentration) plot.

Results and discussion

Oxidation of luminol by hydrogen peroxide under alkaline conditions is one of the best known and most efficient chemiluminescent reactions, presenting a quantum yield in water of approximately 0.01 [11]. This reaction occurs under a large diversity of conditions; therefore, the involved parameters should be controlled in designing a specific analytical procedure so as to attain a direct proportionality between light intensity and analyte concentration. Under optimized conditions, the reaction of luminol with H_2O_2 is relatively fast, raising some operational difficulties when this reaction is to be implemented in a discrete method as is the case of the manual use of single cuvette or multiwell luminometers. A strict timing of all analytical tasks, including the addition of reagents and sample measurement, is fundamental to accomplish the required precision and accuracy.

In most circumstances, this is not easily achieved; therefore, it could be considered preferential to use less favorable chemical conditions, thereby favoring accuracy or precision to the detriment of sensitivity or sampling rate. For the same reasons, some analytical applications would make use of the reaction between lucigenin and hydrogen peroxide that is slower but is more easily implemented and controlled.

Implementation of the investigated chemiluminescent reactions in the MPFS in Fig. 1 required optimization of several parameters, such as flow rate, sample/reagent volumes and concentrations, reaction coil length, pH, available time for reaction development, and composition of the carrier stream, to maximize the gap (Δh) between the emission of light provided by the blank and that provided by the antioxidant solution under evaluation, reflecting the inhibition percentage.

Influence of reagent concentrations

The luminol concentration was investigated within 0.5×10^{-3} and 2.0×10^{-3} mol L^{-1}, and the highest Δh value was obtained for 1.0×10^{-3} mol L^{-1} luminol. Beyond this value, both the blank and the analytical signals decreased, resulting in a pronounced lessening of recorded peak heights. The H_2O_2 concentration was tested within 0.5×10^{-3} and 2.0×10^{-3} mol L^{-1}, and increasing the oxidant concentration led to a slight enhancement in sensitivity. Because the blank signal exhibited a maximum value for 1.0×10^{-3} mol L^{-1} H_2O_2, this concentration was selected for the subsequent experiments. Because the chemiluminescent reaction occurs under alkaline conditions, the influence of alkalinity was investigated. To this end, distinct KOH concentrations (0.05–0.4 mol L^{-1}) were tested and the Δh values were not significantly affected by the KOH concentration. It should be noted that increasing the alkalinity increased the blank value up to 0.3 mol L^{-1} KOH (highest Δh) before it decreased. The KOH concentration was then selected as 0.1 mol L^{-1}. Concerning reaction between lucigenin and hydrogen peroxide, lucigenin concentrations within 0.5×10^{-3} and 4.0×10^{-3} mol L^{-1} were evaluated. An increment of the analytical signals was observed for concentrations up to 3.5×10^{-3} mol L^{-1}, and the system response approached stabilization beyond this value. Hydrogen peroxide concentrations were investigated within 0.5 and 3.0 mol L^{-1}. These concentration values were substantially higher than those used for the reaction with luminol because the reaction is slower and less sensitive. It was verified that an H_2O_2 concentration lower than 2.0 mol L^{-1} did not provide sufficient oxidant concentration for quantitative complete lucigenin oxidation, resulting in two-peak recording. On the other hand, an H_2O_2 concentration higher than 2.5 mol L^{-1} did not produce a significant increase in the analytical signal, dictating the selection of 2.5 mol L^{-1} H_2O_2 for the subsequent experiments. Experiments were carried out at pH values within 7.2 and 7.6, permitting verification of the pronounced effect of pH on the light intensity. Considering these results, a pH of 7.4 was selected for further experiments.

Effect of system geometry

The sample volume, established as the number of sample pulses, was always a multiple of the micropump stroke volume (8 μl). The investigated values were 32, 48, 64, and 80 μl, corresponding to 4, 6, 8, and 10 pulses, respectively. These sample volumes were evaluated by using different coil lengths because this is one of most significant parameters influencing sample dispersion and homogenization of the reaction zone. Length of the reaction coil is also important because it can determine, in combination with the flow rate, whether the maximum chemiluminescent emission occurs before, within, or after the detector flow cell. This parameter should be optimized according to the reaction kinetics to ensure that maximum light emission was attained when the reaction zone passed through the detector flow cell, thereby ensuring a high detection response that guarantees enhanced sensitivity. Because oxidation of luminol and lucigenin exhibited different reaction rates (much higher for luminol), shorter reaction coils for luminol (5–30 cm) and longer coils for lucigenin (10–150 cm) were evaluated.

Results for luminol showed that for less than 10-cm reaction coils sample/reagent mixing was inadequate,

Table 1
Inhibition percentages related to luminol and lucigenin

CL reagent	Antioxidant	Inhibition curve[a]	r^2	IC_{50}
Luminol	Trolox	$I\% = 46.76 \log C + 110.20$	0.998	2.0×10^{-4}
	Ascorbic acid	$I\% = 18.19 \log C + 76.415$	0.991	1.7×10^{-4}
	Resveratrol	$I\% = 41.41 \log C + 49.293$	0.991	4.4×10^{-3}
Lucigenin	Trolox	$I\% = 79.10 \log C + 219.48$	0.999	2.8×10^{-3}
	Ascorbic acid	$I\% = 55.25 \log C + 188.29$	0.998	1.7×10^{-3}

Note. Table refers to the proposed MPFS in Fig. 1. IC_{50} values are expressed in mol L^{-1}. Data are based on six replications.
[a] Equation of the curve relating $I\%$ and $\log C$ (antioxidant concentration).

resulting in poor repeatability of the analytical signals, whereas for a 30-cm reactor maximum chemiluminescent emission occurred before the reaction zone reached the detector. This affected the sensitivity of the determination because it decreased the gap between sample and blank signals. A 20-cm reactor provided a suitable sample residence time inside the analytical path in terms of both sample homogenization and reaction development. Regarding lucigenin, it was verified that improved results were obtained with a 50-cm coil.

Concerning sample volume, a parameter closely related to coil length, it was verified that with luminol there was a very slight increase of the analytical signal with the sample volume up to 48 μl (6 pulses) and a subsequent signal stabilization. This value corresponded to the highest difference between sample and blank recorded peak heights. On the other hand, sample volumes higher than 64 μl led to the appearance of doubled peaks as a consequence of the inadequate sample/reagent mixing and incomplete reaction zone homogenization. Lucigenin exhibited a similar behavior, but the analytical signal increment was much more pronounced in relation to luminol. However, it should be emphasized that for identical sample volumes, the analytical signal provided by luminol was higher relative to that provided by lucigenin. An important difference between performance of both chemiluminogenic compounds is that luminol blank values were nearly unaffected by variations in introduced volume, whereas for lucigenin an 80-μl volume yielded an analytical signal threefold higher than the signal attained with 32-μl introduced volume. As expected, when 150-cm reactors were assayed for lucigenin, the highest analytical signals were obtained with the utmost sample volume because the increment in the sample reaction time was counterbalanced by increased sample dispersion and an undesired dilution effect prevails.

Influence of flow rate was evaluated by setting pulse intervals (resting time between two consecutive pulses) of 0.5 to 0.2 and 0.8 to 0.2 s for the luminol and lucigenin trials, respectively, corresponding to flow rates of 0.96 to 2.4 and 0.6 to 2.4 ml min^{-1}. For luminol, the blank and sample signals were practically unaffected by flow rate variations. As a compromise between reproducibility and sampling rate, a pulse interval of 0.3 s corresponding to a flow rate of 1.6 ml min^{-1} was considered most appropriate. For lucigenin, both the blank value and the analytical sensitivity underwent a slight increase as the flow rate decreased, probably as a consequence of the increased reaction time. Moreover, for higher flow rates, reproducibility was deteriorated. A 1.2-ml min^{-1} flow rate (0.4-s pulse) was then selected.

Inhibition percentage values are presented in Table 1. Regardless of the chemiluminogenic reagent, ascorbic acid exhibited the highest antioxidant activity, followed by Trolox and resveratrol. The procedure involving lucigenin was much less sensitive relative to that using luminol; therefore, higher concentrations of the tested antioxidants were needed. Consequently, and in view of poor solubility of resveratrol, the corresponding IC_{50} value was not determined. The developed methodology was also evaluated in the determination of the antioxidant activity of several ascorbic acid pharmaceutical preparations as well as of a tea extract. The obtained results (Table 2) confirmed the results obtained with the model compounds showing that the procedure involving luminol was much more sensitive that the one using lucigenin.

Approximately 160 samples (for luminol) and 70 samples (for lucigenin) can be run per hour with the proposed systems, and the consumption of sample and reagents per determination was low: sample, 48 μl;

Table 2
Inhibition percentages related to luminol and lucigenin of distinct pharmaceutical formulations and tea extract

CL reagent	Sample	IC_{50}
Luminol	Redoxon	1.57×10^{-4}
	Cecrisine	1.15×10^{-4}
	Vit C Alter	2.24×10^{-4}
	Gincoben	3.67×10^{-3}
Lucigenin	Redoxon	1.51×10^{-3}
	Cecrisine	1.33×10^{-3}
	Vit C Alter	6.75×10^{-3}
	Gincoben	0.96

Note. IC_{50} values are expressed in mol L^{-1} except for tea extract (Gincoben), which is expressed in mg ml^{-1}. Data are based on six replications.

luminol, 85.1 μg; lucigenin, 86.6 μg; hydrogen peroxide, 158.3 μg (luminol), and 446.5 μg (lucigenin). Precise measurements were always obtained (RSD typically $<2.0\%$, $n = 10$), and the analytical system was stable for 8 h operation periods, with baseline drift being not detectable. Solenoid micropumps exhibited good robustness in that no variations in performance were noted during the development of this project.

Conclusions

A fully automated flow-based procedure for total antioxidant capacity determination was developed, enabling a high sampling rate and favorable characteristics of precision, accuracy, and sensitivity. The procedure takes advantage of the chemiluminescent reactions of luminol or lucigenin with hydrogen peroxide. The proposed procedure provided fast sample/reagent mixing and exhibited high flexibility, ensuring a ready system adaptation to the requirements of chemiluminescent reactions without requiring any physical reconfiguration except with regard to reaction coil length. Whenever required, it could be easily coupled with automated sample pretreatment systems. The approach is simple and easily operated, being applicable to the analysis of food, pharmaceutical, and biological samples.

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