Antioxidant Assay for Fruit Juices by Using a Chemiluminescence Method Based on the Co(II)EDTA/luminol/H_2O_2 System

DIANA CRISTEA[1], CLAUDIA VALENTINA POPA[2], SIMONA CARMEN LITESCU[3], ILEANA CORNELIA FARCASANU[2], ANDREI FLORIN DANET[1]*

[1] University of Bucharest, Department of Analytical Chemistry, 90-92 Sos. Panduri, 050657, Bucharest, Romania

[2] University of Bucharest, Department of Organic Chemistry, Biochemistry and Catalysis, 90-92 Sos. Panduri, 050657, Bucharest, Romania

[3] National Institute for Biological Science, Centre of Bioanalysis, 296 Spl. Independentei, 060031, Bucharest, Romania

It is studied a batch analytical method based on the system Co(II)EDTA/luminol/H_2O_2 for the determination of antioxidant capacity of different types of sample. The method is based on the oxidation and chemiluminescence of luminol under the influence of reactive oxygen species produced in a Fenton type reaction of hydrogen peroxide with Co(II). The antioxidants from a sample react with the reactive oxygen species and decrease the chemiluminescence signal. It was drawn a calibration graph by plotting the ratio of chemiluminescence intensity in the absence and in the presence of an antioxidant and the concentration of a standard antioxidant (gallic acid). The calibration graph is linear in the domain 10^{-6} – 10^{-4} M galic acid. The method was applied for the analysis of three types of fruit juices. It has been done a comparison between the results for antioxidant capacity determination obtained with the developed method and the literature data for similar types of samples. The acuracy of the method was verified by using standard addition method.

Keywords: chemiluminescence analysis; Co(II); luminol; total antioxidant capacity; fruit juice

It is well known that oxidation damages various biological substrates and causes many diseases including diabetes [1], inflamation [2], Parkinson's disease [3], Alzheimer's disease and cancer. An antioxidant in a broad way, is *any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate* [4]. A simpler definition would be "any substance that delays, prevents or removes oxidative damage to a target molecule" [5]. In the following we will refer only to antioxidants which are relevant to biologic systems. Recently, research associated with natural antioxidants has increased dramatically in various fields, including biochemistry, food chemistry, food biology, plant chemistry and medicinal plants.

Biological antioxidants include enzymatic antioxidants (e.g., superoxide dismutase, catalase, etc.) and nonenzymatic antioxidants such as oxidative enzyme inhibitors (e.g., cyclooxygenase), reactive oxygen species (ROS) scavengers, and transition metal chelators. Conventional methods for the determination of antioxidant capacity can be roughly classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) [6]. HAT-based assays include inhibition of induced low-density lipoprotein auto-oxidation, oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP). ET-based assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), trolox [38] equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), assay using a Cu(II) complex as an oxidant (CUPRAC) and 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) method. None of the methods mentioned above has general applicability.

Methods concerning the determination of antioxidant capacity for different types of samples have been extensively reviewed [6, 7-16].

The chemiluminometric methods are generally more sensitive than the conventional methods used for ROS and for antioxidant determination. In principle, ROS form with the chemiluminometric reagents excited species that generate chemiluminescence radiations. Any compound that reacts with the initiating radicals inhibits the light production. In this manner it is possible to quantify the antioxidants in a sample. The most widely used chemiluminometric reagent to trap oxidants and convert weak emissions into intense, prolonged, and stable light emission is luminol [13]. Luminol is extensively used to detect the activity of natural and synthetic antioxidants [17], of biologic fluids [18, 19], of various plant extracts [20, 21] and recently was reported an optimized and validated method to evaluate total reactive antioxidant potential [21].

Parejo et al [22, 23] and Giokas et al [24] were the first which have described the application of Co(II)EDTA/luminol/H_2O_2 system for determination of antioxidant capacity. The coupling of FIA technique with chemi-luminescence detection [24, 25] or other detection techniques [26, 27], have the advantage of high sample throughput and a low consumption of reagents and sample. However for the analysis of a small number of sample it is better to use a *batch* method of analysis. The purpose of this paper was to investigate the Co(II)EDTA/luminol/H_2O_2 system utilized in a new *batch* method of analyis for the determination of antioxidant capacity. The mentioned system have been investigated already by the authors of this papers in a flow injection analysis assembly [28, 29]. The new developed assay for antioxidant capacity determination, reported in this paper clearly improve the

* email: andreidanet@yahoo.com; Tel.: +40(0)21-3051455

performance of the Co(II)EDTA/luminol/H_2O_2 method [30, 31] by using a calibration graph instead of the IC_{50} values for assesement of antioxidant capacity.

Experimental part

Reagents and solutions

Boric acid, $CoCl_2.6H_2O$ (Reactivul, Bucharest), 30% H_2O_2 (w/v) (Chimopar, Bucharest), ethylenediaminetetraacetic acid (EDTA) disodium salt (Loba Chemie), 5-amino-2,3-dihydronaphtalazine-1,4-dione, luminol (Fluka Chemie AG), gallic acid (Sigma), NaOH (Chemapol) were used.

- 0.1 moles/L sodium borate buffer, *p*H 9.0 was obtained from a 0.1 moles/L boric acid solution, by adjusting the *p*H with a 10% NaOH solution.

- $3x10^{-3}$ moles/L Na_2EDTA solution in 0.1 moles/L boric acid solution.

- $3x10^{-3}$ moles/L Na_2EDTA solution in borate buffer 0.1 moles/L containing $CoCl_2$ in the ratio Co(II)/ EDTA = 0.8.

- 3.4 $x10^{-4}$ moles/L luminol in solution of 0.1 moles/L borate buffer.

- Stock solution of 1 moles/L hydrogen peroxide from which the working solution of $3x10^{-4}$ moles/L hydrogen peroxide containing 2×10^{-4} moles/L EDTA for stabilization was prepared. EDTA was employed to complex traces of metallic ions which catalytically decompose H_2O_2.

- Stock solution of 10^{-2} moles/L in water gallic acid from which the working solutions with the concentrations of 10^{-4} – 10^{-6} moles/L were prepared .

All solutions were prepared in double distilled water. Hydrogen peroxide working solutions were prepared on a daily basis. The other solutions were kept in the refrigerator (+4°C) for maximum three days after preparation and were brought to room temperature just before measurements.

Apparatus

The chemiluminometric measurements were done with the Turner Biosystems 20/20n apparatus controlled by a computer.

Working procedure

All the determinations were done in 1.5 mL Eppendorf tubes. Before starting the measurements, two volumes of $3x10^{-3}$ moles/L Na_2EDTA solution in 0.1 moles/L borate buffer containing $CoCl_2$ in the ratio Co(II)/EDTA = 0.8 were mixed with one volume of 3.4 $x10^{-4}$ moles/L luminol solution in solution of 0.1 moles/L borate buffer. This solution was named ***working solution*.** Then 350 μL of *working solution*, 350μL of 0.1 moles/L sodium borate buffer, *p*H 9.0 and 25 μL of analyzed samples or standard solution of gallic acid were introduced in an Eppendorf tube. The mixture was thoroughly stirred in the Eppendorf tube.

350 μL of solution of hydrogen peroxide $3x10^{-4}$ moles/L were there introduced in the tube, mixture was thoroughly stirred with the tip of the automatic pipette and then the tube was inserted in the chemiluminometer for measurements.

Results and discussions

Principle of the method

The chemiluminescence reaction of luminol in an alkaline medium in the presence of hydrogen peroxide and Co(II)EDTA can be explained as follows. Hydroxyl radical, $HO^{\bullet}$ which it is a strong oxidant, forms in the reaction medium by the following Fenton reaction:

$$H_2O_2 + Co^{2+} \rightarrow Co^{3+} + HO^{\bullet} + HO^{-}$$

Because the Co(II) ions are complexed with EDTA, the concentration of free Co(II) ions in the reaction medium is very low and population of generated $HO^{\bullet}$ able to oxidise luminol is also very low. In these conditions, the chemiluminescence signal of luminol is low but it lasts much longer in comparison with the chemiluminescence signal obtained in the absence of EDTA in the reaction medium.

The generally accepted mechanism of luminol chemiluminescence is shown in figure1 [32, 33]. The luminol anion undergoes a single electron oxidation (by $HO^{\bullet}$) in the alkaline solution in order to form a diaza quinone which is subsequently oxidized by peroxide or superoxide to give 3-aminophthalate in an excided state which emits light at 425 nm.

If an antioxidant is introduced in the reaction medium this will react with hydroxyl radicals or other oxidants present in the medium and the chemiluminescence signal will decrease. The decrease of chemiluminescence signal depends on the antioxidant concentration. By representing the decrease of chemiluminescence signal *versus* the antioxidant concentration it is possible to draw a

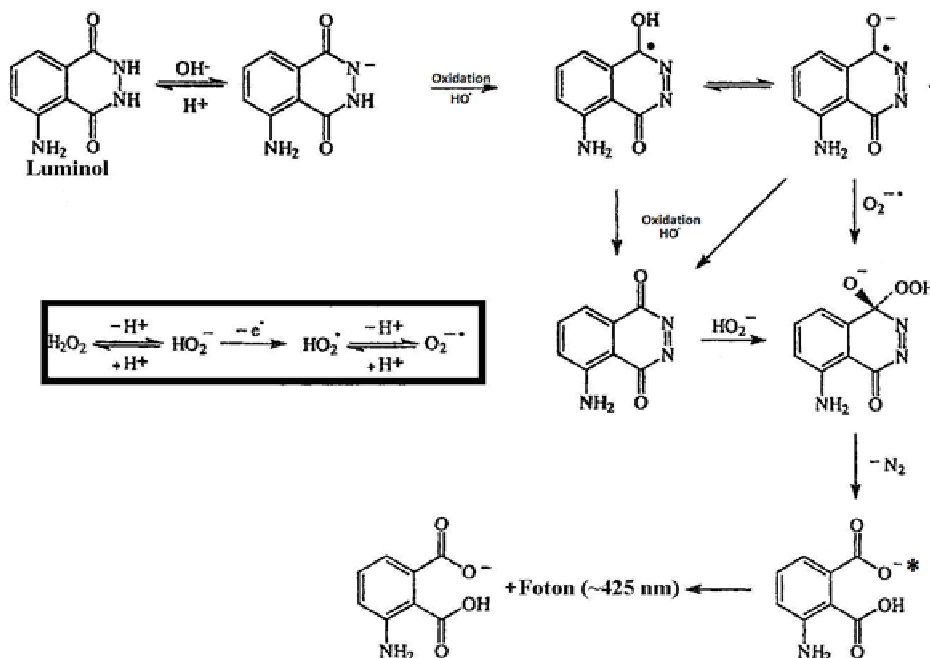

Fig.1. Proposed mechanism for chemiluminescence reaction of luminol

calibration graph and then to determine the concentration of an antioxidat in a sample.

Shapes of the chemiluminescence signals

Shapes of chemiluminescence signals registered for no antioxidant present in the reaction mixture and for analysis of standard solutions of gallic acid of different concentrations are presented in figure 2. As can be seen in figure 2 the shape of chemiluminescence signals remains more or less similar with that obtained in the absence of gallic acid, but the maximum chemiluminescence signal decreases with increasing the gallic acid concentration.

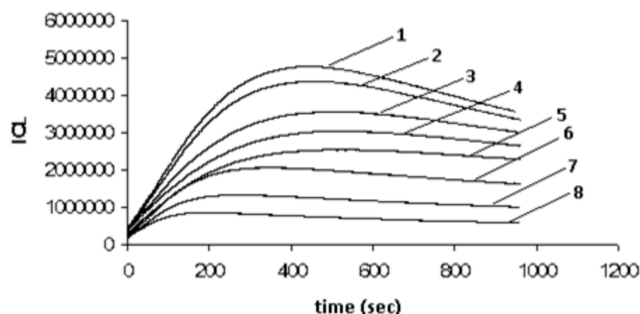

Fig. 2. The shape of the analytical signal registred in the absence and in the presence of different cocentrations of gallic acid. 1) without gallic acid; 2) 10^{-6} M; 3) $2.5x10^{-6}$ M; 4) $5x\ 10^{-6}$ M; 5) 10^{-5} M; 6) $2.5x10^{-5}$ M; 7) $5x10^{-5}$ M; 8) 10^{-4} M gallic acid. Samples were prepared according to the working procedure

The reproducibility of the determinations was very good. The relative standard deviation (RSD) was less than 4% for a set of determinations for the concentrations of gallic acid within 10^{-6} – 10^{-4} M.

Calibration graph

By plotting the I_0/I ratio *versus* gallic acid concentration (where I_0 is the maximum chemiluminescence intensity in the absence of an antioxidant and I is the maximum chemiluminescence intensity in the presence of an antioxidant in the analyzed sample) it is possible to obtain a linear calibration graph. The values I_0 and I were measured at the same time on the curves representing I_{CL} *versus* time. The equation of the calibration graph is:

$$I_0/I = 55601C_{gallic\ acid}(M) + 1.14$$

Coefficient of determination is $R^2 = 0.9975$. The calibration graph is linear in the domain 10^{-6} – 10^{-4} M galic acid.

Antioxidant capacity determination for some fruit juices

The following natural fruit juices were analyzed: concentrated apple juice, orange juice Fruttia (lot LTY 06:1514/05) and orange juice Noki (lot 02325/11L). Each sample was correspondingly dilluted in order to register a chemiluminescence signal that can be interpolated in the calibration graph. Each sample was analyzed five times. The antioxidant capacity was calculated as mM (milimoles/1000 mL) equivalents of gallic acid. The obtained results are listed in table 1.

As can be seen in table 1, the results obtained in this study are in good agreement with the previously published data.

Determination of antioxidant capacity in apple juice by using the standard addition method

The procedure was as following: 1 mL of apple juice and then different quantities of standard gallic acid solutions were introduced in four calibrated flasks with volumes of 10 mL. The flasks were then filled up to 10 mL with distilled water. After homogenization, I_0/I ratio was determined for each solution by using the *Working Procedure*. The ratio I_0/I ratio plotted *versus* concentration of standard gallic acid introduced in each flask is shown in figure 3.

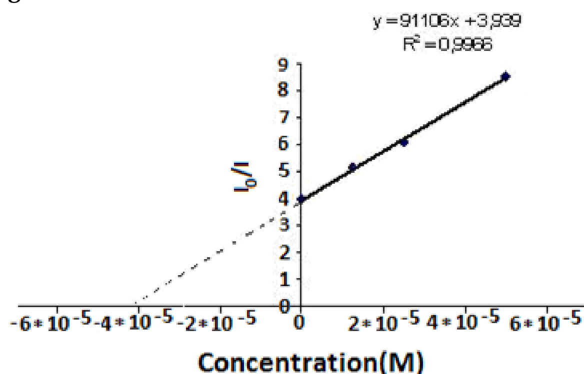

Fig. 3. Graph obtained by plotting I_0/I *versus* concentration of standard solutions of gallic acid introduced in the samples.

As can be seen from figure 3 by plotting I_0/I *versus* gallic acid concentration a straight line is obtained which continued till the intersection with abscissa gives a point that corresponds to the antioxidant capacity of apple juice expressed in gallic acid equivalents. The determined antioxidant capacity was of 4.35 mM equivalents of gallic acid which is close to 3.42 mM equivalents of gallic acid

Table 1
RESULTS OBTAINED FOR THE DETERMINATION OF ANTIOXIDANT CAPACITY OF THREE TYPES OF NATURAL JUICES AND COMPARISON WITH LITERATURE DATA

Sample	Dilution	I_0/I	Gallic acid equivalent, mM	Literature data TEAC* values (mmoles Trolox/kg or mM Trolox) or IC_{50} **
Orange juice Noki	1/50	1.34	0.413 ± 0.041	Apple 3.43 mmol/kg and orange 8.49 mmol/kg, fresh weight fruit, by $ABTS^{•+}$ *** method [34]. Soft beverages apple juice and orange juice 1.83 mM and respectively 3.02 mM, by $ABTS^{•+}$ method [34]. Soft drink 1.74 mM, orange juice 6.63 mM and 3.19 mM, by $ABTS^{•+}$ method [35]. Orange juice 2.22 mmoli/Kg, by $ABTS^{•+}$ method [36]. Apple juice 1.31-1.59 mM, by $ABTS^{•+}$ method. IC_{50}** = 2.66 µg/mL gallic acid determined with the studied method, which is close to 5.1 µg/mL for quercetin and to 2.1 µg/mL for BHA (butylated hydroxyanisole) determined with a similar method [30, 31]. 7.3 mmoles /Kg fruit (limes) by FRAP**** [37]
Orange juice Noki	1/100	1.63	0.358 ± 0.33	
Orange juice Fruttia	1/50	4.33	2.40 ± 0.21	
Orange juice Fruttia	1/100	2.19	2.27± 0.19	
Concentrated apple juice	1/100	3.20	3.53 ± 0.31	
Concentrated apple juice	1/200	1.95	3.32 ± 0.24	

* Trolox equivalent antioxidant capacity; ** IC_{50} is the amount of sample necessary to decrease by 50% the chemiluminescence intensity; *** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); **** ferric reducing antioxidant power assay.

for apple juice determined by using the calibration line. By using the standard addition methods for the determination of apple juice antioxidant capacity the matrix effects were eliminated. The conclusion that a small matrix effect is present in the determination of antioxidant capacity of apple juice could be drawn. So, in order to obtain accurate results the standard addition method for this type of sample is recommended.

Conclusions

A chemiluminescence method based on the system Co(II)EDTA/luminol/H_2O_2 for the determination of antioxidant capacity of different products was developed. In the absence of an antioxidant in the reaction medium a chemiluminescence signal is registered which rise to a flat maximum and then decreases. In the presence of an antioxidant the values of the chemiluminescence signal decrease. All the measurements were done on the flat maximum of the chemiluminescence signal.

Optimum experimental conditions for drawing a calibration graph by using gallic acid were established. The calibration graph is linear within 10^{-6} – 10^{-4} M gallic acid concentration domain. The developed method was applied for the determining the antioxidant capacity of three types of commercialy available juices. The accuracy of the method was verified by using standard addition method for the analysis of apple juice.

The results obtained in this study for antioxidant capacity determination are in good agreement with the ones previously published in literature, by using similar [30,31] or different methods [34-37] for corresponding products, as shown by the comparison presented in table 1. Thus, chemiluminescence can be successfully used in food industry for assessing the antioxidant content of natural fruit juices and soft drinks, being an accurate, rapid and relatively cheap method.

Owing to the complexity of the oxidation-antioxidation processes, no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample. A multimethod approach is necessary in the antioxidant activity assessment. Independently of the chosen method, suitable reference antioxidants should be tested for comparison. A combination of rapid, sensitive, and reproducible methods, preferably requiring small sample amounts, should be used whenever an antioxidant activity screening is designed.

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