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Determination of the antioxidant activity of flavonoids in red Cayena and Roselle flowers by chemiluminescence

José A.Murillo Pulgarín[1]*, Luisa F.García Bermejo[1], Armando Carrasquero Durán[2]
[1]Department of Analytical Chemistry and Foods Technology, University of Castilla-La Mancha, 13071 Ciudad Real, (SPAIN)
[2]Department of Chemistry, Universidad Pedagógica Experimental Libertador, Maracay, (VENEZUELA)
E-mail: acarrasquerod@gmail.com
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Abstract : This study proposes a simple and fast methodology for the estimation of the antioxidant activity (AA) of aqueous extract of flowers, based on the inhibition of the luminol chemiluminescence in a FIA system. At the optimum operational conditions the AA of several common phenolic antioxidants were measured in terms of the EC_{50} (concentration needed to reduce in 50% the CL of blank) by plotting the percentage of CL inhibition (% Inh) against the logarithm of the antioxidant concentration, the results were explained on basis of structure-reactivity relationships. The method was evaluated determining the antioxidant activity of dried flowers of *Hibiscus Rosa Sinenis* and *Hibiscus Sabdariffa,* which were extracted with ethanol and water to dissolve the biophenols, flavonoids and anthocyanins. The AA was measured by the proposed method agreed well with the results obtained by the DPPH test suggesting that it can be used as a complementary method for the evaluation of hydrophobic substances with antioxidant properties.

INTRODUCTION

Antioxidants have become one of the most important topics in human health because of the high exposition to free radicals. Although the molecular oxygen is essential for the survival of all aerobics organisms, the reactive oxygen species (ROS), produced by the oxygen metabolism are harmful for living organisms and they have been considered as promoters of several diseases including cancer, cardiovascular or neurological[15].

The polymorphonuclear are the cell mediators that produce reactive oxygen species (ROS) as an initially protective mechanism against inflammation[5], but the production of these species can damage both the target and the surrounding cells. In consequence, both the ROS release and the inflammation are involved in the pathogenesis and progression of inflammatory diseases. Under these adverse circumstances, ROS secretion is followed by lipid peroxidation, amino acid oxidation, protein fragmentation and DNA damage[9]. For that

reason, natural antioxidants are being studied in medicine as a means of limiting diseases associated with the oxidative stress and the damage that it may cause.

In general, the antioxidants can be divided into two groups depending on their mechanism of action: chain breaking antioxidants or preventive antioxidants[17]. The first group comprises those molecules that reduce the rate of chain initiation and also, they can interfere with the chain propagation. The group of preventive antioxidants includes chelators of metal ions that prevent the uncontrolled formation of free radicals and activated oxygen species catalyzed by these metal ions.

In these groups are included the plant polyphenols, which exhibit multifunctional properties, such as reducing agents, hydrogen donating antioxidants and singlet oxygen quenchers. Therefore, this ability to act as antioxidants makes them useful in protecting the human body against those free radicals by different mechanisms including the radical scavenging[10].

The evaluation of substances with potential antioxidant effects requires reliable, simple and fast analytical methods. Many *in vitro* procedures are employed, for instance, the free radical scavenging of Mediterranean and aromatic herbs were evaluated by Parejo et al.[13] using the DPPH assay, superoxide-nitro-blue tetrazolium (NBT), the coupling hypoxanthine/xanthine oxidase and the luminol chemiluminescence. In addition, the β- carotene bleaching test and Folin-Coicalteau method were used by these authors to determine the antioxidant activity and the total phenolic content in the samples evaluated. In other work, Atoui et al.[4] used the Folin-Coicalteu procedure to determine the total polyphenolic content in tea and herbal infusions, while the antioxidant activity was measured by the DPPH method and by chemiluminescence assay.

The basis of the chemiluminescence (CL) assay using luminol is the measurement of the change in the emission intensity of the electronically excited phthalate intermediates in the presence of the antioxidant, because usually an attenuation of emission (quenching or inhibition) is observed. Those substances which lower the CL intensity without affecting the total light-output are classed as inhibitors, but if the quantum yield is also suppressed they are called as quenchers. In general, the antioxidant mechanism may involve the removing of reactants through chemical interaction or complex formation or by acting as radical scavengers[14].

The procedures for based on the CL for the determination of antioxidant activity have the advantage of low detection limits, wide linear dynamic ranges and the speed of response in the determination of reactive oxygen species in a variety of biological systems, providing a more rapid approach for the measuring of the antioxidant activity compared with other methods[3]. Also, the CL measurements are often used to determine initial radical products of lipid oxidation[8] and for the quantification of the superoxide radical in biological systems.

The natural biophenols, including flavonoids and anthocyanins, present in many flowers, have chemical structures very convenient for radical scavenging, and their ability to delocalize the unpaired electron of the resulting free radical has been show to be directly proportional to their antioxidant capacity[6]. The method proposed in this work was based on the inactivation of the free radicals produced during the decomposition of hydrogen peroxide in a basic solution. In this method, transition metals were not employed because flower flavonoids can act as ion quelators and the aim of this work was to propose an analytical procedure for the evaluation of the ability of flower flavonoids for breaking the chain reactions.

MATERIALS AND METHODS

Reagents and solutions

All of the chemicals used in this work were of analytical grade and used without further purification. Water was obtained from a Milli-Q purification system. Luminol was obtained from Fluka (Spain). Sodium perborate was supplied by Sigma-Aldrich (Spain). A stock solution 1 mM of luminol was prepared in 0.1 mol L^{-1} borate buffer at pH 10.5, this solution was stored for 24 hours before to use in order to have stable and reproducible CL signals.

A 5 mM solution of sodium perborate was prepared daily and was used to supply the H_2O_2 required dor the luminol oxidation. Synthetic antioxidants Gallic acid (GA), Caffeic acid (CA), Propylgalate (PG) and tertbuthylhydroquinone (TBHQ) were obtained from Sigma (Germany).

Manifold and general procedure

A three line FIA manifold detailed in figure 1 was used for all experiments. It consisted in a peristaltic pump (Gilson Miniplus-3) to propel the reagents and carrier stream (Milli-Q water) at a constant flow rate (5 mL min^{-1}). The first channel was used for the solutions that contained the natural or synthetic antioxidants, while the second was used to propel the carrier (Milli Q water). The luminol solution (1 mmol L^{-1}) was manually injected to the carrier stream using a six port injection valve (250 µL sample loop). These solutions merged at a T-piece before enter to the detector device. The third channel carried the perborate solution directly to the detector cell, where it was mixed with the luminol-antioxidant solution.

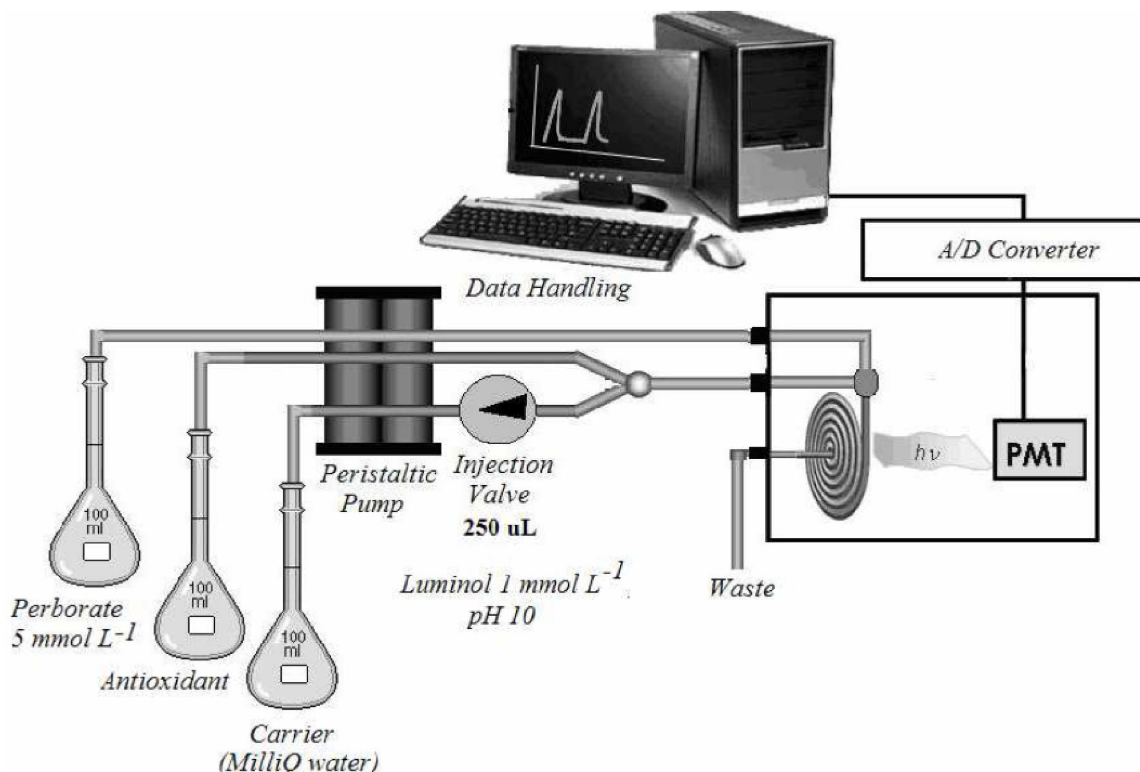

Figure 1 : System for the determination of the antioxidant capacity

The CL detector employed was a Camspec Chemiluminescence Detector CL-2 (photosensor module Hamamatsu 45773-20 spectral response from 300 to 900 nm; spiral-type flow cell, volume 120 µL; Sawston, Cambridge). It was connected to a computer by a digital analogical converter. CL intensity data against time were acquired using the Clarity version 2.4.1.77 (Data Apex) software to obtain the values of maximum CL intensity.

Evaluation of the antioxidant activity of synthetic antioxidants

To evaluate the proposed CL method, the antioxidant activity of the following synthetic antioxidants: GA, CA, TBHQ and PG was measured using the system described above and the results were interpreted on the basis of structure-reactivity relationships. Thus, 100 µg mL^{-1} stock solutions of each antioxidant were prepared in ethanol (5 %) and working solutions in the range between 0 and 100 µM were prepared by dilution in water. A blank solution which did not contain any antioxidant was lso prepared. By plotting the CL intensity against the time, the maximum (I) intensities for blank and samples were measured and the percentage of inhibition (%Inh) in each case was determined using the following equation:

$$\%\mathrm{Inh} = \frac{I_{blank} - I_{sample}}{I_{blank}} \times 100$$

Procedure to determine the EC_{50} for the flower extracts

Flowers of Red Cayena (*Hibiscus Rosa Sinenis*)

were recollected from public gardens from Maracay City, Venezuela, while dried Roselle flowers (*Hibiscus Sabdariffa L.*), known in Venezuela as *Flor de Jamaica* were purchased from a naturist store. The flower extracts were prepared as follows: 100 mg of dried flowers were macerated with 5 mL of ethanol 95 % in 125 mL Erlenmeyer flasks and then sonicated by 15 min. The supernatants were filtered through Whatman 1 filter paper into 100 mL volumetric flasks and the volumes were made up with the ultrapure water.

Aliquots form 0.2 to 2 mL of flower extracts were put into 25 mL volumetric flasks and then filled with the ultra pure water and then. These solutions were used without any other treatment. The % Inh were determined in the same way described for synthetic antioxidants. The EC_{50} values were obtained by plotting the logarithm of the extract concentration in each solution against the % Inh.

Determination of antioxidant activity (DPPH Test)

The determination of free radical scavenging was made using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH) as an alternative procedure in order to compare the results with those obtained by the proposed CL method. Five aliquots of 0.5 mL of a freshly prepared 0.1 mM DPPH radical (Sigma) were added to five test tubes. Aliquots of the flower extracts between 0.1 and 2.0 mL, depending on the antioxidant power, were added to the test tubes and the volumes were made up to 2 mL with Milli Q water. The reaction mixtures were shaken and the absorbance was measured after 30 minutes at 515 nm in a Beckman DU70 UV/Visible spectrophotometer.

The radical scavenging activities (RSA) were calculated as indicated in the equation.

$$\mathbf{RSA\ (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of the control, which consisted in 0.5 mL of MilliQ water and A_1 is the absorbance of the sample. By plotting the RSA values against the extract concentrations in each test tube the inhibition curves were represented and used to obtain the EC_{50} value.

Determination of total biophenols (TBP), anthocyanins and flavonoids

In order to explain the differences in the antioxidant activity of the two types of hibiscus flowers, the concentrations of bioactive compounds such as phenols, flavonoids and anthocyanins were determined. The spectrophotometric Folin-Coicalteu method was used to determine TBp as described by Arnous et al.[2]. Briefly 25 mL of Milli-Q water were added into a 50 mL volumetric flask and suitable amounts of the flower extracts were also added to obtain absorbance values in the range of the calibration curve. One mililiter of the Folin-Coicalteu reagent was added and, after 3 minutes, 2 mL saturated Na_2CO_3. The volume was made up and the absorbances were measured two hours later at 725 nm. Results were expressed as mg of gallic acid equivalents (GAE).

On the other hand, the quantification of anthocyanins content was made by pH-differential spectrometry[12]. Two aliquots of 1 mL of the flower extract were transferred to two 25 mL volumetric flasks. The first flask was filled with a buffer solution pH 1 (KCl in HCl) while the second was filled with the buffer solution at pH 4.5 (acetate/acetic acid). The absorbance was calculated as follows:

$$A = \left(A^{510nm}_{pH1} - A^{700nm}_{pH1}\right) - \left(A^{510nm}_{pH4.5} - A^{700nm}_{pH4.5}\right)$$

Were A represents the absorbance measured at 510 and 700 nm at the two pH conditions. The percentage of total anthocyanins (% w/w) was calculated using the molar absorbance of delphinidin-3-glucoside $\varepsilon = 23700$ and the molecular weight 518.5 g mol^{-1}.

The total flavonoid concentration was determined using the aluminium chloride colorimetric method[7]. Standard solutions of quercetin between 20 and 200 $\mu g\ mL^{-1}$ were prepared in 80 % ethanol, 0.5 mL of each solutions were mixed with 0.1 mL 10 % $AlCl_3$, 0.1 mL of 1 M sodium acetate, 1,5 mL of 95% ethanol and 2.8 mL of distilled water. After incubation at room temperature for 15 minutes the absorbance was read at 415 nm and a calibration curve was made. A blank solution was prepared using water instead of the quercetin solution. Similarly, 0.5 mL of flower extracts were reacted with $AlCl_3$ for the determination of flavonoids as described above.

RESULTS AND DISCUSSIONS

Optimization of the system luminol-perborate-antioxidant

The effects of the luminol concentration and borate buffer pH on the chemiluminescence emission was studied, results showed that luminol concentrations below 1 mmol L^{-1} produced small peaks for blanks and antioxidant samples, while higher concentrations produced wide peaks but not increased the maximum chemiluminescence emission. Also, a pH of 10 of the borate buffer used to dissolve the luminol was the optimum to obtain reproducible signals.

According to the procedure proposed, the luminol is incorporated to the system through the injection of a determined volume into the carrier stream. In such way, sample loops of 10, 100 and 250 and 500 µL were tested and, results showed that the higher and sharper peaks were obtained when 250 µL of perborate are injected to the system.

On the other hand, the perborate was used as a source of the H_2O_2 to give the free radicals that oxidize the luminol molecule to produce the electronically excited phtalate. Concentrations between 1 and 10 mmol L^{-1} were tested, obtaining the highest CL signals with an optimum concentration of 5 mmolL^{-1}, which was used in all further experiments.

It has been well established that the luminol CL emission depends greatly on the rate of reagents mixing, for that reason, the reagents flow rate was other factor to be optimized. Records obtained using flow rates between 2 and 6 mL min^{-1} showed that the CL emission increases and the peaks were less wide when the flow rate increased, achieving maximum values when the peristaltic pump propelled the reagents at 5 mL min^{-1}. Faster flow-rates leaded to small peaks because the maximum emission was reached when the reagents were out of the detector cell.

Under the optimum conditions described above the fiagrams in figure 2 were obtained for the determination of EC_{50} values of Gallic and Caffeic acids. Before the injection of the luminol a low and stable base line is obtained because there is not any CL emission. When this reagent was injected and mixed with the perborate solution at the detector cell, a fast increase of the CL emission was observed achieving a maximum intensity value to fall again to the baseline, producing a CL peak. In this figure, the first three peaks of each series corresponded to the blank solutions and were used to establish the maximum emission or I_{max}. In the presence of the antioxidant solutions, similar peaks can be observed, but the CL intensity diminished as the concentration of the antioxidant increased. Evidencing the influence of the antioxidant over the luminol oxidation by the free radicals generated during he decomposition of the perborate. The last three peaks in both antioxidants represented the inhibition effect of the higher concentration that almost inhibited completely the luminol oxidation.

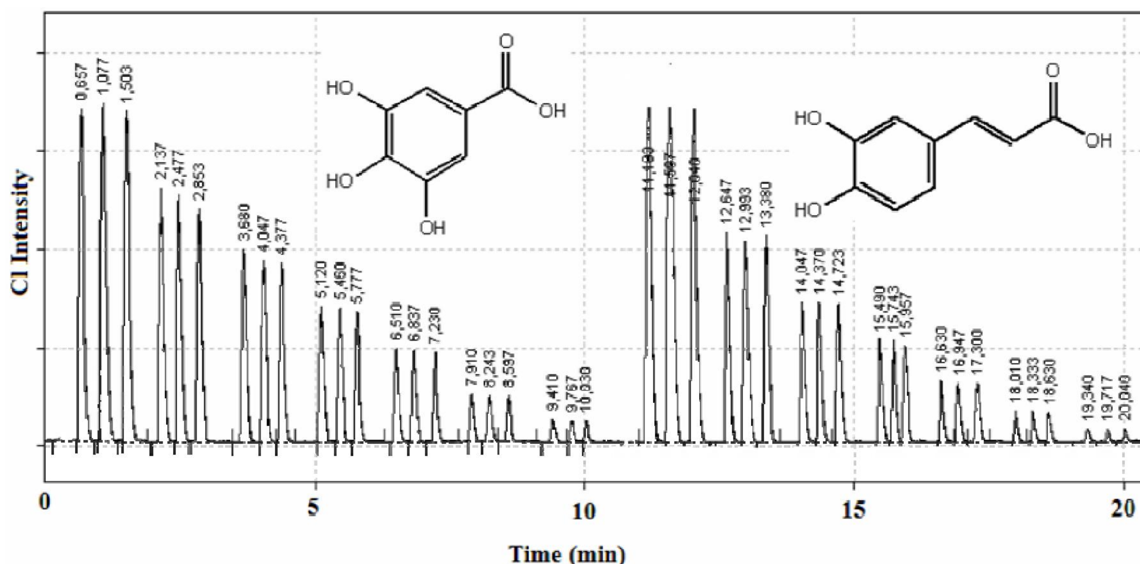

Figure 2 : Typical fiagram for the determination of the AA of gallic and caffeic acids under the optimized conditions.

The influence of the antioxidants concentration on the chemiluminescence emission was represented by the following logarithmic functions:

$$\%Inh = 19.58 Ln[Gallic\ Acid] + 30.21 \quad R^2 = 0.9851$$

$$\%Inh = 14.05 Ln[Caffeic\ Acid] + 53.40 \quad R^2 = 0.9949$$

These equations establish linear dependences for the concentration range between 1 and 16 µmol L^{-1} for both antioxidants. Similar equations were obtained by Wang et al.[20] in the determination of gallic acid in olive fruits using a CL procedure based on the luminol oxidation.

The EC_{50} were calculated giving values of 2.74 and 0.79 µmol L^{-1} for gallic and caffeic acids. The repeatability was tested by five consecutive determinations of the EC_{50} using different luminol, perborate, gallic or caffeic acid solutions, obtaining $EC_{50\ (Gallic\ acid)}$ = 2.80 ± 0.25 µmol L^{-1} and $EC_{50\ (Caffeic\ acid)}$ = 0.81 ± 0.07 µmol L^{-1}, representing relative standard deviations (RSD) of 8.9 and 8.6 % respectively. On the other hand, the reproducibility was evaluated by determination of EC_{50} for the same gallic acid solutions during five consecutive days, obtaining a final value of 2.95 ± 0.15 µmol L^{-1} ($RSD_{interday}$= 5.1 %).

Measurement of the EC_{50} for synthetic antioxidants

In order to test the influences of the molecular structure and reactivity on theproposed CL method, the inhibition curves for four synthetic antioxidants (figure 3) were obtained by plotting the % Inh against the molar concentration and, these curves were used to determine the EC_{50} by interpolation.

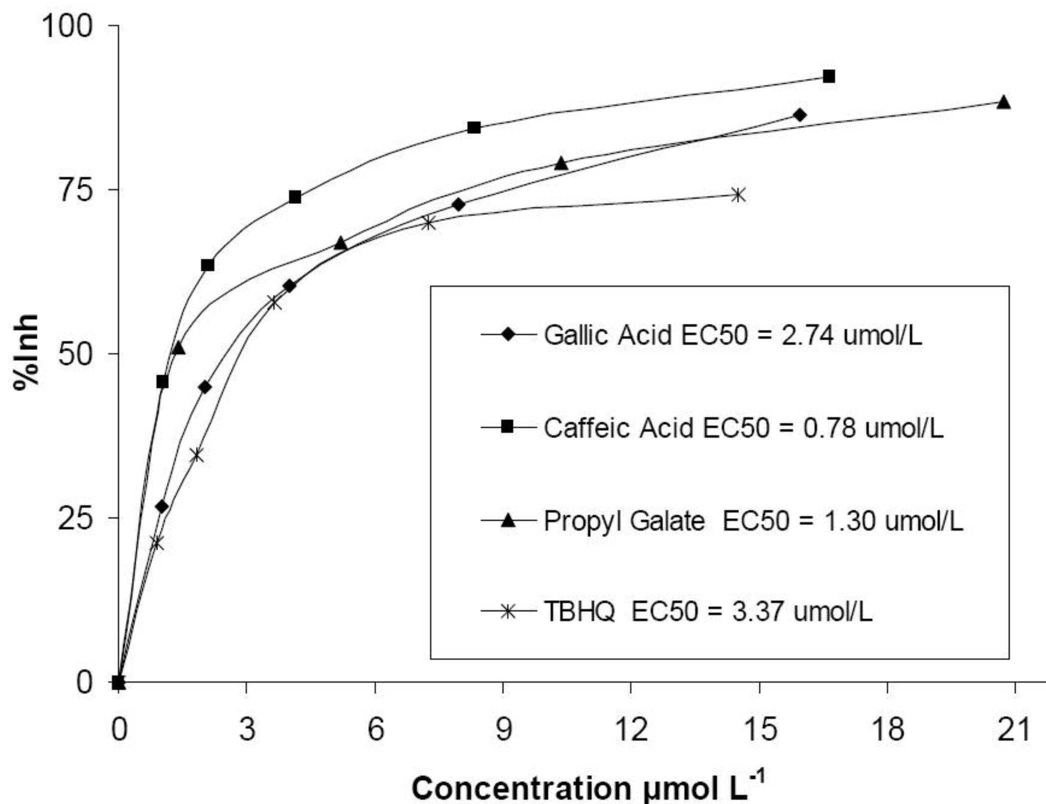

Figure 3 : Inhibition curves for synthetic antioxidants

Low values for EC_{50} implied higher antioxidant capacity, which was related with the stabilization of the unpaired electrons of the phenolic free radicals generated when the antioxidant inactivate the radicals $^{\bullet}OH$ that came from the decomposition of the hydrogen peroxide. The unpaired electron can be delocalized in the several resonance structures associated to the conjugated double bonds of the aromatic rings. In consequence, the concentrations of free radicals available to attack the luminol molecules are low affecting the number of the excited molecules responsible for the chemiluminescence emission.

For example, the caffeic acid, a potent antioxidant commonly used in many food systems, produced the

lowest EC_{50} value, that can be explained on the basis of the orthodihydroxyl functionality in the ring catechol, which is the responsible for its antioxidative and free radical scavenging activity[18]. Also, thee presence of the electron-donating hydroxyl group at the *ortho* position increases the rate of H-atom transfer to peroxyl radicals, while the unsaturated 2-3 double bond of the side chain contribute to maximize the stabilization of the phenolic radical, by the delocalization of the unpaired electron in the resonance structures.

The propylgalate despite of having a third hydroxyl group on the aromatic ring, lacks of the double bound side chain and, for that reason, its antioxidant activity is slightly low if compared with the caffeic acid. The gallic acid possesses three electron-donating hydroxyl groups in the positions *meta* and *para* with an acid carboxylic functional group. This molecular structure present a minor number of resonance structures to stabilizing a free radical with a consequent increase in the value of $EC_{50.}$

The molecule of TBHQ only presents two OH groups in *para* positions with a tertbuthyl group that has a minor contribution in the stabilization of the free radical, this structural difference with respect the other antioxidants is the responsible for the higher value of the EC_{50}, which is more than three times higher compared with the caffeic acid.

Antioxidant activity of flowers extracts

The antioxidant activity of the hibiscus flowers was determined by the method described above. According to the results showed in figure 4, the Red Cayena exhibits the greatest antioxidant activity with an EC_{50} value of 6.6 μg mL^{-1}, while the value for Roselle flowers was 28.1 μg mL^{-1}. These results suggest that the variety *Rosa Sinensis* has the higher radical scavenging activity when compared with other *Hibiscus* variety A similar trend was observed when the DPPH test was applied. In this case the EC_{50} values were 0.20 and 2.83 mg mL^{-1} *Hibiscus Rosa* and *Hibiscus Sabdariffa*.

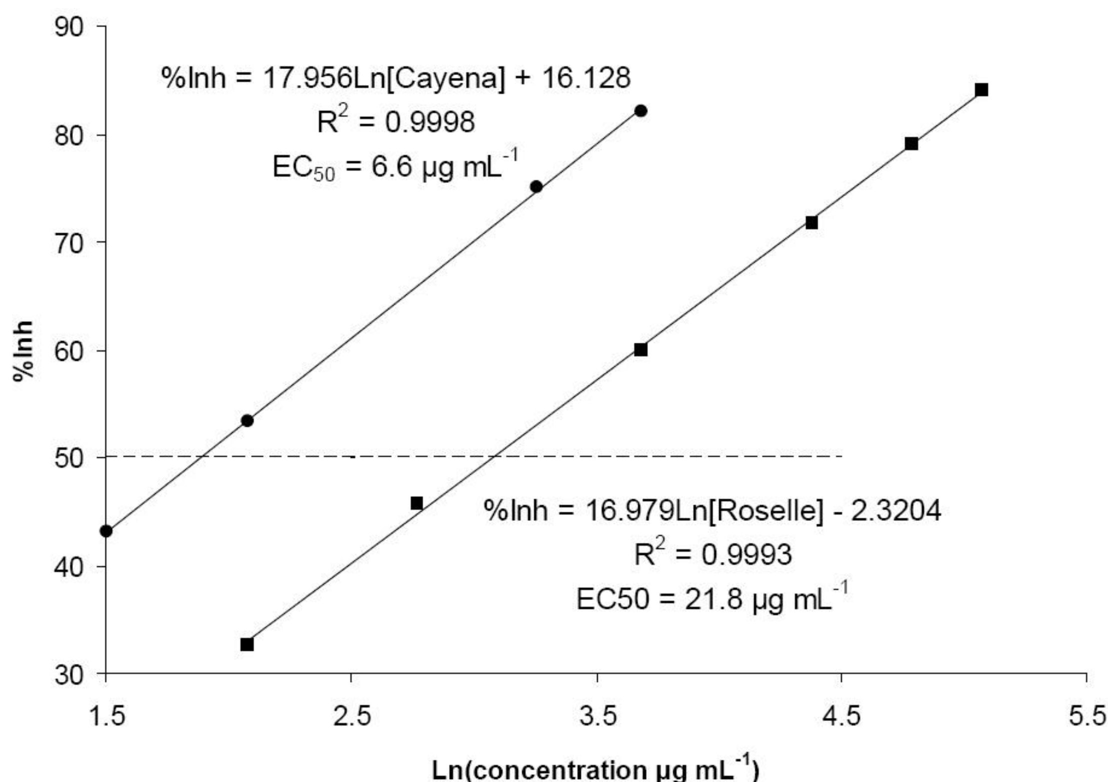

Figure 4 : Dependence of %Inh with the logarithm of flower extract concentrations.

The chemical analyses of the flower samples used in this work revealed that the extract obtained from *Hibiscus Rosa Sinensis* have a concentration of biphenols, flavonoids and anthocyanins higher than those found for the Roselle flowers (TABLE 1); for example, the concentration of flavonoids in Red Cayena was 257 mg g^{-1} on dry basis, while in Roselle flowers the concentration was only 27.5 mg g^{-1}. On the other

hand, the total biphenols concentrations determined by the Folin method included not only the flavonoids, but also other phenols and polyphenols that can be extracted under the conditions of the present experiment. The analysis of *Hibiscus Rosa* extracts given a concentration of 49.5 mg g^{-1} against the 10.7 mg g^{-1} obtained for Roselle flowers.

TABLE 1 : Concentration of bioactive compounds in flowers of *Hibiscus Rosa Sinensis and Hibiscus Sabdariffa*

Bioactive Compounds	*Hibiscus Rosa Sinensis*	*Hibiscus Sabdariffa*
Biophenols	49.5 mg g^{-1}	10.7 mg g^{-1}
Anthocyanins	0.77 mg g^{-1}	0.40 mg g^{-1}
Flavonoids	257 mg g^{-1}	27.5 mg g^{-1}

These differences in the chemical compositions may explain the results in the RSA determination. Thus, Puckhaber et al.[16] found that *Hibiscus Rosa Sinenis* flowers contain the flavonol quercetin along with the cyanidin, being this last the anthocyanin responsible for the red color of the flowers in acidic solutions. Both compounds belong to the groups of bioactive compounds with strong antioxidant activities and many other important properties, as for example hair growth promoting[11]. On the other hand, the flavonoids such as delphinidin-3-sambuboside and cyanidin-3-sambuboside, have been found in petals of Hibiscus *Sabdariffa* by Ali et al.[1].

The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. For example, Van Acker et al.[19] reported that for a good antioxidant capacity a catechol moiety on ring B of flavonoids is required. Also, the 3-OH group in combination with a C2 C3 double bond.

The presence of hydroxyls in the flavonoids molecules is of fundamental importance to understand the antioxidant and radical scavenging activity. For that reason the effect of the commonly used shift reagents on the UV spectrum was study. Flower extracts were obtained in methanol and the position of the peaks of maximum absorption were determined after the addition of sodium metoxide, sodium acetate and $AlCl_3$.

The UV spectrum of the methanolic solution of *Hibiscus Rosa Sinensis* supported the flavone or flavonol 3-*O*-substituted structures with a maximum at 328 nm associated with the cinnammoyl functionality, while a second band at 276 by the presence of the benzoyl ring (TABLE 2). The addition of sodium metoxide produced a bathochromic shift of the band I, which is an indication of the presence of hydroxyl groups in the flavonoid molecule.

TABLE 2 : UV-visible absorption peaks of flavonoids in MeOH and their shifts in different solvents

Reagent	*H. Rosa Sinensis*		*H. Sabdariffa*	
	Band I	Band II	Band I	Band II
CH_3OH	328	276	326	272
CH_3ONa	380	278	382	278
CH_3COONa	334	276	326	276
$AlCl_3$ + HCl	330	276	332	274

The sodium acetate is a base weaker than sodium metoxide and is used to detect the presence of the most acidic hydroxyls such as those present in the 3, 4′ and 7 positions. The addition of this base produced a shift of the band I from 328 nm to 334 nm which is consistent with the presence of the OH in position 7, which may be associated with the antioxidant activity determined in the Red Cayena extracts. Finally, the addition of $AlCl_3$ did not produce any displacement discarding the presence of a flavonol with OH in the positions 3 or 5.

For the Roselle flower extracts the methanolic band at 326 nm indicates the presence of a flavone. The shift in presence of sodium metoxide reveals the existence of hydroxyl groups, but not located at the positions 3, 4′or 7, because no displacements were detected when the sodium acetate was used as shift reagent. The addition of $AlCl_3$ produce a slight changes in the maximum of absorption, probably due to the presence of a 3 or 5 hydroxylated flavonol.

These results show differences in the molecular structure of the flavonoids present in both kind of flowers, which can be related with the antioxidant activities determined in this work. Therefore, the proposed method was able to detect such differences in terms of the ability to inactivate the free radicals.

CONCLUSIONS

The proposed CL method is able to distinguish be-

tween two sources of natural antioxidants in fast a simple way, that only require the dissolution of the antioxidants in an aqueous-EtOH dissolution with out any additional treatment. The above results demonstrate that the antioxidant potential, expressed in terms of EC_{50}, was dependent on the molecular structure of the pure phenolic compounds and a similar situation was observed in the complex phenolic mixtures of teas, wines or grape seed. The proposed FIA system, based on the attenuation of the luminol CL can detect such differences, providing a simple, sensitive and accurate method for the determination of the antioxidant potential which was associated to the TBp content.

The proposed method was easy to apply and fast, requiring a few minutes to complete the analysis of a single sample. Aditional advantages of this procedure, such as absence of sophisticated equipment, highly cost reagents or pretreatments of the samples, results in a more economic and environmental friendly method that can be used as a complement of other methods for the monitoring of the antioxidant activity of food samples.

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