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8 **Antioxidant capacity automatic assay based on inline photogenerated**
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10 **radical species from L-glutathione-capped CdTe quantum dots**
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Abstract

This work aimed at the development of a methodology implemented in an automatic flow system for determination of the antioxidant capacity in food samples, based on the luminol oxidation by inline photogenerated radical species from cadmium telluride nanoparticles capped with L-glutathione. Radical species were generated inline by a high-power visible light obtained by Light Emitting Diodes (LEDs) assembled in a multipumping flow system (MPFS). The use of visible light instead of UV radiation allowed the development of a new methodology for antioxidant capacity determination, more environmentally friendly and to circumvent the risk for UV photo-induced degradation of sample antioxidant compounds. Additionally, it was theoretically predicted the formation of superoxide radical species considering the variation of the redox potential with the size of CdTe QDs and the values of redox potential of the oxidizing and oxidable species present in the irradiated medium. The obtained results of Trolox equivalent antioxidant capacity (TEAC) from the analysis of commercial beverages were compared with the results of ABTS and DPPH batch assays through the Spearman's-Rho correlation coefficients and no correlation was found (for ABTS: $\rho = 0.2$, $p < 0.6$ and for DPPH: $\rho = 0.5$, $p < 0.1$, respectively) since the mechanism of action of the proposed methodology was based on the scavenging capacity of ROS species rather than the reduction of a colored oxidant. An analytical linear response range between 0.0001 and 0.005 mmol L⁻¹ of trolox and a limit of detection of 0.00005 mol L⁻¹ was found. The QDs based MPFS methodology allowed a determination rate of about 79 h⁻¹, a total waste generation of 20.5 mL h⁻¹ and the consumption of 0.100 mg h⁻¹ of QDs and 2.1 mg h⁻¹ of luminol.

Keywords: antioxidant capacity; radicals photogeneration; quantum dots; multipumping; superoxide; light emitting diode.

1. Introduction

Reactive oxygen species are a group of radicals produced by aerobic metabolism, exposure to UV radiation or environmental pollutants, through the partial reduction of molecular oxygen originating, for example, superoxide anion radical, hydroxyl radical and peroxy radical. These species can impair or severely interfere with the normal functioning of the bioprocesses in the living tissues. The ROS species can cause oxidative modification of biomolecules and bioprocesses playing an important role in ageing and in the development of diseases such as cancer, neurodegenerative disorders, arteriosclerosis, oxidative stress, etc. [1]. Nevertheless, scientific studies have proved that a proper food diet offers chemoprotective effects due to the antioxidant activity of polyphenolic compounds and some vitamins present in those foods. Despite the fact the human bodies defense system is capable of generate natural antioxidants against those pathological ROS species, these are nowadays probably insufficient considering the variety of pathways that lead to the formation of radicals. This way, it is perfectly justifiable the numerous methodologies found in the scientific literature dealing with assays for the determination of the antioxidant capacity of purified compounds or food samples. These methodologies enable the seriation of food stuffs accordingly with their antioxidants content.

The quantum dots (QDs), nanocrystalline colloids of semiconductor materials, are of proved excellence when used as chemical probes since by reacting with other substances have their distinctive photo-physical and chemical properties changed [2]. Additionally,

the QDs are capable of generating free radical species upon irradiation in aqueous media [3], through the absorption of photons with energy equal to or greater than the energy of the band gap by a process of photoactivation [4]. This means that when the QD is exposed to an electromagnetic radiation in the ultraviolet [3, 5, 6] or visible region [4, 7] of the spectrum, this can promote a displacement of an electron from the valence band to the conduction band forming a particle called exciton. The exciton formed is endowed with redox properties dependent on the values of the two surrounding energy bands being perfectly capable of reducing the molecular oxygen to ROS [3]. The yield in the generation of radical species depends largely on the nanoparticle size: for the same reaction conditions the higher the QDs size the higher the production of highly reactive radical species [4]. This fact allows to use low concentrations of QDs for high yields of radical generation and thus, contributes for the direct reduction of the environmental contamination with chemical wastes.

However, taking into account the difficulty in monitoring separately the antioxidant species present in a sample and also considering the possible interactions between the different antioxidants, several methodologies have been developed with the aim of evaluating the total antioxidant capacity in food samples, such as wine, natural juices and soft drinks rather than the specific antioxidant capacity. Moreover, these methodologies must comprise a wide variety of mechanisms of action through which the antioxidant capacity occur, like for example, the inhibition of generation and scavenging capacity against ROS/RNS, reducing capacity, metal chelating capacity, activity as antioxidative enzyme and inhibition of oxidative enzymes [8]. As expected, the available approaches for the antioxidant capacity must be focused in one particular mechanism of action. This way, these assays can be considered divided in two main categories, dependent on the type of the oxidant species: (i) scavenging capacity assays for specific ROS/RNS and (ii)

1 scavenging capacity assays for stable, non-biological radicals and evaluation of total
2 reduction capacity. Overall, the total antioxidant capacity of agro-foods comprises the
3 general contributions of all the mechanisms or species with antioxidant properties present
4 in the food stuffs. Considering the growing needs of obtaining fast information about
5 antioxidant properties, some methodologies have been implemented in automatic methods
6 aiming for a large scale antioxidant quality control. These automatic procedures enable the
7 industry to control the addition of artificial antioxidants to some food stuffs for their
8 nutritional enrichment and also protection to self-deterioration.
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10 The automatic flow methodologies constitute a valuable asset for the quantification of low
11 concentrations of compounds in samples of different natures. Its easy configuration and
12 control combined with all the advantages of an automatic analytical system, documented in
13 the scientific literature in the past 40 years, contributes to reduce errors arising from the
14 human execution of the analysis while assuring at the same time high determination rates
15 with precision and accuracy.
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17 The implementation of a quantum dots based analytical methodology into an automatic
18 flow system for the production of free radical species results in a new methodology for the
19 determination of substances with antioxidant capacity. The antioxidants subject has been
20 increasing in recent years, largely due to the constant advertising of such compounds as
21 food and dietary supplements constituents, but also because of the importance they have
22 for the pharmaceutical industry where they are used as preservatives and stabilizers of
23 pharmaceutical formulations.
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25 A bibliographic survey in the scientific literature revealed only two works dealing with the
26 photogeneration of radical species from quantum dots, for the determination of substances
27 with antioxidant properties, namely, epinephrine [4] and ascorbic acid [7] in
28 pharmaceutical formulations, both methodologies implemented in an automatic flow
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system. Additionally, it can be found one work involving the photobleaching of L-cysteine-capped CdTe QDs with radical species photogenerated by UV irradiation of the nanoparticles, for the fluorometric determination of the antioxidant capacity of tea samples, in batch assays [6].

Consequently, to our knowledge, this work is the first to propose a new analytical method for the determination of the antioxidant capacity of food samples based on the photogeneration with visible radiation of radical species from CdTe quantum dots capped with L-glutathione and using luminol as a chemiluminescence probe.

2. Experimental

2.1 Reagents and samples

All solutions were prepared with double deionized water (specific conductivity $\leq 0.1 \mu\text{S cm}^{-1}$) and chemicals of analytical grade were used. All solutions were prepared daily and maintained protected from the light.

The working standard solutions of CdTe QD were obtained through the dilution of stock solutions with concentrations of 10.0 and 5.0 $\mu\text{mol L}^{-1}$. For such, it was dissolved: 4.90 mg of QDs with $\varnothing=1.3 \text{ nm}$ in 25.00 ml of water for a concentration of 10.0 $\mu\text{mol L}^{-1}$, 11.56 mg of QDs with $\varnothing=2.8 \text{ nm}$ in 25.00 ml of water for a concentration of 10.0 $\mu\text{mol L}^{-1}$ and 13.22 mg of QDs with $\varnothing=3.4 \text{ nm}$ in 25.00 ml of water for a concentration of 5.0 $\mu\text{mol L}^{-1}$.

The working solutions of sodium hydroxide were obtained through dilutions of a stock solution with concentration fixed at 2.0 mol L^{-1} , prepared by dissolving 80.0 g of NaOH in 1000 ml of deionized water.

The trolox solutions were obtained by dilution of a stock solution with concentration of 0.01 mmol L⁻¹ in 0.01 mol L⁻¹ NaOH, prepared by the dissolution of 2.50 mg of trolox (Fluka[®]) in 1 liter of a 0.01 mol L⁻¹ NaOH solution. Additionally, the working trolox standard solutions had the content of NaOH offset to a final concentration of 0.005 mol L⁻¹.

The solutions of luminol were obtained through dilutions of a stock solution of luminol with a concentration of 0.01 mol L⁻¹ prepared in 0.0175 mol L⁻¹ NaOH. This stock solution was prepared by dissolving 177.1 mg of luminol (Fluka[®]) in 100 ml of water. Also, upon dilution of the stock solution aiming at a concentration of luminol of 0.0015 mol L⁻¹, the concentration of NaOH was normalized for a final value of 0.06 mol L⁻¹ NaOH.

The food samples consisted of 15 juice beverages of 5 different brands, commercially available, namely: “Brand 1” mango-orange, “Brand 1” tomato, “Brand 1” carrot-manga, “Brand 1” pear, “Brand 1” orange, “Brand 1” red fruits, “Brand 1” tutti-frutti, “Brand 2” red fruits, “Brand 2” mango, “Brand 2” lemon, “Brand 2” peach, “Brand 3” red fruits, “Brand 4” pomegranate and red berries, “Brand 4” green tea and “Brand 5” lemon. All samples had to be diluted in the proportion 1:4000 using deionized water before insertion in the developed MPFS for determination of the antioxidant capacity. Additionally, the concentration of NaOH in the diluted samples was fixed at 0.005 mol L⁻¹.

2.2 Apparatus

The developed flow system consisted in four solenoid micro-pumps (model 120SP, Bio-Chem Valve Inc, Boonton, NJ, USA), responsible for the propulsion, insertion and commutation of the reagent and sample solutions. Three micro-pumps had a fixed stroke volume of 10 µL and one micro-pump had a stroke volume of 50 µL. The automatic

control of the micro-pumps was made through a micro-computer Intel Pentium[®] and a homemade software developed in Microsoft Visual Basic 6.0[®]. The solenoid devices were actuated by a homemade power controller and controlled by the PC parallel communication port. All the flow tubing was of PTFE with 0.8 mm internal diameter and were also used connectors and confluence points in acrylic constructed in the laboratory.

The photo-excitation unit (PEU) consisted of a reactor coil of PTFE tube with 0.8 mm internal diameter placed between two LED lamps of high-power (Parathom[®] R50 40 daylight) emitting white light of high efficiency.

A luminescence detector of the brand Jasco, model FP-2020 Plus, Easton, MD, was used to monitor the chemiluminescent signal and was equipped with a modular flow cell formed by a helical PTFE tube of internal diameter 0.8 mm i.d., with an internal volume of 100 μ L, placed in front of a highly sensitive photomultiplier. The analytical signal was recorded on a strip chart recorder, model Linseis L 250E.

2.3 Flow manifold

The MPFS used for the implementation of the analytical methodology consisted in four solenoid micro-pumps $P_1 - P_4$ (figure 1) whose pulse time and sequence of activation was pre-established and controlled by computer. Each micro-pump was responsible for the manipulation of one reagent solution, in a total of four reagent solutions used: a solution of QDs (P_1 , 10 μ L/stroke), water as carrier (P_2 , 50 μ L/stroke), the sample solution (P_3 , 10 μ L/stroke) and the luminol solution (P_4 , 10 μ L/stroke).

(Figure 1)

1 Preceding the analytical cycle, all the flow tubing was filled with the reagent solutions by
2 actuation of the corresponding micro-pump.
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4 The analytical cycle started by the simultaneous insertion at the confluence point X_1 of pre-
5 set number of pulses of the QDs and sample solutions using the merging zones approach
6 [9], through the activation of the micro-pumps P_1 and P_3 with a pulse time fixed at 250 ms.
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8 After the sample insertion, the micro-pumps P_1 and P_3 were deactivated and the micro-
9 pump P_2 responsible for the insertion and propulsion of the carrier (water) was activated at
10 a pulse time of 250 ms, using a number of pulses sufficient to ensure the transport of the
11 sample zone through the photo-excitation unit and up to the confluence point X_2 .
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14 At the confluence point X_2 , the luminol solution was inserted together with the sample
15 zone through the simultaneous activation of the micro-pumps P_2 and P_4 , with a pulse time
16 of 250 ms. Aiming at the fast monitoring of the chemiluminescence signal, the reaction
17 zone was propelled towards the detector through the activation of the micro-pump P_2 with
18 a pulse time of 250 ms.
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21 Considering that the life time of the ROS species is very low, partly due to their high
22 reactivity that even allows the occurrence of self-quenching between radicals, the length of
23 the tube between the confluence point X_2 and the detection unit was only the necessary (3
24 cm) for the physical connection between the mentioned parts. Additionally, considering
25 that also the flow rate at this stage had to be high for the reasons mentioned before, the use
26 of a micro-pump with a stroke volume of 50 μL allowed the rapid CL detection of the
27 reaction zone. This way, together with the advantage of using an automatic control of all
28 the analytical parameters, it was guaranteed the detection of the signal at its highest CL
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2.4 ABTS and DPPH radicals assays

The reference procedures performed were two different assays, based on the radicals ABTS^{•+} and DPPH[•].

For the ABTS radical assay [10], the radical solution was prepared by mixing equal volumes of an ABTS aqueous stock solution of concentration 0.007 mol L^{-1} with $2.45 \times 10^{-3} \text{ mol L}^{-1}$ of potassium persulfate. This mixture was allowed to stand for 12 – 16 hours at room temperature protected from light. Then, five working standard solutions of ABTS radical with concentrations comprised between 30 and $250 \text{ } \mu\text{mol L}^{-1}$, were prepared in acetate buffer ($\text{pH} = 4.6$, $5 \times 10^{-2} \text{ mol L}^{-1}$). These solutions were used to establish a linear correlation between the concentration and the absorbance at the wavelength of 734 nm.

For the DPPH radical assay [10], a stock solution in ethanol containing $600 \text{ } \mu\text{mol L}^{-1}$ of the radical was prepared and kept protected from light at room temperature. Afterwards, for the sample analysis, five working standard solutions of DPPH radical with concentrations comprised between 25 and $250 \text{ } \mu\text{mol L}^{-1}$ were prepared in a 50 % (v/v) ethanol solution. The linear correlation between the radical concentration and the absorbance at 517 nm was also performed.

After proper dilution of the samples, the antioxidant capacity was calculated through the absorbance values at 734 nm and 517 nm of the diluted samples and the equations of linear regressions by extrapolation.

2.5 Synthesis of quantum dots

The CdTe QDs capped with reduced L-glutathione (GSH) as stabilizing agent, were synthesized following the modified method of Quian *et al.* [11]. In the synthesis of CdTe QDs the following reagents were used: cadmium chloride (CdCl₂), sodium telluride (Na₂TeO₃) and GSH in a molar ratio of 1:0.2:1.2 (Cd:Te:GSH), together with an excess of sodium borohydride (NaBH₄), trisodium citrate and water. The pH of the solution was adjusted to 10.5 with a solution of 1 mol L⁻¹ NaOH and the solution was maintained under reflux at a constant temperature of 100 °C during the growth of the nanocrystals [12]. The QDs size was tuned by varying the heating time. After the aqueous synthesis of the QDs, ethanol was added to remove the excess of precursors by precipitation followed by centrifugation. The QDs were dried and stored at 4 °C under N₂ atmosphere, to preserve their photoluminescence characteristics.

2.6 Characterization of quantum dots

Taking into account the influence of the QDs size on the photochemical properties of the nanocrystals, that parameter is of paramount importance in their reactivity and hence, on the analytical signal of the proposed methodology.

The size of the nanoparticles (Ø) was determined taking into account the obtained wavelengths of maximum absorption corresponding to the first transition (λ) using the following equation [13]:

$$\text{Ø} = (9.8125 \times 10^{-7})\lambda^3 - (1.7147 \times 10^{-3})\lambda^2 + 1.0064 \times \lambda - 194.8$$

Based on the value of Φ , it is possible to determine the molar absorptivity (ϵ) through the equation $\epsilon = 3450 \times \Delta E \times \Phi^{2.4}$, where ΔE represents the transition energy corresponding to the first absorption peak expressed in eV.

The molar concentration (c) expressed in $\mu\text{mol L}^{-1}$ is then calculated using the Lambert-Beer law represented by the equation $A = \epsilon \times c \times L$, where L is the optical path length in centimeters, and A is the absorbance value.

Accordingly with the figure 2A, the CdTe-GSH QDs of different sizes showed wide absorption bands with absorption maxima for the first excitonic transition at 486, 515 and 564 nm, determining the sizes of 1.3, 2.8 and 3.4 nm, respectively. The fluorescence spectra of the QDs represented in Figure 2B exhibited narrow and symmetric emission spectra with maximum emission wavelengths at 503, 546 and 603 nm. Additionally, the obtained Full Width at Half Maximum (FWHM) values (< 55.0 nm) of the QDs emission bands demonstrated that all prepared nanocrystals are homogeneous and nearly monodisperse.

(Figure 2)

Additionally, in order to obtain useful information about the crystal structure and also to confirm the mean particle size of the as-prepared QDs, a X-ray powder diffraction (XRD) technique was performed. The XRD profiles of the as-prepared QDs showed broad peaks typical of the zinc blend crystalline structure that are attributed to the (1 1 1), (2 2 0) and (3 1 1) planes. The Figure 3 shows the XRD patterns for the GSH-CdTe QDs of size of 3.4 nm where it can be observed the characteristic peaks of zinc blend cubic CdTe.

The QDs diameters were calculated using the XRD patterns and by applying the Scherrer equation:

$$\varnothing = k\lambda/(\beta\cos\theta)$$

in which λ corresponds to the wavelength of the X-ray source (1.5405 Å), k is an empirical constant equivalent to 0.9, β is the FWHM of the diffraction peak, and θ is the angular position. The obtained diameters of all the as-prepared QDs using the XRD technique were in compliance with those calculated by applying the empirical formula proposed by Yu *et al.* [13].

(Figure 3)

3. Results and discussion

3.1 Mechanism of reaction

As already mentioned in the scientific literature it is well-established that the quantum dots when irradiated with electromagnetic radiation with energy equal or superior to the nanoparticles band gap (bg) causes the electron delocalization from the valence band (vb) to the conduction band (cb) forming thus, the exciton. The formed exciton can trigger redox reactions with surrounding species within a specific redox potential range (inferior to the QDs band gap) originating radical species.

Redox reactions that originates reactive oxygen species can be triggered depending on the redox potential of the chemical species present on the surrounding medium and on the valence and conduction band energies of the QDs. Additionally, the valence (E_{vb}) and conduction (E_{cb}) band energies depend on the size of the nanoparticles [14]. Indeed, the

work of Kovalenko *et al.* [15] revealed that for quantum dots of size comprised between 3.0 and 6.6 nm, the band-gap energy (E_g) varies between 2.24 and 1.74 eV, respectively, supporting the established fact of the direct dependence of the optical band gap and band edge energies with the QD size.

Considering the following assumptions: (i) for CdTe QDs of size 3.2 nm the E_{cb} , E_{vb} and E_g are -1.21 V, 0.91 V and 2.12 V with reference to a normal hydrogen electrode (NHE), respectively [15]; (ii) for molecular oxygen and hydroxide ions the redox potential are $E_{(O_2 \rightarrow O_2^{\bullet-})} = -0.33$ V (NHE) and $E_{(OH^- \rightarrow \cdot OH)} = 2.8$ V (NHE) [16]; (iii) for the oxidation of H_2O to $\cdot OH$ the redox potential is 1.8 V [17], then, one can conclude that, in the present work when using quantum dots of size 3.4 nm, only superoxide ($O_2^{\bullet-}$) radicals are generated upon irradiation of the QDs with visible light, since the E_{cb} of the 3.4 nm CdTe QDs is sufficient for the oxygen reduction.

Thus, some assays were conducted to ascertain the influence of the presence of molecular oxygen in the solutions for the photogeneration of superoxide radicals from QDs. The automated process of radical generation before and after oxygen removal from the solutions was tested, following the method of nitrogen purging by Butler *et al.* [18].

The obtained results demonstrated a decrease of the radical generation upon 20 minutes of oxygen removal from the involved solutions. The complete inhibition of the analytical signal was not achieved, since a total oxygen removal was not attained [18]. It was demonstrated the dependence of the photogeneration of superoxide radicals from the presence of molecular oxygen in the solutions.

The radicals $O_2^{\bullet-}$ generated upon the photoactivation of the QDs can be used to oxidize a chemiluminescent probe, like for example, luminol that was applied in the developed work. In this case, in alkaline medium the probe luminol is oxidized by the ROS species and originates an excited state of the 3-aminophthalate ion. When returning to its ground

state it occurs the emission of light, which determines the chemiluminescent (CL) analytical signal.

The application of a strong antioxidant, namely trolox, as a substance capable of scavenge the free radicals formed by the photoactivation of QDs, prevents the reaction of ROS with luminol, inhibiting the CL analytical signal.

In figure 4 is represented the overall scheme of the analytical methodology developed in this work.

(Figure 4)

3.2 Optimization of the chemical parameters

The multipumping flow methodology [19] combines low-cost of implementation, operation and maintenance, high versatility and ease of automation with the total control of all the parameters influencing the development of the chemical reaction. The characteristic pulsed flow in MPFS assures an improved homogenization of the solutions within the flow tubing ensuring fast reaction development, homogenization and detection in chemiluminescence measurements, because light is usually generated by very fast reactions that require a fast and efficient sample/reagent mixing almost in front of the detector. These characteristics are also important when radical species (ROS) are involved due to their extremely short time of existence. Thus, the developed method for the determination of the antioxidant capacity was implemented in a MPFS and the optimization of all the analytical parameters was conducted through the automatic system.

The optimization of all the parameters affecting the analytical determination of the antioxidant capacity through the proposed automatic MPFS-based methodology was

performed aiming at the highest variation in the chemiluminescent quenching, expressed in %. This value was calculated by $100-(P \times 100/B)$, representing P and B the analytical signal obtained in the presence and absence of the sample solution (or trolox standard solution) containing antioxidants. In the absence of antioxidants the resultant chemiluminescent signal corresponds to the highest value possible, considering that in these conditions there is not present any antioxidant capable of reacting with the photogenerated free ROS that thus, are at their maximum concentration value for the fixed analytical conditions. In the presence of antioxidant compounds the CL signal diminishes because there are less available ROS to react with the CL probe luminol.

The chemical parameters influencing the ΔCL (%) were investigated and the optimum values were later used for the analytical determination of the antioxidant capacity of food samples. The studied chemical parameters involved the size of QDs and the solutions concentrations of the QDs, luminol and sodium hydroxide.

3.2.1 Size and concentration of quantum dots

The optimization of the size of the QDs was performed aiming at the highest rate in the generation of ROS, assessed by its influence in the CL analytical signal. For this purpose, 3 different sizes of the nanoparticles (NPs) were tested, namely, 1.3, 2.8 and 3.4 nm. Additionally, and performed simultaneously with the last assay, the influence in the sensitivity (through ΔCL , in %) of the concentration of each one of the QDs tested was assayed by preparing the following QDs solutions: for the QDs of smaller size (1.3 nm) the concentrations studied were 2.5, 5.0 and 7.5 $\mu\text{mol L}^{-1}$; for the QDs of 2.8 nm the concentrations used were 0.5, 0.75 and 1.0 $\mu\text{mol L}^{-1}$; finally, for the QDs of 3.4 nm the concentrations of the solutions were 0.05, 0.15 and 0.25 $\mu\text{mol L}^{-1}$.

The concentration values of QDs was adjusted for the amount of radicals generated to circumvent the saturation of analytical signal.

A solution of luminol with a concentration of 0.002 mol L^{-1} prepared in 0.04 mol L^{-1} NaOH and a NaOH solution of concentration 0.01 mol L^{-1} , together with two standard solutions of trolox (0.000075 and $0.001 \text{ mmol L}^{-1}$) were used for the optimization of the size and concentration of the QDs.

Overall, the results revealed that the higher the size of the QDs the highest rate in the generation of ROS was obtained, and also, for each size of QDs, the higher its concentration the highest ROS generation was achieved. These results obtained with the GSH-CdTe QDs were consistent with the results obtained by Ribeiro et al. [4] for the CdTe QDs capped with MPA (3-mercaptopropionic acid). This QDs size-radical generation dependence is a function of several factors, like for example, occurrence of surface traps (less pronounced on QDs of higher sizes) and variations on the surface-to volume ratio and band-gap energy.

Thus, the QDs of 3.4 nm were selected for the following assays, considering that these allowed to work with lower concentrations of the NPs assuring at the same time high sensitivity in the methodology.

For the QDs of size 3.4 nm , the higher $\Delta\text{CL} (\%)$ magnitude was observed when using a QDs concentration of $0.15 \text{ }\mu\text{mol L}^{-1}$.

In conclusion, with the aim of ensuring the best compromise between the maximum generation of ROS and the highest $\Delta\text{CL} (\%)$ it was decided to use QDs of size 3.4 nm and with a concentration of $0.15 \text{ }\mu\text{mol L}^{-1}$.

3.2.2 Concentration of NaOH

1 In the developed methodology the concentration of NaOH has strong influence in the
2 generation of ROS during the photoactivation of QDs, as well as, in the luminol redox
3 reaction that originates the formation of the 3-aminophthalate (light emitting species) that
4 occurs in alkaline medium.
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9 This way, simultaneously it was tested the influence in the CL analytical signal of the
10 concentration of NaOH during the generation of ROS and during the detection stage. For
11 this purpose, solutions of 0.002 mol L⁻¹ luminol were prepared in different concentrations
12 of NaOH: 0.005, 0.01, 0.015, 0.02, 0.04 and 0.06 mol L⁻¹. For each solution of
13 luminol/NaOH it was also studied the influence of NaOH concentration during the
14 photoactivation of QDs, by using solutions of NaOH with concentrations comprised
15 between 0.001 and 0.1 mol L⁻¹.
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34 From the obtained results, compiled in the figure 5, it was possible to verify an increasing
35 production of ROS during the photoactivation for concentrations of NaOH up to 0.01 mol
36 L⁻¹. In relation to the influence of NaOH during the luminol redox reaction it was observed
37 that in general the higher the NaOH concentration the higher the CL analytical signal.
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43 The maximum analytical signal was verified when using 0.005 mol L⁻¹ and 0.06 mol L⁻¹ of
44 NaOH in the sample and luminol solutions, respectively.
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51 **3.2.3 Concentration of luminol**

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56 Despite the fact that the concentration of luminol is not related with the radicals'
57 generation, it directly determines the extension of the CL reaction that originates light
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1 emission influencing thus, the sensitivity of the analytical methodology. Then, its
2 concentration was evaluated for values comprised between 0.0005 and 0.0025 mol L⁻¹. The
3 analytical signal was analyzed in relation to the CL quenching effect, ΔCL (%), of a
4 standard solution of trolox.
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9 The results revealed an increase in the ΔCL (%) for luminol concentrations from 0.0005 to
10 0.0015 mol L⁻¹. Thus, a solution of luminol with concentration 0.0015 mol L⁻¹ was chosen
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19 **3.3 Optimization of the physical parameters**

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24 The optimization of the physical parameters that influences the determination of
25 antioxidant capacity through the developed MPFS was conducted involving studies of the
26 influence in the ΔCL signal of the volume of the sample and luminol solutions, as well as,
27 the flow rate during the stages of QDs irradiation (time for irradiation) and the
28 chemiluminescent signal detection (time for detection). For this purpose a solution of QDs
29 of 3.4 nm was used with the concentration fixed at 0.15 μmol L⁻¹ and the solution of
30 luminol applied for the detection reaction had a concentration fixed at 0.0015 mol L⁻¹
31 prepared in 0.06 mol L⁻¹ of NaOH. In addition, it was used as sample a standard solution of
32 trolox with concentration of 0.001 mmol L⁻¹ prepared in 0.005 mol L⁻¹ of NaOH.
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49 **3.3.1 Volume of QDs, sample and luminol**

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54 The influence on the analytical signal of the QDs, sample and luminol volumes was
55 studied by varying the number of pulses of the reagent solutions inserted in the MPFS.
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With this purpose, and by using the optimized values for the chemical parameters, the variation of chemiluminescence signal ($\Delta\text{CL} \%$) was monitored when inserting 4 to 12 pulses of sample volume ($[\text{trolox}] = 0.001 \text{ mmol L}^{-1}$) with the same number of pulses of QDs solution, through the merging zones approach. The results (Figure 6) revealed a slightly increase of about 4 % in the ΔCL up to 8 pulses, while for a higher number of pulses the variation was not significant. This way, 8 pulses of sample was selected for the following assays, corresponding to a sample volume of 80 μL .

Additionally, by analyzing the same figure, which also contains represented the results obtained when varying the number of pulses of the luminol solution between 4 and 12, one can conclude that the highest variation in the chemiluminescent signal was attained when inserting a number of 10 pulses of luminol solution, corresponding to a volume of 100 μL of luminol solution.

(Figure 6)

3.3.2 Flow rate during irradiation and detection

The time for the QDs irradiation and the one required for the detection stage had an accentuated influence in the yield of the radicals' photo-generation and on the residence time of the radicals within the flow system immediately prior to detection, respectively. The optimization of these two parameters, considering the short lifetime of the generated radicals due to self-deactivation reactions, is of utmost importance. This way, the optimization of the time of irradiation and for detection was conducted independently in order to obtain the best analytical conditions for the formation of radicals and prevention of their auto-deactivation before reaction with luminol.

1 The time of irradiation of the mixed solution of sample and QDs is determinant in the
2 extension of the generation of ROS, thus limiting the amplitude of the chemiluminescence
3 signal obtained through the oxidation of luminol by these highly reactive species. The
4 influence of the time of irradiation in the analytical signal was studied by varying the flow
5 rate of the solutions during the irradiation stage. In a MPFS methodology, the flow rate is
6 easily controlled in real-time through the time of pulse used to control the actuation of the
7 micro-pumps responsible for the solutions propulsion, taking into account the volume of
8 solution per stroke. This way, aiming at the study of the influence on the ΔCL signal of the
9 irradiation time, it was varied the pulse time (100 - 750 ms) of the micro-pumps
10 responsible for the sample and QDs insertion into the flow system and of the micro-pump
11 responsible for the propulsion of the sample zone through the irradiation reactor coil.
12 These results allowed to conclude that the optimum flow rate for radical generation from
13 GSH-CdTe QDs was higher than the one used with MPA-CdTe QDs [4], favoring thus, the
14 determination rate of the proposed methodology.
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16 By analyzing the obtained results represented in the figure 7 it was concluded that the
17 highest ΔCL was achieved when using a pulse time of 250 ms, during the stage of insertion
18 and irradiation of the sample zone.
19

20 (Figure 7)

21 The influence of the flow rate during the transport of the reaction zone towards the
22 detector, that is, the flow rate immediately after the irradiation and during the detection
23 stage is of extreme importance considering the transient signal originated by
24 chemiluminescent reactions whose kinetic is very fast and additionally, due to the short
25 lifetime of the radicals species that oxidize the CL probe. Again, the flow rate during the
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transport of the reaction zone towards the detector and during the CL detection is the determining factor of the residence time of the reaction zone through the luminescence detector, conditioning the intensity of the measured signal.

This way, it was conducted an assay, using the optimized conditions of the parameters affecting the methodology and a trolox solution with concentration of $0.001 \text{ mmol L}^{-1}$, in which the time of pulse during the detection stage was varied between 125 ms and 500 ms (10.9 and 4.6 mL min^{-1} , respectively).

After the analysis of the obtained results (Figure 7), it was concluded that the highest ΔCL % and also the most reproducible readout, was obtained when using a pulse time of 250 ms during the detection stage, fixing thus the flow rate in this analytical stage at a value of 7.5 mL min^{-1} .

3.4 Analysis of samples

(Table 1)

Upon the optimization of the chemical and physical parameters of the MPFS (Table 1), it was established an analytical linear response range between 0.0001 and $0.005 \text{ mmol L}^{-1}$ of trolox prepared in 0.005 mol L^{-1} of NaOH (supplementary figure 1). The calibration curve was represented by the equation $\Delta\text{CL} = 46 (\pm 3) \times \text{Log} [\text{Trolox}] + 200 (\pm 8)$ ($R = 0.9953$, $n = 5$), in which ΔCL was the chemiluminescence signal quenched expressed in percentage, and the concentration of trolox expressed in mmol L^{-1} . The limit of detection calculated from the equation of the calibration curve [20] was approximately $0.00005 \text{ mmol L}^{-1}$.

The accuracy of the developed methodology based in the MPFS, aiming to evaluate its potential for the routine laboratory determinations of the antioxidant capacity of beverages

1 in the food industry, was assessed by determining the antioxidant capacity of 15 samples of
2 beverages of 5 different brands (“Brand 1” – “Brand 5”) purchased in commercial
3 establishments (Table 2).
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7 Some compounds present in the samples can influence the optical properties of the QDs
8 and, consequently, the photogeneration of radical species from the QDs. Aiming at the
9 clarification of the possible influence of those compounds in the proposed methodology, it
10 was conducted a fluorometric assay by monitoring the luminescence of CdTe QDs ($\varnothing$ =
11 3.4 nm; λ_{ex} =564 nm and λ_{em} =603 nm) in the absence and presence of samples diluted
12 4000x. The results revealed that a difference in the fluorescence signal not superior to 3 %
13 was obtained.
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24 In this study, the comparison of the profile of the antioxidant capacity of the samples was
25 carried out from the values of the antioxidant capacity equivalent to trolox concentration.
26 This way, after establishing the calibration curve, it was possible the calculation of the
27 antioxidant activity of the samples, through the interpolation of its analytical signal (ΔCL
28 %), being the end result of antioxidant capacity expressed in mmol L⁻¹ of trolox (TEAC).
29 All samples had to be diluted prior to determination due to the high sensitivity of the
30 developed procedure. The obtained results were compared with those found by conducting
31 assays in batch, namely the ABTS and DPPH radical methodologies [10], because these
32 are the most often used methods and well-established in the literature for the evaluation of
33 the antioxidant capacity. All results obtained from the MPFS, ABTS and DPPH
34 methodologies are compiled in the table 2.
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53 (Table 2)
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The obtained results revealed different antioxidant capacity (AOC) profiles between the batch assays, indicating that the conclusions about the AOC should be performed considering the mechanism of the conducted assay. This way, for comparison purposes the Spearman's-Rho correlation coefficients [21] were calculated in order to establish the relationship between the antioxidant capacities determined by the different methods. Comparing the results between the ABTS and DPPH assays the value of ρ was 0.736 and the value of p was < 0.006 , demonstrating a strong correlation between both methods. The antioxidant capacity measured by ABTS and DPPH assay did not showed correlation with the proposed MPFS methodology (for ABTS: $\rho = 0.2$, $p < 0.6$ and for DPPH: $\rho = 0.5$, $p < 0.1$, respectively). These results were predictable considering that the determination of the AOC by all methods is based on different mechanisms of action: the ABTS and DPPH radical assays are based on electron transfer and involves reduction of a colored oxidant [22] while the proposed QDs based MPFS methodology involves the scavenging of superoxide radicals originated inline and its following reaction with luminol (chemiluminescence probe). The proposed methodology prove to be very useful as an alternative methodology aiming at the antioxidant capacity determination specifically for the superoxide radical.

A profile of the AOC with respect to ROS scavenging of the tested samples was obtained by a thorough evaluation of the obtained results by the QDs based MPFS methodology. This way, it was possible to conclude that the beverages with higher antioxidant capacity were the "Brand 1" pear and the "Brand 1" red fruits. On the other hand, the samples with inferior quantity of antioxidants were the "Brand 4" pomegranate and red berries and "Brand 5" lemon. All the other samples presented antioxidant capacity values very similar between themselves.

1 These results are related with the nutritional constitution of the samples, since in the case
2 of beverages with high quantities of red fruits and pear [23, 24] these contain higher
3 concentrations of antioxidants. Additionally, since these beverages are considered
4 equivalent to natural fruit juices, the quantity of nectar fruit is high and, therefore, the
5 amount of antioxidant compounds capable of scavenging ROS in those beverages is higher
6 in relation to the samples that are only formed by fruit extracts. It is also important to
7 emphasize the fact that all of the samples analyzed had on its nutritional information label
8 the indication of addition of antioxidant compounds to prevent or delay its rapid
9 deterioration. Thus, there is the possibility of these non-natural antioxidants have
10 influenced the analysis of the antioxidant capacity of the samples.
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24 The evaluation of the precision of the proposed methodology for the antioxidant capacity
25 determination was performed through the repeated analysis of each beverage sample (5
26 consecutive determinations for each sample). The obtained results revealed a good
27 repeatability taking into account the calculated concentration ranges for a confidence level
28 of 95 % (Table 2).
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36 Additionally, the proposed MPFS allowed a determination rate of about 79 h^{-1} , a waste
37 generation of only 20.5 mL h^{-1} and low reagents consumption (0.100 mg h^{-1} of QDs and
38 2.1 mg h^{-1} of luminol). The presented values contemplated only the volumes of the
39 reagents, excluding the volume of deionized water used as carrier in the automatic MPFS.
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49 **3.5 Figures of merit**

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54 In the literature, a scientific work can be found, by Hemmateenejad et al. (2012) [6],
55 involving the use of QDs for the AOC determination, despite the mechanism of reaction is
56 different. In that work, the authors used L-cysteine CdTe QDs for radical photo-generation
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1 upon ultraviolet irradiation, which caused the photobleaching phenomenon of the NPs. The
2 presence of antioxidant substances inhibited the photobleaching process, and thus, the
3 determination method was based on the monitoring of the fluorescence of the QDs.
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6 In our work the determination of AOC was based on the chemiluminescence signal
7 induced by the oxidation of luminol by radical species, which on the other hand were
8 inhibited by antioxidant substances. The radical species were generated by irradiation of
9 GSH-CdTe QDs with visible light. The table 3 contains a thorough comparison of different
10 parameters (comprising the figures of merit) between the QDs based MPFS methodology
11 and the photobleaching based methodology [6] for the AOC determination.
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22 In our work the use of chemiluminometric detection allowed to obtain LOD values lower
23 than when using spectrofluorometric detection, since the LOD in the former detection is
24 determined only by reagents purity and not by instruments limitations.
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28 The use of a homemade visible light photo-excitation unit based on LEDs in our work
29 confers some advantages comparatively to the use of UV light in the photobleaching based
30 methodology. In fact, the use of a high-power lamp based on LED technology allows the
31 analyst not to be exposed to dangerous UV radiation emitted from UV low-pressure lamps.
32 Additionally, it was concluded that visible radiation is perfectly sufficient to the QDs
33 photo-excitation, without the risk of causing molecular photo-degradation as when using
34 the UV lamps. In food samples, there is the risk of its content in natural antioxidants be
35 altered upon exposition to UV radiation, considering the use of UV radiation in numerous
36 works for promoting the photo-degradation of substances applied in contaminated water
37 treatments. The use of UV radiation in the advanced oxidative processes (AOP) for the
38 photo-degradation of xenobiotics in contaminated waters can constitute a source for the
39 formation of oxidative species from surrounding medium, which would be a cumbersome
40 in the methodologies for determination of antioxidant capacity.
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Also, the variation of temperature when using UV lamps constitutes another risk factor for the degradation of natural antioxidants that consequently are not quantified in the AOC determination and also, without the analyst be aware of. Concluding, the use of UV radiation in analytical methods for the AOC determination might constitute a risk taking into account the possible photo-degradation of the antioxidants content in the samples upon exposure to the radiation. The use in our methodology of white LEDs emitting visible radiation that are cold lamps, circumvent the main drawbacks of UV radiation and fill the required standards of environmental friendliness.

The implementation of the developed reaction scheme in an automatic flow-based system (MPFS) allowed the determination of AOC with a throughput of about 79 determinations per hour, being this value associated with the ease of automation with all its advantages inherent like for example, minimal operator attention without influencing the quality of the analysis. Additionally, the use of solutions volumes in the order of microliters resulted in a very low consumption of reagents, e.g., the amount of QDs per determination was only 1.27 μg . Our work involved the consumption per determination of 80 μL of QDs solution with a concentration of $0.15 \mu\text{mol L}^{-1}$ corresponding to a quantity of 1.2×10^{-11} mol of NPs; whereas, in the photo-bleaching based procedure the consumption was of 3000 μL of QDs solution with a concentration of 32 nmol L^{-1} corresponding to a quantity of 9.6×10^{-11} mol of NPs per determination.

(Table 3)

4. Conclusions

1 In the developed QDs based MPFS methodology the radical species were generated by a
2 high-power visible light obtained by Light Emitting Diodes (LEDs), circumventing the risk
3 of using UV radiation that can cause the photo-degradation of the antioxidant substances in
4 food samples (through advanced oxidative processes) and/or uncontrolled radical
5 generation originated from QDs or the surrounding medium. By analyzing the redox
6 potential of some chemical species present in the aqueous solutions used in the work it was
7 predictable that only superoxide radical species were generated from the irradiation with
8 visible light of CdTe QDs of size 3.4 nm.
9

10 The implementation of the methodology in an automatic multipumping flow system
11 allowed an unpaired control of the sample and reagents manipulation with high
12 determination rates and good figures of merit, despite the extremely short lifetimes of the
13 radical species and the high kinetics of the chemiluminescence processes. Additionally, the
14 use of higher sizes of CdTe QDs (3.4 nm) allowed to work with low concentrations of the
15 nanoparticles, since the higher the size the higher production of ROS species. The use of
16 low concentrations of QDs did not impair the good figures of merit obtained in the
17 methodology.
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19 The methodology in this work revealed to be a valuable method for the evaluation of
20 antioxidant capacity of food samples due to antioxidants substances that act by scavenging
21 and neutralization of radical species, like for example, superoxide radicals.
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Captions for figures

Figure 1 – Diagram of the multipumping flow system. P_1 , P_2 and P_4 , 10 μL solenoid micro-pumps; P_3 , 50 μL solenoid micro-pump; X_1 and X_2 , confluence points; LED-PEU, photo-excitation unit; D, chemiluminescence detector; QDs, quantum dots: size particle of 3.4 nm with a concentration of $0.15 \mu\text{mol L}^{-1}$ in water; S, sample: trolox in 0.005 mol L^{-1} NaOH; C, carrier: H_2O ; L, luminol: $0.0015 \text{ mol L}^{-1}$ in 0.06 mol L^{-1} NaOH; W, waste.

Figure 2 – Normalized light absorption and emission spectra of the different sizes of QDs. (----) QD size = 1.3 nm; (—) QD size = 2.8 nm; (-.-.) QD size = 3.4 nm.

Figure 3 – XRD patterns for the GSH-CdTe QDs of size of 3.4 nm.

Figure 4 – Scheme of ROS generation by photoactivation of GSH-capped CdTe QDs and its participation in the chemiluminescence reaction with luminol.

Figure 5 – Influence on the analytical signal of the concentration of NaOH in the ROS generation and luminol reaction. Luminol $0.0015 \text{ mol L}^{-1}$ prepared in: - NaOH 0.005 mol L^{-1} ; - NaOH 0.01 mol L^{-1} ; - NaOH 0.015 mol L^{-1} ; - NaOH 0.02 mol L^{-1} ; - NaOH 0.04 mol L^{-1} ; - NaOH 0.06 mol L^{-1} .

Figure 6 – Influence on the analytical signal of the number of pulses. ■ – QDs + sample solutions; ● – Luminol solution.

Figure 7 – Influence on the analytical signal of the pulse time (ms). ■ – Irradiation stage;
● – Detection stage.

Table 1 - Compilation of all the parameters and values studied, as well as, the selected values, for the optimization of the developed MPFS based methodology for the determination of the antioxidant capacity.

Parameters	Studied values	Selected Values
QDs diameter (nm)	1.3 - 3.4	3.4
Concentration of QDs ($\mu\text{mol L}^{-1}$)	0.05 - 7.5	0.15
Concentration of luminol (mol L^{-1})	0.0005 - 0.0025	0.0015
Concentration of NaOH (in sample) (mol L^{-1})	0.001 - 0.1	0.005
Concentration of NaOH (in luminol) (mol L^{-1})	0.005 – 0.06	0.06
Number of pulses of sample	4 - 12	8
Volume of sample (μL)	40 - 120	80
Number of pulses of luminol	4 - 12	10
Volume of luminol (μL)	40 - 120	100
Time of pulse during irradiation (ms)	100 - 750	250
Time of pulse during detection (ms)	125 - 500	250
Flow rate during detection (mL min^{-1})	10.9 - 4.6	7.5

Table 2 – Antioxidant capacity of samples determined by CdTe QDs-MPFS, ABTS and DPPH assays, expressed in mmol L⁻¹ of trolox (TEAC).

Samples	MPFS Methodology TEAC mmol L ⁻¹	DPPH methodology TEAC×10 ⁻⁷ mmol L ⁻¹	ABTS methodology TEAC×10 ⁻² mmol L ⁻¹
“Brand 1” carrot-mango	3.4±0.1	12.3±0.1	13.1±0.5
“Brand 1” orange	2.20±0.05	n/a	10.5±0.2
“Brand 1” mango-orange	1.76±0.04	13±1	13.6±0.9
“Brand 1” pear	5.7±0.3	12.7±0.1	7.6±0.4
“Brand 1” tomato	2.41±0.07	6.1±0.4	5.7±0.6
“Brand 1” tutti-frutti	3.5±0.1	11.5±0.6	17±2
“Brand 2” exotic fruits	n/a	9.0±0.5	10.2±0.2
“Brand 2” red fruits	2.01±0.08	4.4±0.4	n/a
“Brand 2” lemon	1.8±0.1	7.4±0.2	7.8±0.3
“Brand 2” mango	1.63±0.06	6.6±0.8	7.5±0.1
“Brand 2” peach	2.31±0.06	9.0±0.5	8.4±0.2
“Brand 3” red fruits	1.35±0.04	7.0±0.2	7.8±0.2
“Brand 4” pomegranate and red berries	1.26±0.07	7.0±0.5	9.0±0.5
“Brand 4” green tea	3.0±0.1	34.4±0.4	41.9±0.5
“Brand 5” lemon	1.05±0.01	7.0±0.8	n/a

(i) Mean ± *t*0.05 (Student's *t* test) × (S/ $\sqrt{n}$).

Table 3 – Comparison of different parameters (comprising the figures of merit) between the QDs based MPFS methodology and the photobleaching based methodology [6] for the AOC determination.

	MPFS	Photobleaching ([6])
Detection method	Chemiluminescence	Fluorescence
Radiation	Visible	Ultraviolet
Equation of Linear Dynamic Range	$\Delta\text{CL} = 46 (\pm 3) \times \text{Log} [\text{Trolox}] + 200 (\pm 8)$	$\% \text{Inib} = 23.567 \times [\text{Trolox}] + 137.78$
Linear range with trolox	0.0001 - 0.005 mmol L ⁻¹	0.0333 - 0.333 mmol L ⁻¹
Correlation coefficient	0.9953	0.9914
Limit of detection (LOD)	0.00005 mmol L ⁻¹	n/a
Throughput	79 h ⁻¹	n/a
Precision (RSD)	< 5 %	< 5 %
QDs size	3.4 nm	3.5 nm
Constitution of QDs	L-Glutathione CdTe QDs	Cysteine CdTe QDs
Concentration of QDs	0.15 μM	32 nM
Volume of QDs/det.	80 μL	3 mL
Quantity of QDs/det.	1.2×10^{-11} mol	9.6×10^{-11} mol
Amount of QDs/det.	1.27 μg/det.	n/a

n/a – not available

Figure_1
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Figure_2

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