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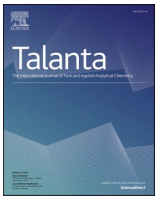

Fluorescent enzymatic assay for direct total polyphenol determination in food-related samples

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ABSTRACT

A direct and simple fluorescent assay for the total polyphenol determination based on the bioconjugate formed between the laccase enzyme (*TvL* from *Trametes versicolor*) and carbon nanodots (CD) is developed. One of the most used reactions for the determination of phenols is based on the enzymatic reaction of their oxidation to quinones. In this work, CD has been biofunctionalized with *TvL* (*TvL-CD*) and employed as a fluorescent label to follow the enzymatic reaction. The bioconjugate was formed and characterized by spectroscopy and microscopy. The optimal *TvL-CD* ratio was established. The reaction between the bioconjugate and a phenolic compound such as gallic acid (GA) was followed by monitoring the fluorescence bioconjugate decrease due to the quenching effect of the quinones generated in the enzymatic reaction. These studies confirm that bioconjugation does not inhibit the enzymatic activity and the fluorescence decrease during the enzymatic reaction is mainly due to an electron transfer processes. Based on these results, a new method for the quantitative determination of polyphenols measured as GA concentration is developed. The detection and quantification limit was found to be 7.4 and 25 μM, respectively. Subsequently, the method has been applied to the direct determination of GA in wine, juice, and rice leaf extracts.

1. Introduction

Polyphenols are found to be the most abundant antioxidant in our diet, mainly they are present in tea, coffee, red wine, and fruit juices. Other sources that also contribute to the total polyphenol intake are vegetables, cereals, and legumes. They have been proven to have several health benefits, including potent antioxidant and anticancer activity. In, particular, gallic acid (GA: 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3$-COOH)) is an important polyphenol, which widely exists in gallnuts, tea leaves, grapes, hops and oak bark [1,2]. It is a strong antioxidant with many biological properties [3,4] and with significant applications in food [5–7], in pharmaceutical industry [8] and medicine [9]. Hence, the availability of rapid methods for its selective and sensitive determination as well as for polyphenols in general it is a great deal of interest. A wide variety of analytical methods for the determination of gallic acid have been developed, such as High Performance Liquid Chromatography (HPLC) [10], UV–vis [11], chemiluminescence [12,13] and electrochemical methods [14–18].

Usually, quantification of polyphenols is carried out by the Folin-Ciocalteu method [19], which has low sensitivity and specificity. Other methods developed such as biosensors involve laccase or tyrosinase enzymes, being laccase/polyphenol oxidase perhaps the most extensively employed. The biggest advantage of laccase is it does not require peroxide as a co-substrate and any co-factor for the biocatalytic reaction. Hence, it has been used in many electrochemical biosensors for polyphenols detection [20,21]. However, this enzyme has scarcely been employed combined with nanomaterials for the development of fluorescent methods, despite the simplicity and high sensibility of these

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methods. For example, Akshath et al. used quantum dot-laccase enzyme system for detection of polyphenols [22].

Carbon dots (CD) is a new class of carbon nanomaterial that have attracted considerable interest since they were discovery in 2004 [23–25]. Compared to traditional semiconductor quantum dots, CD show several advantages, such as low toxicity, high solubility, strong and tuneable luminescence, high biocompatibility and photostability [26,27]. Therefore, CD have been widely applied in different fields such as in biosensing [28], optoelectronic devices [29], bioimaging [30] and drug delivery systems [31].

CD possess unique spectral properties, such as high emission quantum yield, sharp emission spectra, broad absorption spectra and photostability, and some advantages as easy and eco-friendly method of synthesis compared to quantum dots. Therefore, they can be excellent candidates to develop simple and fast analytical methods for the detection of very different analytes based on the change in their fluorescence intensity. However, to the best of our knowledge, CD have never been used after bioconjugation with enzymes to follow enzymatic reactions with application in fluorescent assays to detect analytes involved in these reactions.

It has been reported that CD surface adsorbed electron acceptors like quinones can lead to an efficient electron transfer resulting on the quenching of the CD fluorescence [32]. Since laccase enzyme converts polyphenols to quinones (see Scheme 1), CD can be employed as optical labels to detect polyphenols [33]. Therefore, in this paper we report on CD biofunctionalization with laccase to develop a direct and sensitive method for total polyphenols determination by combining the unique optical properties of CD and the specificity of laccase. The ensuing fluorescent assay, which is simple, sensitive, and reproducible, was used here to determine gallic acid as model analyte.

2. Experimental

2.1. Reagents and apparatus

D-Fructose, urea, GA, sodium carbonate, Folin-Ciocalteu reagent, glucose, ascorbic acid, and citric acid were obtained from Merck. Sodium sulfite was acquired from Panreac and sodium acetate and glacial acetic acid were provided by Fluka. Laccase enzyme (*TvL*, EC 1.10.3.2 from *Trametes versicolor*) lyophilized powder (34 U/mg) was purchased from Fluka. *TvL* stock solutions were prepared weekly in 10 mM acetate buffer solution (pH 4.5) (AcB) and the aliquots were stored at −20 °C until use. All the solutions were prepared using ultrapure water obtained from a Millipore Milli-Q system.

UV–Vis absorption spectra were performed on the PharmaSpec UV-1700 spectrometer (Shimadzu) operating from 200 nm to 1100 nm. Low volume quartz cells with 1.0 cm optical path were used.

Emission spectra were recorded using a Cary Eclipse spectrofluorometer (Varian). Fluorescence lifetime measurements were made using a 405 nm ps pulsed diode laser (LDH - D - C - 405 PicoQuant) with a PDL828 (PicoQuant) controller with FWHM <70 ps?as the excitation source. Emission was dispersed by an Acton SP2500 spectrophotometer and detected using a low dark current photomultiplier (PMA 06, PicoQuant), covering a spectral range between 220 and 650 nm (extended transit time <50 ps, FWHM), connected to a HydraHarp - 400 TCSPC event timer with 1ps resolution. Quartz cuvettes with an optical path of 1.0 cm were used for all emission measurements.

An inverted microscope Axiovert 200 (Zeiss) coupled to a CCD monochrome camera was used to obtain the fluorescence images. A SPECTRA-X (LUMENCOR) and a 20X/0.45 Plan/Apochromat Ph 1 (Zeiss) were used as the illumination source and objective, respectively. As excitation and emission filters, DAPI (340/25) and DAPI (444/36) were used, respectively. The images were processed with the FIJI software.

2.2. Procedures

2.2.1. Synthesis of CD

CD were synthesized following a previously published microwave-assisted "bottom-up" procedure [34], using as starting materials fructose and urea, and fully characterized by SEM, TEM, XRD, FTIR, among others, to asses that we have obtained the desired nanomaterial. Results agree well to those previously published [34].

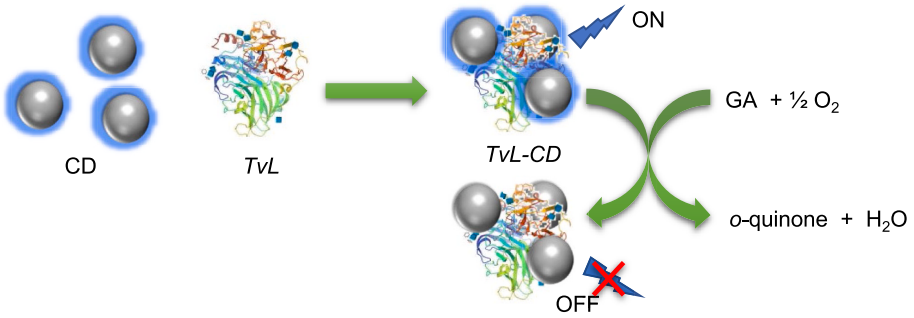
Briefly, adequate amounts of both precursors were left to react in a domestic microwave oven for 4 min at 750 W. The resulting dark brown solution containing CD was centrifuged, filtered with a nylon filter of 0.20 μm and dialyzed using a dialysis membrane with a range of molecular weight cut-offs of 0.1–0.5 kDa (Spectrum Laboratories) for 24 h. To estimate the concentration of the CD stock solution, 1.0 mL of this solution was heated to dryness. From the weight of the brown solid, a final concentration of CD of 12.7 mg/mL was calculated.

2.2.2. Preparation of the TvL-CD bioconjugate

The bioconjugate of *TvL* and CD was prepared by mixing 50 μL of CD solution (0.12 mg/mL in AcB) and 50 μL of the stock solution of *TvL*, allowing them to interact with gentle stirring for 1 h at room temperature. Subsequently, the bioconjugate was purified and the excess of reagents were removed by centrifugation at 9520 RCF (10,000 rpm) during 10 min at 4 °C using an Amicon Ultra Centrifugal Filter (Millipore). The *TvL-CD* pellet was resuspended in AcB and it was stored at 4 °C keeping stable for one month.

2.2.3. Determination of GA using the TvL-CD bioconjugate as fluorescent probe

GA was used as substrate of the *TvL* enzyme because this analyte is commonly used as a standard in the quantification of the total content of polyphenols in food. A standard solution of GA (10.0 mM) was freshly prepared in AcB. GA standard solutions of different concentration were obtained by serial dilution of the stock solution. For the determination of GA, 100 μL of *TvL-CD* were mixed with 100 μL of GA of different concentration (from 1.0 μM to 2.5 mM). After mixing thoroughly, the mixture was left at room temperature for 15 min. Then,



Scheme 1. Schematic representation of the developed bioconjugate fluorescent probe for GA detection.

photoluminescence (PL) was measured at 405 nm using an excitation wavelength of 320 nm, and the emission intensity was used for analytical purposes.

2.2.4. Determination of total polyphenols in samples

The total content of polyphenols was determined in wine, orange juice and in rice plant leaves using the developed optical method. White wine and orange juice from a local store were analyzed directly after dilution in AcB. However, before analyzing, rice plant leaves were pretreated as follows: 0.1500 g of leaf were weighed and placed in an agate mortar. Polyphenols were extracted with three times of 95% methanol. These extracts (3 mL) were collected and stored for 48 h in darkness. Then, they were centrifuged at 7711 RCF (9000 rpm) for 15 min. Finally, the supernatant was collected and stored at 4 °C until the analysis [35].

To determine the total content of polyphenols, increasing volumes of the GA standard solution (0.300 mM) were added to 10 μL of sample and diluted with AcB to a final volume of 100 μL. Next, 100 μL of this solution were added to the *TvL-CD* bioconjugate and incubated 15 min at room temperature and the PL signal was recorded at 405 nm using an excitation wavelength of 320 nm. The added volumes of GA results in analyte concentrations that are within the linear range of the method (between 30 and 135 μM). The results obtained for all the samples were validated by the Folin-Ciocalteu method [36].

3. Results and discussion

The use of fluorescent bioconjugates prepared from enzymes and nanomaterials, such as CD, allows the design of new sensitive and simple fluorescent methods based on the combination of the unique optical properties of CD and the specificity of enzymatic reactions.

In this work we employed CD prepared from non-toxic materials using a microwave-assisted carbonization method. After characterization by different techniques [34], we conclude that CD consist of nanoparticles with an average size of 5.5 nm and a high content of nitrogen, as demonstrated by TEM and elemental analysis, respectively. The Z potential of as-prepared CD in aqueous solution was found to be +90 mV, which confirms no aggregation. XRD showed that the CD present a nucleus with a predominantly amorphous structure. In addition, from the interpretation of the FTIR spectrum it was deduced that the core is surrounded by different functional groups, such as hydroxyl, carboxyl and amino, among others.

The presence of nitrogen groups in the CD structure confers unique optical properties to this nanomaterial, improving their photoluminescence and electronic structures significantly [37]. The UV–vis spectrum shows two absorption bands at 212 nm and 280 nm, and a strong absorption between 300 and 450 nm, without a defined band (Fig. S1(a)). The fluorescence spectrum (Fig. S1(b)) shows an emission band at 405 nm when the excitation wavelength is set at 320 nm. These interesting optical properties combined with the specificity of laccase enzyme are exploited as fluorescent probe for the development of method for direct and simple polyphenols determination in different food-related samples.

3.1. Preparation and characterization of TvL-CD bioconjugate

The negative environment of the Cu T1 site in the *TvL* structure [38,39] suggests that it can bind to the positive CD through electrostatic interactions, resulting the *TvL*-CD bioconjugate. The emission spectra of CD before and after addition of increasing concentrations of *TvL* and incubation at room temperature for 1 h were recorded (Fig. 1). Upon the concentration of the enzyme increases, there is an increase in the emission signal of the bioconjugate, and then its level off. The ratio between CD and enzyme amounts were optimized to obtain the best fluorescent features. Best results were obtained for 0.06 mg/mL CD and 0.074 mg/mL (2.5 U/mL) *TvL* (Fig. 1, inset).

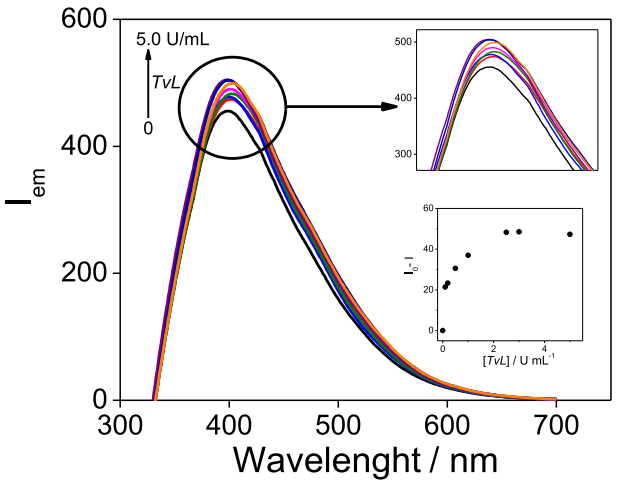


Fig. 1. Fluorescence emission spectra of the bioconjugate prepared as indicated in the experimental section using increasing amounts of *TvL* enzyme in AcB. Inset: Variation of the difference in the fluorescence intensity at 405 nm (before (I_0) and after (I) addition of *TvL*) with the amount of *TvL*. Excitation wavelength: 320 nm.

The as-prepared *TvL-CD* bioconjugate was characterized by fluorescence spectroscopy and microscopy, as well as FTIR. Fig. 2 shows the normalized 3D fluorescent mapping of each of the elements involved in the formation of the bioconjugate: CD, *TvL* and the bioconjugate (*TvL-CD*) after 1 h of incubation at room temperature. The excitation wavelength was set between 280 nm and 400 nm and the emission wavelength was recorded from 300 nm to 700 nm. As can be seen, the emission spectrum of CD has a maximum intensity at approximately 400 nm when the excitation wavelength is 320 nm (Fig. 2A). *TvL* enzyme presents two weak emission signals due to the intrinsic optical properties of the enzyme (Fig. 2B). The first one at 340 nm, when the excitation wavelength is set at 280 nm, is due to the presence of the tryptophan groups of the enzyme. The second band even weaker and centered at 450 nm appears when an excitation wavelength of 340 nm is used, and is ascribed to the presence of copper sites [40]. The bioconjugate (*TvL-CD*) spectrum shows an increase in the photoluminescence intensity, compared to those observed in Fig. 2A and B, with a maximum emission at 370 nm if the excitation wavelength is set at 290 nm (Fig. 2C). This result is consistent with the formation of hybrid material formed by non-covalent interactions between the CD and the T1 site of the *TvL* enzyme.

Taking advantage of the fluorescent properties of the CD, the *TvL-CD* bioconjugate formation was corroborated by fluorescent microscopy. Fig. 2 shows the fluorescence micrographs of CD (D), *TvL* (E) and *TvL-CD* (F) obtained at 444 nm when the excitation wavelength was set at 340 nm. As can be observed, CD image (Fig. 2D) shows blue emitting spots according to the fluorescence of the nanomaterial. On the other hand, as expected the *TvL* enzyme by itself does not present significant fluorescence emission under these experimental conditions (Fig. 2E). The *TvL-CD* bioconjugate displays also blue emitting spots (Fig. 2F) larger than that observed for the CD, suggesting the presence of CD-*TvL* aggregates due to the agglutinating effect of the enzyme.

FTIR was also employed to corroborate the bioconjugate formation. For this purpose, the FTIR spectrum of the *TvL-CD* was compared with those obtained separately for the enzyme and the nanomaterial (Fig. 3). The enzyme spectrum shows a wide band at 3402 cm^{-1} attributed to the amide A and hydroxylic groups. Likewise, the spectrum presents a band at 2927 cm^{-1} assigned to CH symmetric stretching in CH_2 and a band at 1408 cm^{-1} attributed to C–H bending of the CH_3 group. It can also be seen C═O and *N*–H stretching at 1647 cm^{-1} and 1546 cm^{-1}, respectively, assigned to the contributions of amide I and II. Finally, a C–O stretching vibration at 1036 cm^{-1} and bands at 804 cm^{-1} and 1252

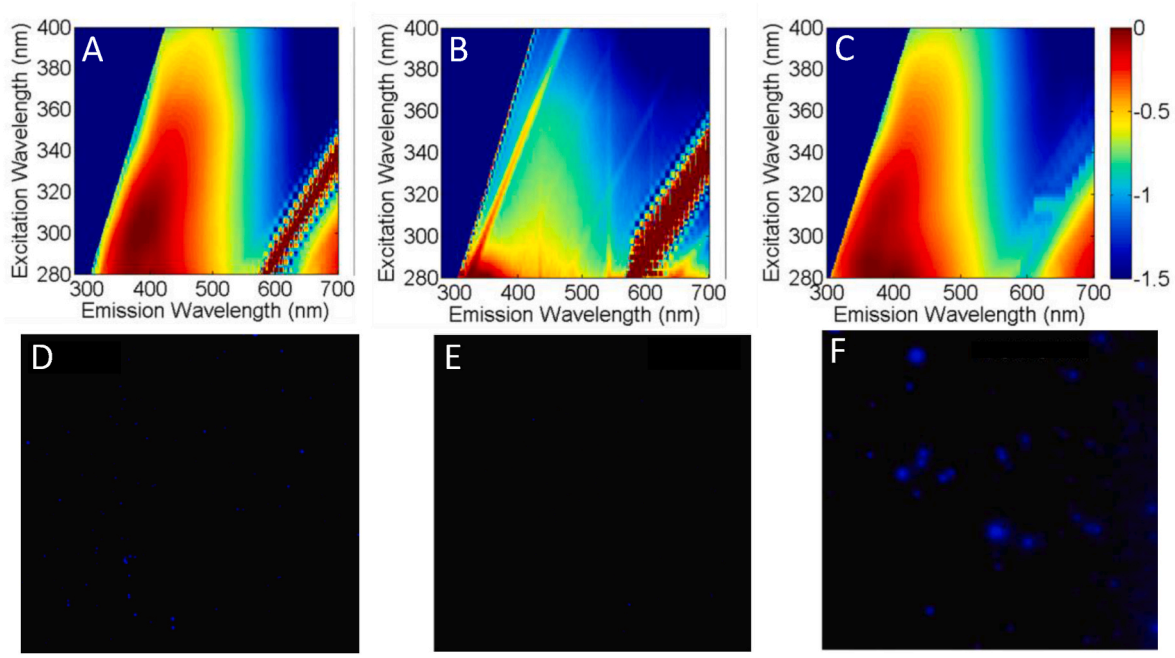


Fig. 2. Normalized 3D fluorescent mapping and fluorescence micrographs of CD (A and D, respectively), *TvL* (B and E, respectively) and *TvL-CD* bioconjugate (C and F, respectively). 20X magnification and excitation wavelength: 340 nm.

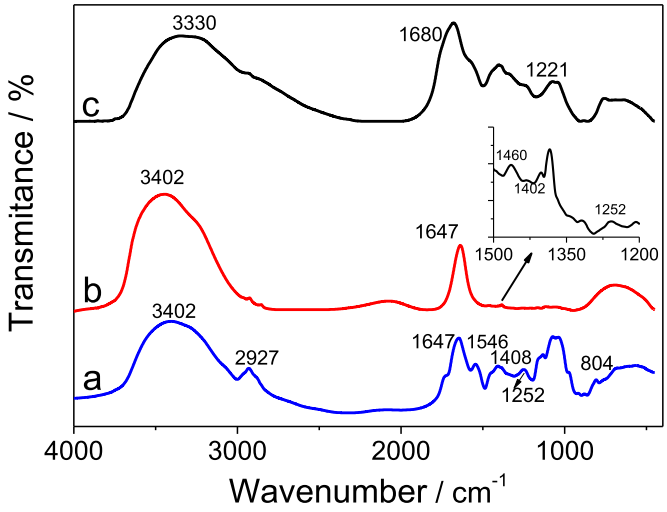


Fig. 3. FTIR spectra of *TvL* (a), *TvL-CD* (b) and CD (c).

cm^{-1} related to the amide III and V, respectively, were observed [41].

On the other hand, the FTIR spectrum of the CD depicts a wide band at around 3300 cm^{-1} corresponding to stretching vibrations of –OH and –NH bonds, suggesting the presence of both hydroxyl and amino groups on CD surface. Moreover, other less intense absorption bands observed at 1050 cm^{-1} and 1221 cm^{-1} can be assigned to bending and stretching vibrations corresponding to C–O bonds present in carboxyl groups [34].

The FTIR spectrum of the *TvL-CD* bioconjugate shows a band around 1252 cm^{-1} corresponding to amide III and other bands about 1402 cm^{-1} corresponding to the C–H bending of the CH_3 group. In addition, it is evident a slight shift (from 1546 cm^{-1} to 1460 cm^{-1}) of the band related to the *N*–H stretching corresponding to the amide II. The presence of the stretching bands of amide I, amide II, amide III, and C–H group that come from the enzyme, in the spectrum of the *TvL-CD* bioconjugate confirms its formation.

3.2. Study of the TvL-CD bioconjugate response to enzymatic substrate

The *TvL* enzyme catalyzes the oxidation of many phenolic substrates by losing one electron and one proton from the phenolic hydroxyl groups to form phenoxy radicals or quinones, involving the reduction of molecular oxygen in aqueous solution in the process. Among the phenolic substrates, GA is a polyphenol obtained from the hydrolysis of tannins, present in many foods from vegetal origin, with very interesting properties due to its antioxidant capacity and is commonly used as a standard in the quantification of the total content of polyphenols [42]. In the presence of the *TvL* enzyme, GA is oxidized to *o*-quinone, as indicated in the Scheme 1. According to this reaction, the amount of quinones generated as a product of the enzymatic reaction will be proportional to the number of GA molecules present in the solution. The progress of the enzymatic reaction can be followed by optical methods, either by the absorption changes that occur at the wavelength at which the product of the enzymatic reaction (*o*-quinone) absorbs or by the quenching of the *TvL-CD* bioconjugate, due to *o*-quinones can act as fluorescence quenchers (see Scheme 1). Therefore, GA was used as enzymatic substrate in the proposed fluorescent method.

Fig. 4 shows the evolution of absorption bands during the GA oxidation to *o*-quinone by the enzymatic reaction catalyzed by *TvL*. The UV–vis spectra of a solution containing only GA (Fig. 4a) has a characteristic low-energy absorption band at 265 nm corresponding to the n-π^* transitions [43]. The *TvL-CD* bioconjugate spectrum (Fig. 4b) shows a wide absorption band at 280 nm attributed to the formation of the bioconjugate between the CD and the oxidized form of the *TvL* enzyme. When GA is added (Fig. 4c) a new band at 400 nm appears, which according to the enzymatic reaction corresponds to the reduced form of the *TvL* enzyme. In addition, a shoulder around 350 nm related to the interaction between the *o*-quinones originated in the enzymatic reaction and the amino groups of the CD is observed [44]. These facts are evidenced by the change in color of the solution from colorless to dark yellow when the substrate (GA) is added (Fig. 4, inset).

Similarly, according to the Scheme 1, the progress of the enzymatic reaction can be followed by the quenching effect of the *o*-quinones, generated in the enzymatic reaction, on the fluorescence of *TvL-CD* bioconjugate. As can be observed (Fig. 5A), after the addition of

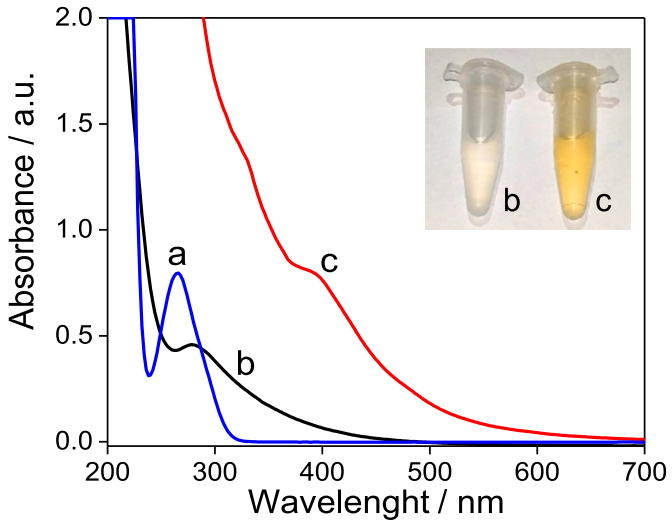


Fig. 4. UV–vis absorption spectra of 0.50 mM GA (a) and the *TvL-CD* bioconjugate in the absence (b) and in the presence (c) of 0.50 mM GA in AcB. Inset: Photographs of a *TvL-CD* solution before (b) and after (c) addition of GA.

substrate, the enzymatic reaction takes place and the presence of the *o*-quinones produces a decrease in fluorescence intensity at 405 nm, using an excitation wavelength of 320 nm. However, the fluorescence spectra of an aqueous solution of CD in the absence and in the presence of 0.10 mM GA (Fig. 5B) shows that the addition of the substrate does not produce variations in the photoluminescence intensity of the CD, indicating that GA does not cause any effect on the fluorescence of the nanomaterial. Therefore, the catalytic oxidation of GA to *o*-quinones can be followed through the decrease that occur in the emission intensity of the bioconjugate when adding the substrate. The quenching can be explained considering an electron transfer mechanism in which a photoinduced electron from the CD present in the bioconjugate is transfer to the quinones, which act as electron acceptors, causing the decrease of the photoluminescence intensity of the bioconjugate.

Additional and valuable information about the mechanism of the fluorescence quenching of the biconjugate after the addition of GA, have been obtained by measuring the fluorescence lifetime. It is the characteristic time in which a molecule remains in its excited state before returning to the fundamental state, and is widely used to study biomolecules, their microenvironment, and their molecular associations [45]. Thus, it provides information on the interactions between the fluorophore and other molecules, as well as on the mechanism by which they are produced.

The fluorescence lifetime decay profiles of *TvL-CD* bioconjugate in the absence and in the presence of 0.15 mM GA give lifetime values of 5.21 ns and 5.05 ns, respectively (Fig. S2). This slight change indicates that there is not non-radioactive energy transfer from the donor (CD) to the acceptor (*o*-quinones) that significantly affects the fluorescence lifetime, suggesting that the energy transfer mechanism (FRET) is excluded [45]. Therefore, as we anticipated, the electron transfer mechanism is probably responsible for the fluorescence quenching.

3.3. Determination of GA

Based on the above explained results, we developed a fluorescent method to determine GA. Thus, at a first step several experimental variables involved in the method were optimized. This optimization study comprised the ratio between CD and enzyme amounts employed in the preparation of the bioconjugate, and the reaction time. Considering as the best result the highest decrease in the photoluminescence intensity of the *TvL-CD* in the presence of 0.15 mM of GA, these were obtained using 0.06 mg/mL CD and 0.074 mg/mL *TvL*. Regarding the incubation time, the fluorescence emission of *TvL-CD* bioconjugate after addition of GA was continuously monitored for 3 h (Fig. S3), obtaining the greatest decrease in signal at 15 min and from then on it practically remains stable. Therefore, 15 min was the incubation time chosen as optimal.

The *TvL-CD* bioconjugate fluorescence decrease after addition of increasing GA concentrations was also studied. Fig. 6A exhibits the calibration curve obtained by representing the difference in the fluorescence intensity measured at 405 nm before (I_0) and after (I) addition of GA versus the amount of GA. The response fits well to a Michaelis-Menten curve, indicating that the fluorescence response is controlled by the enzymatic reaction. The analytical properties of the method were calculated from the linear part of the calibration plot (up to 200 μM; $r^2 = 0.996$), which fits to the equation: $I_0–I = (8.9 \pm 0.2)x10^2$ [GA], mM - (1.5 ± 0.8) (Fig. 6B). Data presented are the average value of three determinations. A sensitivity of $(8.9 \pm 0.2)x10^2$ mM^{-1} was calculated from the slope of the calibration plot. The limits of detection and quantification were calculated as three or ten times, respectively, the standard deviation of the blank divided by the sensitivity. The values obtained were 7.4 and 25 μM, respectively. The repeatability of the proposed method was evaluated by three repeated measurements of different concentration of GA and the relative standard deviation (RSD) was less than 10% in all cases, indicating the reliability of the method.

Although the detection and quantification limits obtained for the determination of GA are not so low (see Table S1), it is important to highlight that the content of this analyte in many food samples is in the mM range, and therefore the method developed would be perfectly valid, as will be demonstrated below. Moreover, the proposed methodology is based on the use of an easily synthesized nanomaterial using ecofriendly procedures and prepared from low cost, non-toxic and natural source precursors. This represents an important advantage compared to the previously described methods that use, among others, gold, palladium, or lanthanide-derived nanomaterials.

With the aim of testing the applicability of the method for the total

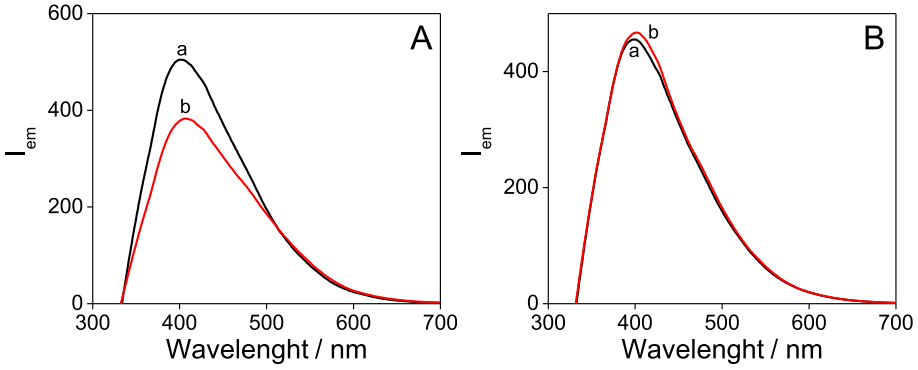


Fig. 5. Fluorescence emission spectra of (A) the bioconjugate *TvL-CD* or (B) the synthetized CD, in the absence (a) and in the presence (b) of 0.15 mM GA in AcB.

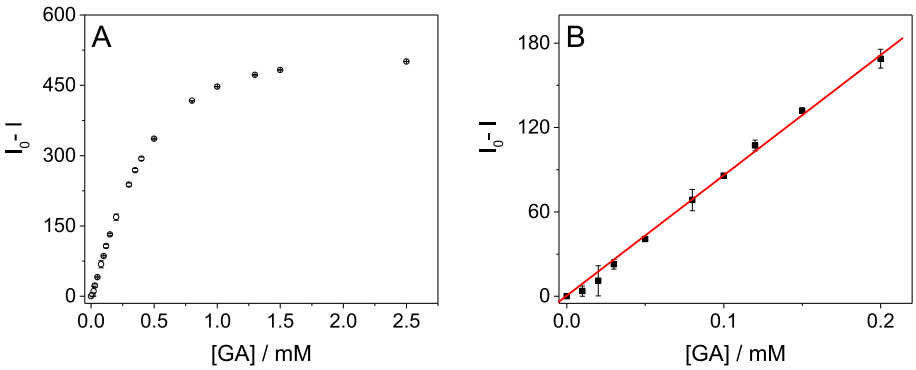


Fig. 6. (A) Calibration curve for GA biosensing, measured at 405 nm (λ_{exc} = 320 nm). (B) Linear part of the calibration curve. Error bars were estimated as the standard deviation of three measurements.

content of polyphenols (expressed as GA concentration) in food-related samples, orange juice, white wine, and rice leaf extracts were analyzed. First, the effect of the most usual potential interfering compounds that can be found in these samples was evaluated. To this end, the assay response to GA in the absence and in the presence of a high (1:0.1) and low (1:0.01) concentration of glucose, fructose, citric acid, ascorbic acid, and sodium sulfite (Table 1). was obtained. As can be seen, the response is not affected by the presence of potentially interfering compounds when these are at a concentration 100 times lower than GA. However, when the interfering compound is at a concentration 10 times lower than GA, the photoluminescence increases around 5–8%, being of 10% for sodium sulfite, which is an inhibitor of the *TvL* enzyme.

3.4. Determination of total content of polyphenols in samples

Finally, the developed fluorescent method was applied to the determination of total content of polyphenols, expressed as GA concentration, in white wine, orange juice and rice plant leaves. All the determinations were carried out using the standard addition method to minimize matrix effects. The results obtained were statistically compared to those obtained by a the Folin-Ciocalteu method, chosen as reference method. It is based on the use of a mixture of phosphomolybdate and phosphotungstate that react with polyphenols present in the sample to form a blue complex that can be quantified by UV–vis spectrophotometry. The results are summarized in Table 2. The average GA concentration value obtained for ten or three measurements in the case of juice and wine or rice leaves, respectively (as listed in Table 2), agrees well with that obtained by the Folin-Ciocalteu assay and the statistical analysis of comparison of the experimental mean of both methods confirms that in the case of the orange juice and rice leaves, there is no significant difference between the two methods at the 5% level, since values of t were less than the critical value. However, in the case of wine the comparison of the two experimental means gives an experimental value of t greater than the critical vale, suggesting that a significant difference between the two results at the 5% level exists. This result can be explained by the presence of sulfite in the white wine samples that results in a significant overestimation of polyphenols content when the Folin–Ciocalteu's assay is used as it has been reported by other authors [46].

In this sense, to highlight the selectivity of the method a recovery study of GA was carried out. For this purpose, the samples were spiked with known amounts of GA and analyzed in triplicate. As can be seen in Table 3, good recoveries were obtained indicating that the developed method is suitable for the determination of total content of polyphenols.

Table 1
Relative *TvL-CD* bioconjugate response to GA in the presence of potentially interfering compounds.

Compound	Relative response[a]	
	1:0.1	1:0.01
Ascorbic acid	1.07	1.00
Citric acid	1.08	1.00
Fructose	1.07	0.99
Glucose	1.05	1.00
Sodium sulfite	1.10	1.02

[a] Relative response = $I_{GA + I}/I_{GA}$. $I_{GA + I}$: response of 0.10 mM GA in the presence of interfering compound; I_{GA}: response of 0.10 mM GA.

Table 2
Determination of total polyphenols as GA concentration in different samples with the developed fluorescent method and with the Folin-Ciocalteu assay.

Sample	Folin-Ciocalteu assay (n = 4)		Developed fluorescent method		
	[GA]/mM	RSD/%	[GA]/mM	RSD/%	N
Orange juice	4.6 ± 0.2	4.3	4.4 ± 0.2	4.5	10
White wine	2.2 ± 0.1	4.5	1.96 ± 0.05	2.0	10
Extract of rice leaves	0.33 ± 0.04	12.1	0.29 ± 0.02	6.9	3

4. Conclusions

In summary, we developed a simple, label-free, and highly selective fluorescent method to determine total polyphenols based on CD conjugated with laccase. CD retain their fluorescence after enzyme bioconjugation and the enzymatic reaction of polyphenol oxidation to quinones can be followed by the quenching effect of quinones generated in the enzymatic reaction. Enzymatically generated quinones adsorb on the CD surface serving as the electron acceptors for CD, resulting in the fluorescence quenching probably due to an electron transfer process.

The method has been successfully applied to determine total polyphenols, measured as GA concentration, in different samples. Results compare well with those obtained from the standard Folin-Ciocalteu method, being the developed method more selective.

Table 3
Determination of GA in different spiked samples with the developed fluorescent method.

Sample	Developed fluorescent method (n = 3)			
	[GA]/μM		Recovery/%	RSD
	added	found		
Orange juice	50	44 ± 5	88	11.4
	100	(1.0 ± 0.1)x10^2	100	10.0
White wine	30	29 ± 1	97	3.4
	100	101 ± 4	101	3.9
Extract of rice leaves	50	45 ± 7	89	15.6
	100	(1.0 ± 0.1)x10^2	100	10.0

Author statement

Encarnación Lorenzo: Conceptualization, Supervision, Writing, Funding acquisition. **Félix Pariente**: Conceptualization. **Lourdes Hernández-Apaolaza**: Conceptualization. **Cristina Gutierrez-Sánchez:** Investigation, Writing, Funding acquisition. **Mónica Revenga-Parra**: Conceptualization, Supervision, Writing. **Mónica Mediavilla**: Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123576.

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