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Peroxidase like activity Prussian blue nanocubes for the polyphenol detection in commercial beverages

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ABSTRACT

The present study describes an efficient method for the determination of polyphenol content in beverages based on a composite material of graphene oxide decorated with Prussian blue nanocubes (rGO/PBNCs). In this method, rGO/PBNCs act as a nanoenzyme with a catalytic activity like peroxidase and produce a colorimetric product in the presence of hydrogen peroxide and tetramethylbenzidine (TMB). To verify the effectiveness of the method, we used two model standards for antioxidants: gallic acid (GA) and tannic acid (TA). The method validation included a comparison of the performance of a natural enzyme and the artificial one (rGO/PBNCs) and two polyphenols in the analysis of commercial beverage samples. After optimization, a pH of 4, ambient temperature (22ºC), a reaction time of 2 minutes and an rGO/PBNCs concentration of 0.01 µg/mL were found to be the most favorable conditions. The detection limits obtained were 5.6 µmol/L for GA and 1.5 µmol/L for TA. Overall, rGO/PBNCs offer advantages over natural enzymes in terms of stability, versatility, scalability and durability, making them attractive candidates for a wide range of catalytic and sensory applications.

KEYWORDS: Phenolic compounds; colorimetric assay; nanoenzymes, beverages

1. INTRODUCTION

Nowadays, the market for healthy food is growing due to trends in consumers' healthy lifestyles [1, 2]. Many of these trends are related to the search for solutions to health problems caused by oxidative stress, in which the formed free radicals (FR) can damage biomolecules and subsequently lead to vascular diseases, cancer, autoimmune diseases, or other conditions linked to oxidative stress. Scientific evidence confirms that foods, including vegetables and fruits, exert an antioxidant effect, that helps mitigate the harmful effects of free radicals within the body [3-6]. In this context, commercial beverages such as wines, teas, and natural juices are also recognised as sources of antioxidants for the body which can benefit human health [7, 8]. Typically, these antioxidant sources contain polyphenols, molecules with strong antioxidant capacity, that can neutralize FR protecting against antioxidative stress however, its consumption requires a balance as excessive consumption can cause side effects [9-12]. Therefore, it is of utmost importance to analyse and inform consumers about the polyphenol content in beverages and foods[13]. Apart from their impact on consumer health, polyphenols also play a crucial role in the organoleptic properties of beverages such as colour, aroma, and taste. Polyphenol phytochemicals are produced by plants to help them resist infections. These bioactive antioxidant compounds can be found in several parts of the plant such as in seeds, fruits, leaves, roots, stems, and flowers) [14, 15]. Considering the enormous diversity of polyphenols, they are classified according to their most important properties.

Regarding food control, polyphenols separation and quantification is a major challenge due to their abundance and the complex nature of different food samples and matrices. Analytical separation methods such as high-performance liquid chromatography (HPLC) [16-21] and capillary electrophoresis[22-24] in conjunction with various detection systems such as UV–Vis [25], fluorescence [26] and mass spectrometry (MS) [27] have already been described. Among these, mass spectrometry is the most effective system for the identification of polyphenols.

While HPLC remains the predominant technique for isolating and measuring individual polyphenols, various spectrophotometric assays are also employed to ascertain the total polyphenol content and consequently, the antioxidant capacity [28, 29]. Generally, the spectrophotometric assays are based on chemical reactions, some of which are based on single electron transfer, such as the Folin–Ciocalteu method (FC) [30-32] and the reducing antioxidant power of iron (FRAP) [33, 34]. Other methods widely used rely on hydrogen atom

transfer mechanisms like the oxygen radical absorbance capacity (ORAC) [35], or an (e.g. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid - ABTS) [36, 37] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [38, 39] methods. However, they have some disadvantages. The DPPH and ABTS assays rely on the scavenging activity of antioxidants against the synthetic free radicals DPPH and $ABTS^{+}$, which do not occur in natural systems, regarding the ORAC assay requires relatively complex instruments such as fluorescence spectrophotometers for detection. Therefore, the development of a simple, reliable, and novel alternative for the assessment of antioxidant radical scavenging activity is still a demand.

Prussian blue (PB) has been widely used as a nanozyme given its strong antioxidant capacity with enzyme-like properties such as peroxidase-like activities, catalase-like activities, and superoxide dismutase-like activities, which play an important role in the regulation of oxidative stress in various diseases [40, 41] In this context, PB has proven to be a versatile and innovative material to be used as a sensing platform. PB nanoparticles have been successfully used in diverse applications, including the detection of H_2O_2 [42], lactate levels [43], and glucose [44]. These analytical efforts rely on the direct catalytic reduction of H_2O_2, a product formed when target analytes interact with oxidase enzymes. This catalytic process, driven by PB, leads to the formation of $^{\bullet}HO$ ([45-48], which triggers the colorimetric reaction of a chromogenic substrate. Monitoring the intensity of the resulting colour change enables precise quantification of the analyte.

Inspired by the advantages of nanomaterials, further enhancements have been undertaken to expand the features of PB materials, thus unlocking their potential for diverse applications. For that, PB and their analogs have been synthesized and integrated with a variety of materials including carbon-based substrates, metal nanoparticles, and conducting polymers, among others. These combinations lead to enhanced stability, higher conductivity, and improved redox reversibility although each strategy exhibits unique advantages and limitations[49] Scientific literature reports ascribed the conjugation of PB with carbon-based materials like carbon nanotubes, graphene/graphene oxide sheets, and analogous structures aiming to increase their conductivity, confer chemical stability, facilitate inclusion of functional groups, and facilitate electron transfers mechanisms.

Graphene production encompasses two main approaches: one entails exfoliation and separation of graphite to obtain graphene, while the other focuses on the growth of small carbon precursors to form graphene. Typically, these methods require reducing agents, that exhibit toxicity and are hazardous to handle or demand expensive high-temperature

processes. In response to these challenges alternative environmentally friendly strategies have been developed, centering their efforts in safer chemicals, microorganisms such as bacteria and yeasts, plant and fruit extracts, as well as protein molecules [50] umar et al. described the production of biographene based on BSA/graphene through a simple method to be applied in cell culture studies that can be embraced by most laboratories.

Overall, PBNCs, so-called nanoenzymes, offer several advantages over natural enzymes in certain applications. They are more stable under harsh conditions, such as high temperatures or extreme pH values, and retain their structure and function, whereas enzymes can denature. They are produced synthetically and can be customised for different purposes so that size, shape and surface properties can be precisely adapted. Unlike natural enzymes, which are limited by their inherent structure, Prussian blue nanocubes offer versatility. In addition, their scalability enables large-scale production through efficient synthesis methods, unlike natural enzymes which often require labour-intensive extraction processes. Nanoenzymes exhibit similar catalytic activity to enzymes and can be modified by adjusting their composition and structure to improve specific reactions. Their longevity allows for continuous use in various applications without significant degradation, unlike natural enzymes which can degrade over time and require frequent replacement. [1-5]

Here, we make use of PB nanoparticles of cubic morphology and size controlled by adjusting the concentration of reactants, combined with biographene to be applied as a sensing material. Remarkably, the use of graphene oxide decorated with PB nanocubes (rGO/PBNCs) for the evaluation of polyphenol content is still unexplored in the existing literature. Our work presents an innovative, cost-effective, and straightforward approach for the colorimetric assessment of the radical scavenging activity of polyphenols that exploits the special properties of rGO/PBNCs. This method is based on the ability of antioxidants to quench $^{\bullet}HO$, a naturally occurring free radical produced by the catalytic interaction between rGO/PBNCs and H_2O_2. In the absence of polyphenols, Fe_3O_4 MNPs catalytically oxidise H_2O_2, leading to the formation of $^{\bullet}HO$. Subsequently, the generated $^{\bullet}HO$ vigorously oxidises a colourless substrate such as 3,3’,5,5’-tetramethylbenzidine (TMB), causing a striking colour change from colourless to blue. However, the presence of polyphenols sequesters $^{\bullet}HO$, reduces the pool of reactive species that can contact the nanoenzyme-based rGO/PBNCs substrate. Consequently, the intensity of the resulting colour is attenuated. This colour shift is the basis for assessing the antioxidant capacity of the samples. As a proof of concept for the suitability of our method, gallic acid (GA) and tannic acid (TA) were used as model standard polyphenols. The

colorimetric analysis of assays was carried out using a UV–vis spectrophotometer. Our results showed that the inhibition of antioxidant properties on the catalytic activity of rGO/PBNCs correlated well with the concentration of both polyphenol standards and the specific type of antioxidant used.

2. Experimental

2.1 Materials & Equipments

Absorbance measurements were performed using a Thermo Scientific Evolution 220 UV-Vis spectrophotometer, and plastic cuvettes with an optical path length of 1 cm and a maximum capacity of 2.5 mL. The absorbance spectra were collected for the wavelength ranging from 500 to 750 nm. The pH value adjustments and measurements were carried out using a Crison GLP 22 pH Metre Potentiometer. TEM Analyses were performed using a Jeol JEM 1400 transmission electron microscope with a scanning transmission electron microscopy (STEM) detector.

2.2 Chemicals and Solutions

Ultrapure water with conductivity values up to 0.054 µS cm^{-1} at 25 °C was used to prepare the aqueous solutions. The chemicals used included phosphate-buffered saline (PBS) (Fisher BioReagents); sodium dodecyl sulfate for Electrophoresis (SDS) (>97 % TCI); hydrogen peroxide (H_2O_2) (35 % Labchem); tetramethylbenzidine (TMB) (≥ 99 % TCI); horseradish peroxidase (HRP) (Sigma); dimethyl sulphoxide (DMSO) (≥ 99.9 % Sigma Aldrich); (±)-Catechin hydrate (CAT) (> 96 % Sigma Aldrich), gallic acid (GA) (≥ 99 % Panreac); tannic acid (TA) (Riedel-de-Haen), potassium chloride (Merck); serum albumin protein (BSA) (Sigma), potassium hexacyanoferrate III ($K_3[Fe(CN)_6$) (Scharlau), Iron(III) chloride ($FeCl_3$) (Fluka); graphite powder (99 % Sigma Aldrich) and polyethyleneimine solution (PEI) (50 % (w/v) in H_2O Fluka).

2.3 Sample preparation

Six commercial beverages of different brands were analysed to assess their antioxidant capacities. All the analysed beverages were purchased from local retailers, in detail the six samples were: (i) flavoured water; (ii) 100 % apple-flavoured fruit juice; (iii) 2020 white wine; (iv) 2020 red wine; and (v) green tea.

2.4 Spectrophotometric Analysis

Firstly, an investigation was conducted to determine the optimum concentrations of the different reaction components. The volumetric proportions for the assay were: 1888 µL of buffer (PBS), 4 µL H_2O_2, 1 µL HRP, 5 µL SDS, 100 µL polyphenol/buffer (corresponding to the acid concentration used in each cuvette), and 2 µL TMB. Throughout the study, some adjustments were made according to the requirements of the system until an optimal completion was reached. The reaction time was also optimised between 2 and 5 minutes and the pH value was adjusted to 7.4. The optimised conditions for the spectrophotometric determination of phenolic content were: a mixture containing 1888 µL PBS (pH 7.4), 1 µL HRP (100 µg/mL), 4 µL H_2O_2 (1 mol/L), 2 µL TMB (0.4 mol/L), 5 µL SDS (1 mol/L) and 100 µL of the reference polyphenol, AG (1000 µmol/L) or AT (1000 µmol/L) or CAT (1500 µmol/L) prepared in disposable plastic cuvettes of 2.5 mL. To optimise the concentrations of the different polyphenols, it was necessary to test different concentration ranges. For GA the concentration range was 0-50 µmol/L and for TA 0-14 µmol/L. All assays were performed in triplicates.

2.5 Synthesis of the nanoenzymes

2.5.1 Preparation of Biographene

Biographe (BioGR) was synthesised following the methodology described by Kumar et al.. Briefly, a suspension of graphite crystals (100 mg/mL) in 200 mL of deionised (DI) water adjusted to pH value 7.0 and containing the BSA protein (3.0 mg/mL) was subjected to shear forces in a kitchen blender for 30 min. Samples were taken every 5 min to monitor the rate of exfoliation, and the mixing process was interrupted to avoid overheating (<30 °C). The absorbance of the suspension at 660 nm was used to quantify the graphene concentration after removing the unfoliated graphite by centrifugation at 1500 rpm for 45 min. The exfoliation experiments were carried out systematically with precise amounts of graphite, BSA, and DI water in a kitchen blender at the blade speed.

2.5.2 Synthesis of the rGO/PBNCs

The composite was synthesised according to a procedure described by Cao et al. [47]. First, a solution containing 10 mL of 5 mmol/L $FeCl_3.6H_2O$ (pH 1.1), 1 mL of 3 % PEI, and 10 mL of 5 mmol/L $K_3Fe(CN)_6$ (pH 1.1) was mixed with 10 mL dispersion of BioGR (6.7 mg/mL, pH 1.1) at a constant stirring during 2 h. The mixture was then refluxed for 3 h at 130 ºC. Throughout the reaction, the colour of the mixture gradually changed from dark yellow to a deep blue, indicating the successful formation of the composite of rGO/PBNCs. At the end of the reaction,

the resulting mixture was centrifuged and the pellet was washed three times with ultrapure water. The final material was then redispersed in 10 mL of water.

2.6. Spectrophotometric analysis

2.6.1 Detection of phenolic compounds with HRP

Colorimetric determination of phenolic compounds was performed in 2.5 mL disposable plastic cuvettes containing HRP (0.03 µg/mL), H_2O_2 (4 mmol/L), TMB (400 µmol/L), PBS pH 4, and increasing concentrations of the target phenolic compounds (GA or TA) according to the different assays. The selected concentrations were based on the work described by Gao et al. [51], with some slight adjustments based on the requirements optical signal of the system. The reaction time and pH conditions were also optimised. For this study, a solution of GA ranging from 10 to 200 µmol/L was prepared from a 4 mmol/L stock solution and a solution of TA ranging from 0.5 to 10 µmol/L was prepared from a 200 µmol/L stock solution. The mixture was incubated at room temperature for 2 min and the absorbance was monitored over time in the UV-Vis spectrophotometer at the λ_{max} of 652 nm. The limit of detection value (LOD) for both standard polyphenols was calculated using the definition three times the standard deviation of the blank divided by the slope of the calibration curve. The commercial beverage samples were analysed using this method to determine the phenolic content relative to GA and TA.

2.6.2 Detection of phenolic compounds with the nanoenzyme rGO/PBNCs

The determination of the phenolic content using an artificial enzyme was carried out by the same procedure described in section 2.6.1. with slight modifications and by replacing the HRP with the synthesised rGO/PBNCs. The assays were also performed in 2.5 mL cuvettes containing rGO/PBNCs (0.01 µg/mL), H_2O_2 (4 mmol/L), TMB (400 µmol/L), and different concentrations of GA and TA described for each assay. The reaction time of the mixture was also analysed and the incubation time was determined at room temperature **(Figure 1).**

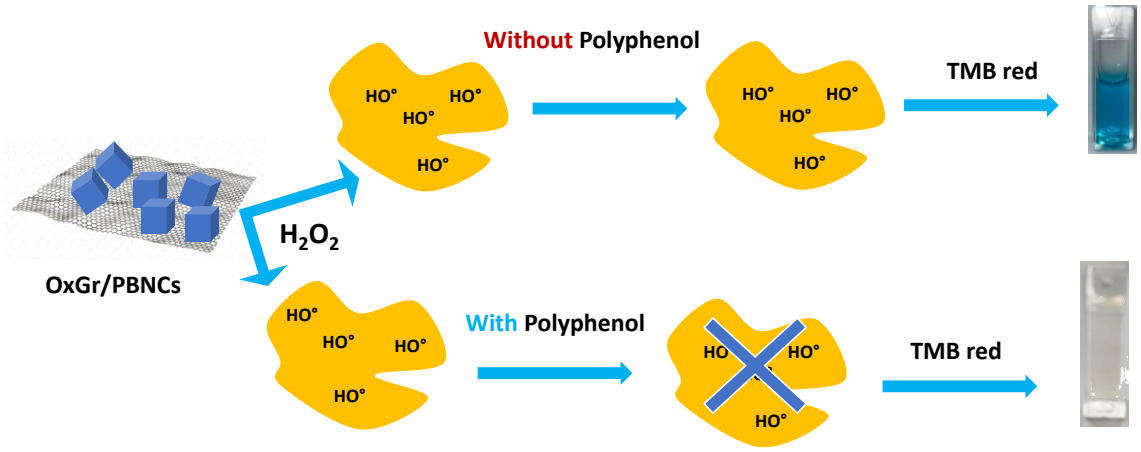


Figure 1 - Colorimetric detection of polyphenols using nanoenzymes like rGO/PBNCs.

The working concentration range for GA was 10-200 µmol/L, prepared from a 4 mmol/L stock solution while for the TA, the working concentration was 1.0–20.0 µmol/L, prepared from a 400 µmol/L stock solution. The absorbance values of the solutions were monitored at the UV spectra (λ_{max}=652 nm) and the LOD for both standard polyphenols was calculated based on three times the standard deviation of the blank divided by the slope of the calibration curve. The developed method was used to determine the phenolic content, especially GA and TA, in the commercial beverages used as samples, as described in section 2.2.

3. Results & discussion

In this work, we present and discuss the development of a colorimetric method for the determination of the phenolic content in commercial beverages using a natural enzyme and the synthesised nanoenzyme rGO/PBNCs to evaluate the feasibility of mimicking the natural enzyme features.

The graphene-based materials obtained by green production were characterized by TEM analysis as shown in **Figure 2**. The obtained BioGR displayed in **Figure 2A** clearly shows the flat transparent graphene sheets at the nanoscale with small wrinkles and folding mostly in their edges. **Figure 2B** shows the composite material, rGO/PBNCs with graphene oxide sheets modified with PBNCs at nanoscale dimensions. The PBNCs have an average size of 47.8±12.0 nm (n=30) and from the synthesis, sphere by-products were also formed. Some regions display PB nanocubes in an aggregated form, **Figure 2.B1** than others, although it is clear that PBNCs are involved by the oxide graphene sheets as desired.

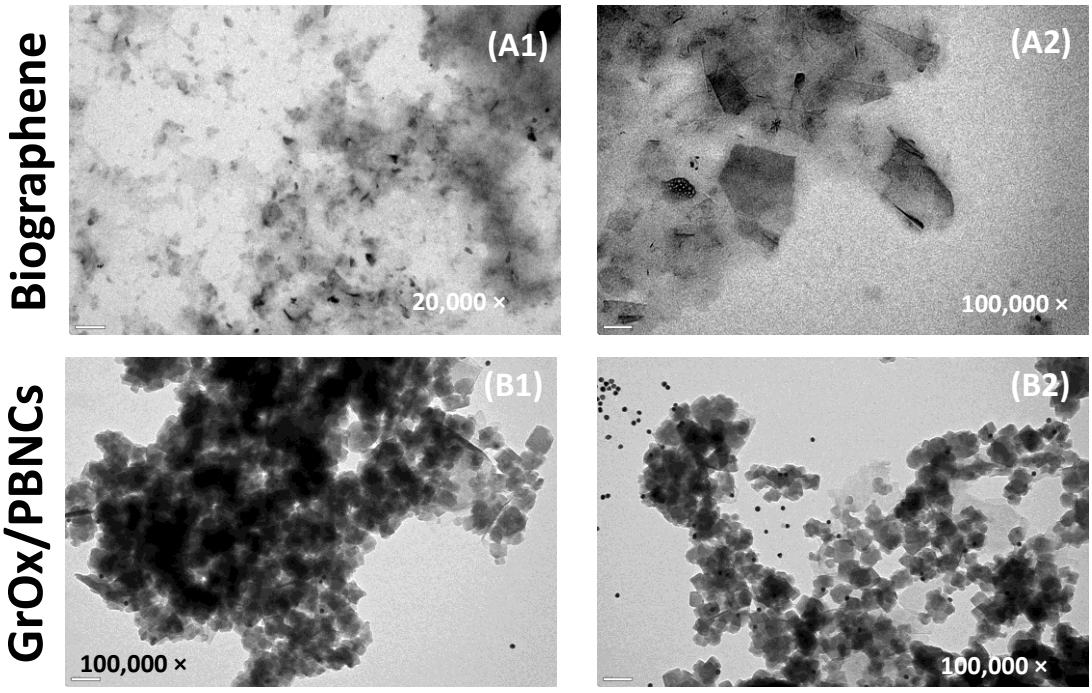


Figure 2 - TEM analysis of (A) biographene and (B) rGO/PBNCs.

3.1. Optimization of HRP colorimetric assay

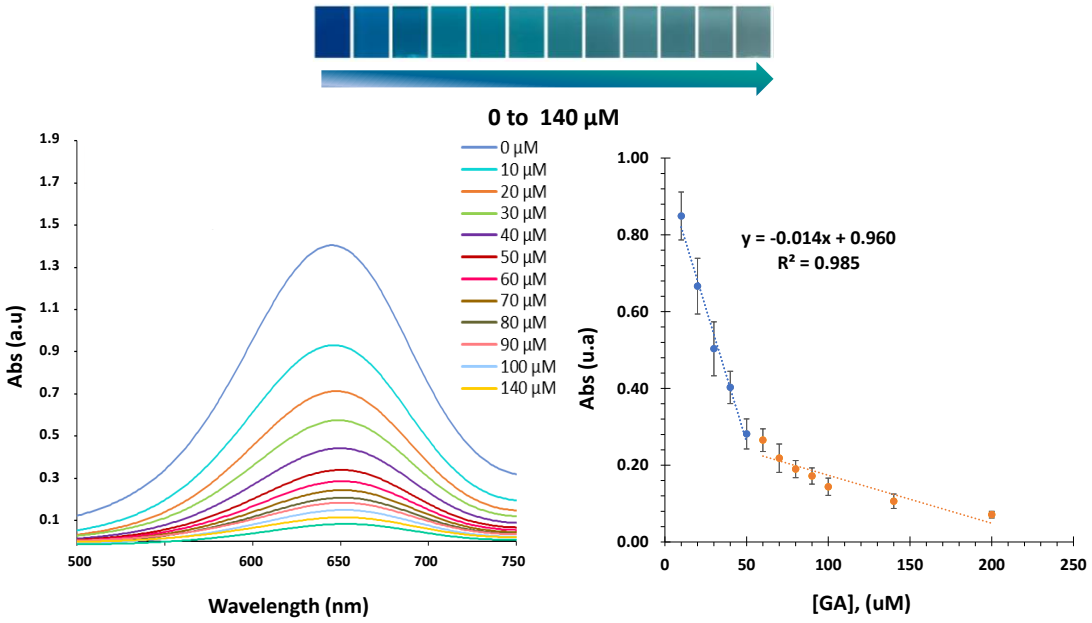
The catalytic activity of HRP in the TMB oxidation reaction was investigated in the presence of different polyphenols, and the respective absorbance was monitored using UV-Vis spectroscopy. First, the tests were performed as described in the study **[64]** using TMB (816 µmol/L), H_2O_2 (8.8 mmol/L), and decreasing concentrations of HRP (0.01, 0.005, and 0.0025 µg/mL) in a medium with pH value between 3.5 and 4.5 and temperatures between 25 and 37 °C. In these assays, the HRP reduces the hydrogen peroxide and leads to the formation of •HO that oxidizes TMB, which in this oxidative state displays a blue colour. At the different HRP concentrations tested under the pH value 4 and room temperature (0.01, 0.005, and 0.0025 µg/mL), the assay showed minimal blue colour development with very low absorbance of 0.06, 0.04, and 0.03 a.u., respectively. The maximum absorbance achieved (0.06 a.u.) was way below the value required for future assessments of the antioxidant capacity of phenolic compounds in the same medium **(Figure S1).** Due to the low absorbance values, slight modifications to the reaction conditions were made. The pH value and temperature were kept at 4 and room temperature (22 °C), although TMB and H_2O_2 concentrations were 2-fold decreased to 400 µmol/L and 4 mmol/L, respectively. Higher concentrations of HRP (0.02, 0.03, and 0.06 µg/mL) were also tested. The absorbance results under these conditions were 0.80, 1.19, and 2.31 a.u., respectively. For the last measurement, absorbance is above 2 a.u.

indicating that the relationship between sample concentration and maximum absorbance peak is no longer linear. In this scenario, the absorbance values obtained with the modifications enabled the increase in absorption, which was compatible with the levels required for further experiments. Thus, it was decided to perform the antioxidant capacity assays with an HRP concentration of 0.03 µg/ml, given the high blue colour intensity with absorption values below 2 a.u.. The reason for the maximum absorbance threshold is related to the measurement noise of the equipment above 1.5 a.u., which could lead to uncertainties in the final result. The optimal concentrations in the reaction to maximize the colourimetric signal were: 400 µmol/L of TMB, 4 mmol/L of H_2O_2, and 0.03 µg/mL HRP. After determining the optimal concentration of the reaction components, an evaluation of the absorbance over time was performed to assess colour stability of reduced TMB. The reaction mixture was analysed for 10 minutes, results shown in **Figure S2**. In the first 5 min, the absorbance showed an increasing tendency which reached a stable plateau. The data also indicates that a reaction time of 2 min was enough to obtain absorbance values higher than 1.5 a.u. and with smaller variations. However, after 3 min particle formation started to be observed, which directly affects the evaluation of the absorbance by increasing scattering.

3.2 Colorimetric Determination of Antioxidant Capacity of Phenolic Compounds - HRP

3.2.1 Gallic Acid Units

The polyphenols GA and TA were selected as standard phenolic compounds given their high prevalence in various foods. The significance of assessing the antioxidant content in products lies in the fact that even though antioxidants possess excellent health-promoting properties, when present in excessive concentrations, they can induce the opposite effect. Therefore, an approach that allows the detection in the µM range is warranted. The HRP is one of the most commonly used enzymes capable of oxidizing phenolic compounds with the advantage that when compared with other enzymes, it is first reduced by polyphenols and only then oxidized by hydrogen peroxide [52] In this part of the study, the phenolic content was determined in the presence of HRP. The concentration range of 10-200 µmol/L of GA was tested and the evolution of absorbance values was monitored at the 2 min time point, as shown in the spectra in **Figure 3A.**



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300 **Figure 3** - Absorption spectra (A) for the reaction containing HRP and GA, and the calibration plot (B) for
301 colorimetric determination of GA in the concentration range from 10 to 200 µmol/L (linear fitting for
302 concentrations range 0-50 µmol/L, in blue).

303 The spectra showed a maximum absorbance peak at 652 nm. Data clearly show a decrease in
304 the maximum absorbance with the increasing concentration of GA due to the antioxidant
305 capacity of this compound. The enzymatic degradation of hydrogen peroxide by the HRP leads
306 to free radicals that oxidize the aromatic amine of TMB, although the presence of AG blocks
307 the formation of oxidized TMB with a blue colour. Thus, increasing GA concentration induces a
308 smaller amount of oxidized TMB and thus a blue colour intensity decreases, as shown in **Figure**
309 **3** on the top. The blue colour is inversely proportional to the concentration of the phenolic
310 compound. The behaviour displays two linear trends, one for concentrations ranging from 0-50
311 and the other from 50 to 200 µmol/L. A linear fit was established between the maximum
312 absorbance peak, at 652 nm, and the concentration of GA for the lower concentration range,
313 0-50 µmol/L **(Figure 3-B)**, with a linear equation Y= -0.0140×+0.960, with an R^2 of 0.985 and a
314 LOD of 6.6 µmol/L.

315 ***3.2.1 Tannic Acid Units***

316 The same approach was repeated for TA with a concentration range of 0.5 -10 µmol/L. The
317 reaction behaviour was also evaluated based on the absorbances of the spectra shown in
318 **Figure 4A**. Considering that the same chromogenic substrate, TMB, was used the maximum
319 absorbance peaks at 652 nm were collected. Similarly to GA, the TA showed decreasing

absorbance intensity for increasing AT concentrations. The most intense blue colour was recorded for the blank, with no TA added, as can be seen in the colour gradient of **Figure 4** on top.

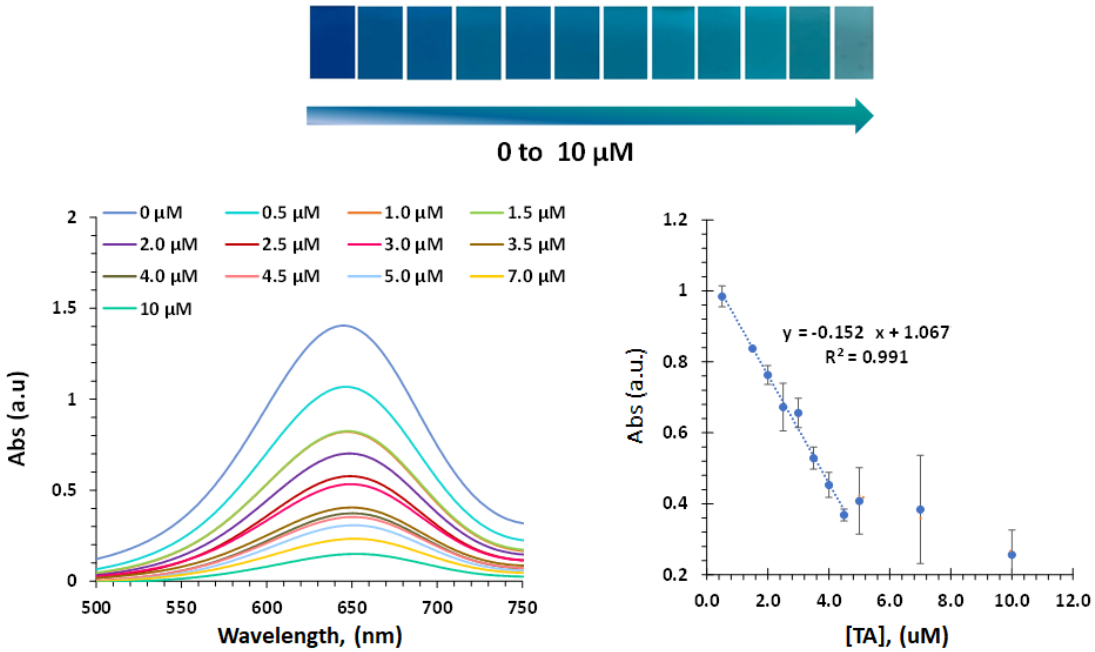


Figure 4- Absorption spectra (A) for the reaction with HRP and TA, and the calibration plot (B) for colorimetric determination of TA from 0.5 to 10 µmol/L.

As expected, TA acts as an antioxidant and induces a significant reduction in the oxidation of TMB and consequently in the intensity of the blue colour. In contrast, samples with lower TA concentrations showed higher blue colour intensities. The correlation between the absorbance peak at 652 nm and the increasing concentrations of TA (**Figure 4B**), was plotted showing a linear fitting with the equation Y= -0.152× + 1.067 and an R^2 of 0.991 and a LOD of 0.41 µmol/L.

The HRP assays with GA and TA displayed similar responses, although a 10-fold sensitivity increase was obtained for the TA, which also enabled approximately a 16-fold smaller LOD. These data indicate that the two single-component polyphenol standards exhibit distinct antioxidant capacities.

3.4 Colorimetric Determination of Antioxidant Capacity of Phenolic Compounds – rGO/PBNCs

3.4.1 Gallic Acid Units

The goal of this work was to replace the typically used HRP with an artificial enzyme in colorimetric-based methods to achieve improved stability, activity, sensitivity, and reproducibility. Thus, we replicate the approach presented in sections 3.2 and 3.3, now using the synthesised nanoenzyme, rGO/PBNCs. Artificial enzymes have advantages over natural enzymes as its easy for large-scale production and their lower cost.

First, to determine if the colorimetric response was triggered in the presence of the artificial enzyme rGO/PBNCs different medium conditions were tested. The combination of PBS, rGO/PBNCs, H_2O_2, and TMB successfully resulted in the oxidation of TMB and the formation of the blue colour (**Figure S3.A**). Controls without H_2O_2, rGO/PBNCs, and both were carried out, showing no colorimetric response (solution remained transparent), as shown in **Figure S3**. When H_2O_2 was excluded from the reaction, the oxidation of TMB could not occur due to the lack of free radicals formation (**Figure S3.B**). In the absence of rGO/PBNCs, there was no catalytic activity and the oxidation of TMB was significantly slower, therefore no colorimetric response occurred for the same reaction time (**Figure S3.C**). As expected, the exclusion of both H_2O_2 and rGO/PBNCs from the reaction also prevented TMB oxidation, and no colorimetric response was observed (**Figure S3.D**). These data confirmed that the artificial enzyme behaved similarly to HRP, thus further studies with different polyphenols were carried out.

In the case of GA, the same working concentration range from 10 to 200 µmol/L was considered, details described in section 2.5.2. The absorbance values with increasing concentrations of GA were evaluated and the results were displayed in **Figure 5A.** The obtained colorimetric responses decrease the blue shade intensity with increasing concentrations of GA as shown in **Figure 5** on the top.

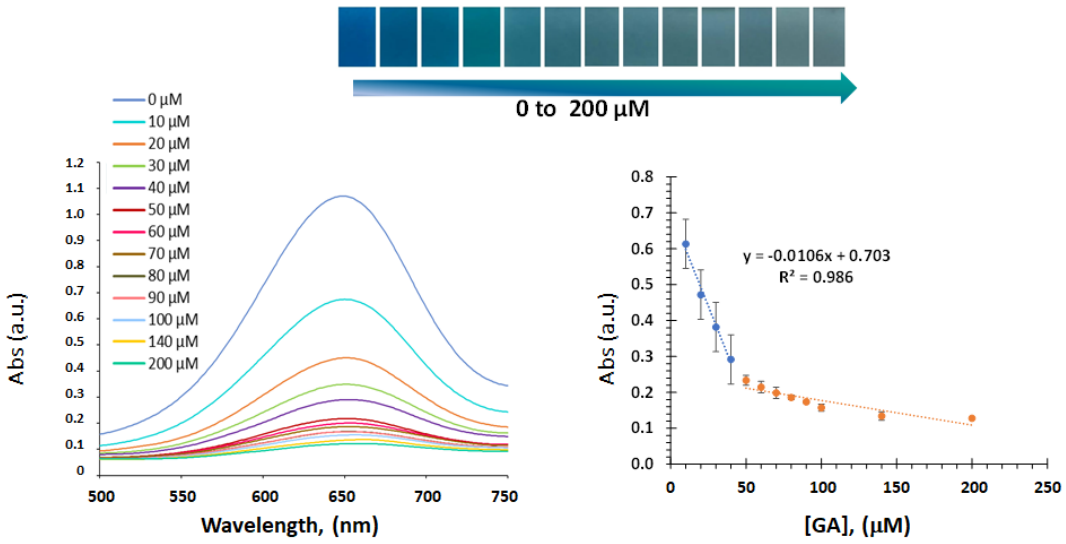


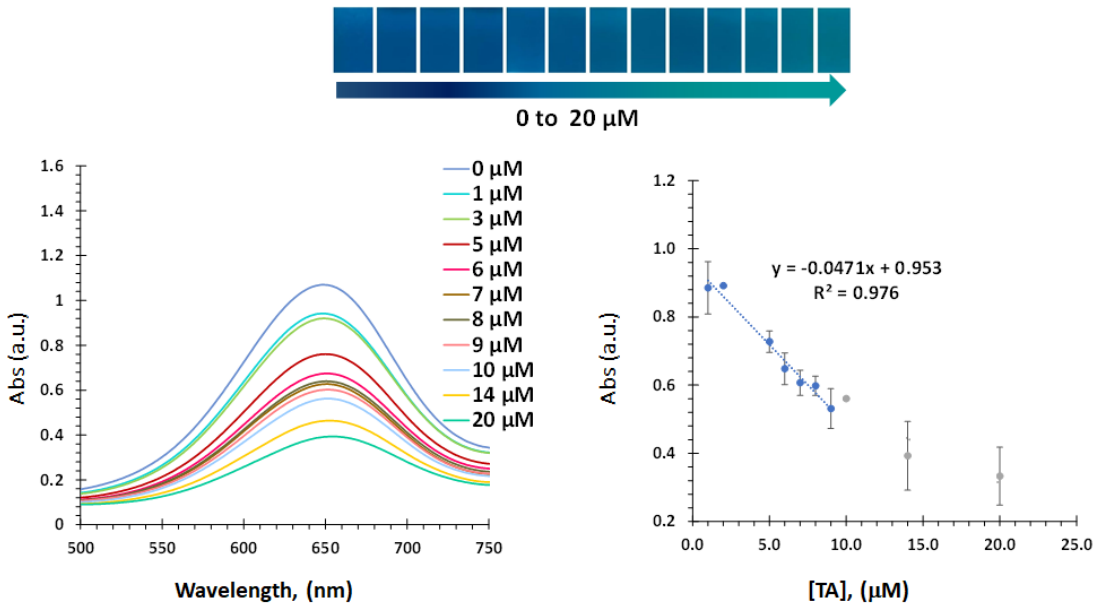
Figure 5- Absorption spectra (A) for the reaction with rGO/PBNCs and GA, and calibration plot (B) for colorimetric determination of GA from 10 to 200 µmol/L (linear fitting for concentrations range 10-50 µmol/L, in blue).

The data confirms that by increasing GA concentration, at 652 nm, the oxidation of TMB is decreased due to GA antioxidant capacity, leading to a decrease in the blue colour intensity. A linear relationship between the maximum absorbance and the GA concentrations was also observed although a plateau was reached at approximately 50 µmol/L **(Figure 5B)**. The linear fitting resulted in the following equation Y=-0.0106× + 0.703 with an R^2 of 0.986 and an LOD of 5.6 µmol/L. When compared to the assay using HRP and GA, the system using the artificial enzyme rGO/PBNCs enables to slight decrease in LOD and sensitivity indicating that the synthesized artificial enzyme is capable of performing even better than the natural enzyme.

3.4.2 Tannic Acid Units

Similarly, this study was conducted with TA and the artificial enzyme rGO/PBNCs. In this case, the TA concentration range was expanded, increasing the upper concentration range from 1.0 -20 µmol/L. The colorimetric signal was monitored based on the maximum absorbance, as shown in the spectra and colour gradient of **Figure 6A** and **top**. As previously, the maximum absorbance decreases with increasing TA concentration, consequently decreasing the intensity of the blue colour of the solution. The assay with TA and the artificial enzyme rGO/PBNCs also showed a linear relationship with increasing TA concentrations, **Figure 6B**, providing the linear equation Y= -0.0471× + 0.9539 with an R^2 of 0.977 and an LOD of 1.51 µmol/L.

In this assay, a larger linear working concentration range was obtained when compared to the equivalent assays using HRP, spanning from 0.1 - 4.5 to 0.1 - 9, respectively. Even though the LoD is slightly higher, it is compensated by the broader working range, which is capable of monitor a larger spectrum of concentrations, thereby providing a significant advantage when testing samples with a wide array of polyphenol content. Nevertheless, the assays with both standard polyphenols showed that the artificial enzyme nanomaterial mimicked the natural enzyme HRP and its intrinsic enzyme-like catalytic activity.



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390 **Figure 6** - Absorption spectra (A) for the reaction with rGO/PBNCa and TA, and linear calibration plot (B)
391 for colorimetric determination of TA from 1.0 to 20 µmol/L.

392 **3.5 Analysis of Real Samples using HRP and rGO/PBNCs**

393 The method developed allows a meaningful assay that provides insights into the antioxidant
394 capacity and indirectly the phenolic content by both HRP and rGO/PBNCs. Thus, as a proof-of-
395 concept the developed method was applied to the analysis of commercial beverages.
396 Commercial beverages have in their compositions different phenolic compounds that
397 contribute as a whole to the total antioxidant capacity. In this case, the samples were assessed
398 as GA and TA equivalents.

399 It is important to emphasise that most foods/beverages have more than a single phenolic
400 compound, which typically includes a mixture that may not have been fully characterised.
401 Furthermore, the same food can often contain different polyphenol compositions within its
402 different species. In addition to this variability, other factors can also influence the phenolic
403 content in foods, such as the degree of ripeness at the time of harvest, environmental factors,
404 processing, and storage. Due to the large number of polyphenols present and the considerable
405 number of factors that can alter their concentrations, comprehensive food composition tables
406 on polyphenol concentrations have not yet been produced. Only part of the information based
407 on direct food analyses is publicly available [53].

408 Therefore, the methods described in sections 2.6.1 and 2.6.2 were used to characterise the
409 samples. According to the results of the HRP method, the beverages with the highest levels of

phenolic compounds (GA and TA) were red wine and green tea (**Figure 7**). This was an expected result as these types of beverages generally have high tannin concentrations and consequently increased antioxidant capacity. The results for the white wine sample showed lower phenolic compound content. This is an expected result considering that white wine production often discards skins, seeds, and stems of the grapes, which have high polyphenol content. For the other samples low polyphenol content was also observed.

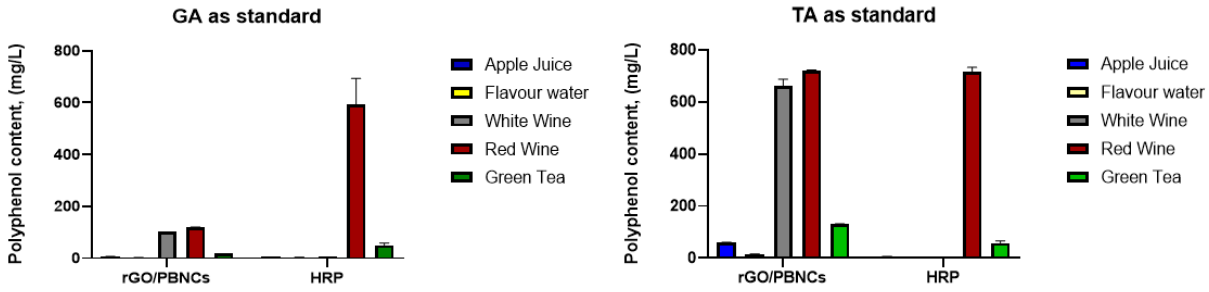

Figure 7 - Phenolic compound concentration levels expressed in terms of GA content (mg GA/L) [left]; and in terms of TA (mg TA/L) [right] using the rGO/PBNCs and HRP-based methods for for different commercial beverages.

The results of the samples analysed by the rGO/PBNCs seemed to show a considerably higher variability between the analyses based on GA and TA, which suggests that a statistical analysis should be considered. The results also highlighted the influence of the polyphenol of reference in the quantification of polyphenol content due to their distinct chemical structures and reactivity.

For a deeper understanding of the results, statistical analyses were employed. Firstly, the variability among the different beverage samples for each method for both TA and GA was assessed through the Kruskal-Wallis test ($P < 0.05$), a non-parametric alternative to the one-way analysis of variance (ANOVA). The results indicate that the HRP method using the TA and GA, showed no significant difference in polyphenolic content except for the Flavoured-water and the red wine samples. The same was observed for the rGO/PBNCs for TA, whereas for GA there was no significant difference in polyphenolic content for all the tested samples. Additionally, disparities between the two analytical methods were investigated using the Wilcoxon signed-rank tests. The Wilcoxon analysis is well-suited for comparing paired data, making it an appropriate choice for assessing the impact of the different methods used for polyphenol quantification. The comparison of the two methods with the two polyphenol references showed no significant differences among them for the analysis of commercial

beverage (rGO/PBNCs vs HRP for GA, P = 0.8125; rGO/PBNCs vs HRP for TA, P = 0.0625, rGO/PBNCs for GA compared to TA P = 0.0625 and HRP for GA compared to TA, P = 0.0625). The data obtained with the proposed method is comparable with the standard HRP method, with samples accounting for 72.8% of the total variance while the method only 7.3%.

The statistical analysis provided comprehensive insights into both the inter-sample variation and the methodological differences indicating that the analysis of these samples using the rGO/PBNCs was successful and this new material can be a great alternative to natural enzymes.

Table 1 - Comparison of the methods to estimate the total polyphenol content

METHOD	STANDARD POLYPHENOL	DETECTION LIMIT	REFERENCE
Modified Ferric reducing/antioxidant power) assay (FRAP)	GA TA	180 and 142 mmol/L (for 4 and 30 min) (30.62 and 24.15 μg/mL) 160 (4 min), 66 (30 min) mmol/L (272.19 and 112.28 μg/mL)	**33**
Capillary electrophoresis connected to a diode array detector	Tyrosol Hydroxytyrosol 4-HFA acid Sinapic acid Gentisic Acid Luteolin	0.666 μg/mL 0.307 μg/mL 0.241 μg/mL 0.484 μg/mL 0.291 μg/mL 0.300 μg/mL	**25**
Folin–Ciocalteu's assay*	Tannin	0.39 mg/mL	**28**
Folin–Denis and Folin–Ciocalteu assays*	gallic acid (-)-epicatechin Rutin	10 μg/mL 10 μg/mL 20 μg/mL	**30**
Peroxidase based biosensor	chlorogenic acid	0.7 μmol/L (0.24 μg/mL)	**55**
Improved Folin-Ciocalteu (FC)* and Prussian Blue (PB) assays for small volumes	Gallic acid	PB = 0.27 μg/mL FC = 0.25 μg/mL	**56**

Modified Fast Blue BB diazonium salt	Chlorogenic acid	<10 µg/mL	**57**
rGO/PBNCs	GA TA	5.60 µmol/L (0.95 µg/mL) 1.51 µmol/L (2.56 µg/mL)	**This work**

*Folin-Ciocalteu assays have reported LOD between 0.006 - 14.2 µg/mL, depending on the calibration and standard used [58]

The proposed rGO/PBNCs-based method exhibits a sensitivity within the same order of concentration as traditional methods, affirming its suitability for successful application for total polyphenol estimation in beverages.

Then the samples were analysed using this method. The phenolic content found in the samples is below the content reported in the literature for the same types of commercial beverages: apple juice ±339 mg equiv. phenolic compound/L; green tea 61-220 mg equiv. phenolic compound/g dry weight; beer 452-875 mg equiv. phenolic compound/L; white wine 191-296 mg equiv. phenolic compound/L; red wine ±1615 mg equiv. phenolic compound/L [54]. Even though the samples seem similar in terms of composition they can differ significantly. Several factors could cause these differences, such as slight time of self, storage conditions, and different maturation of matrix products. This is especially relevant, considering that phenolic compound content is sensitive to such variations, thus for comparison, it is crucial to ensure that samples are analysed simultaneously and with the same method reducing inherent errors from general comparisons.

4. Conclusion

The aim of this study was to develop an enzymatic colourimetric method for the evaluation of phenolic compounds in commercial beverages and to replicate it with an artificial enzyme. The experimental parameters were initially based on literature studies and were later optimised to meet the specific requirements. A pH of 4, room temperature and a reaction time of 2 minutes were set as optimal conditions for both methods. Concentrations of 0.4 mmol/L H2O2 and 4 mmol/L TMB were found to be optimal, with an enzyme concentration of 0.03 µg/mL for the HRP method. The developed system successfully detected phenolic compounds with LODs of 6.62 µmol/L and 0.41 µmol/L .

In the rGO/PBNCs method, the selected nanomaterial showed intrinsic HRP-like activity, which was confirmed by colour development in the presence of enzyme and H_2O_2. A concentration of 0.01 µg/ml was used for rGO/PBNCs, with reaction conditions similar to those of the HRP

method. The phenolic compounds were determined with LODs of 5.6 µmol/L for GA and 1.5 µmol/L for TA respectively.

The results obtained in the evaluation of commercial beverages with these methods are promising for future practical applications in the determination of phenolic compounds. However, further optimisation of the methodology is required, especially for sample evaluation with rGO/PBNCs. A valuable addition to the method development would also be the evaluation of the kinetic parameters using the Michaelis-Menten constants for the substrates TMB and H2O2 to evaluate the affinity of rGO/PBNCs and compare them with HRP.

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