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Functionalization of micropipette tips with hydrophobin-laccase chimera and application to the electrochemical determination of caffeic acid in tea samples

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ABSTRACT

Performing on-site measurements is one of the main challenges of current (bio)chemical analysis. Micropipette tips, which are among the most common utilities in laboratories, can be useful and cheap elements for designing out-of-the-box innovative analytical biodevices. Furthermore, most of the analytical properties of these bioplatforms depend on the immobilization of the recognition element (e.g., enzymes). In this context, we have recently designed a chimeric protein in which laccase PoxA1b from the fungus *Pleurotus ostreatus* has been genetically fused to the class I hydrophobin Vmh2. The ability to self-assemble at hydrophobic-hydrophilic interfaces of hydrophobin, allows an easy laccase immobilization. In this work, the Vmh2-PoxA1b fusion protein was immobilized into common polypropylene micropipette tips to develop an innovative enzymatic bioplatform that can produce fast results in a simple way. As proof of applicability, the platform was applied to the determination of caffeic acid by enzymatic oxidation and further electrochemical reduction of the product on a screen-printed electrode, achieving a limit of detection of $1.4{\cdot}10^{-6}$ M. It was then used for determining caffeic acid in tea samples.

1. Introduction

The development of analytical devices that combine high sensitivity, low-cost instrumentation and fast response is still a challenge. Portable and easy-to-use devices can offer several advantages in many application fields if compared to conventional techniques that often are time-consuming and expensive in terms of instrumentation. Thanks to the possibility of creating miniaturized electrochemical cells with cheap materials, electrochemical sensors are representing an interesting alternative to the traditional methods. These devices, such as those based on the use of screen-printed electrodes (SPEs), require a small volume of sample and are often single use. This avoids electrode fouling (Weese et al., 2019) and crossed contamination between samples and, moreover, their mass-production increases the precision of the measurement. As regards the use of cheap materials for the fabrication of these devices, for instance, many paper-based biosensors (Bordbar et al., 2021) have been developed. Indeed, paper materials allow to overcome some limitations through their valuable advantages such as disposability, flexibility, large surface-to-volume ratio, and biocompatibility. When these characteristics are combined with those of bioreceptors (e.g., DNA, antibodies or enzymes), selective and sensitive biosensors can be obtained (Chen & Shamsi, 2017). Indeed, due to the specificity of the interaction bioreceptor/analyte, detection in complex matrices can be approached.

Commercial tips represent another interesting cheap material largely used in the daily life of laboratories. Micropipette tips allow to work accurately with low sample volume, and, thanks to their low-cost production, they can be discarded to avoid contamination. Apart from dispensing liquids, their use in other out-of-box applications has also been reported. Thus, they have been used to perform electrochemical measurements acting as containers of electrolytes or electrodes (da Silva et al., 2010) or as part of the body of working or reference electrodes (Sharma et al., 2018). Furthermore, they were filled with resins to purify and concentrate proteins, peptides, or oligonucleotides prior to mass

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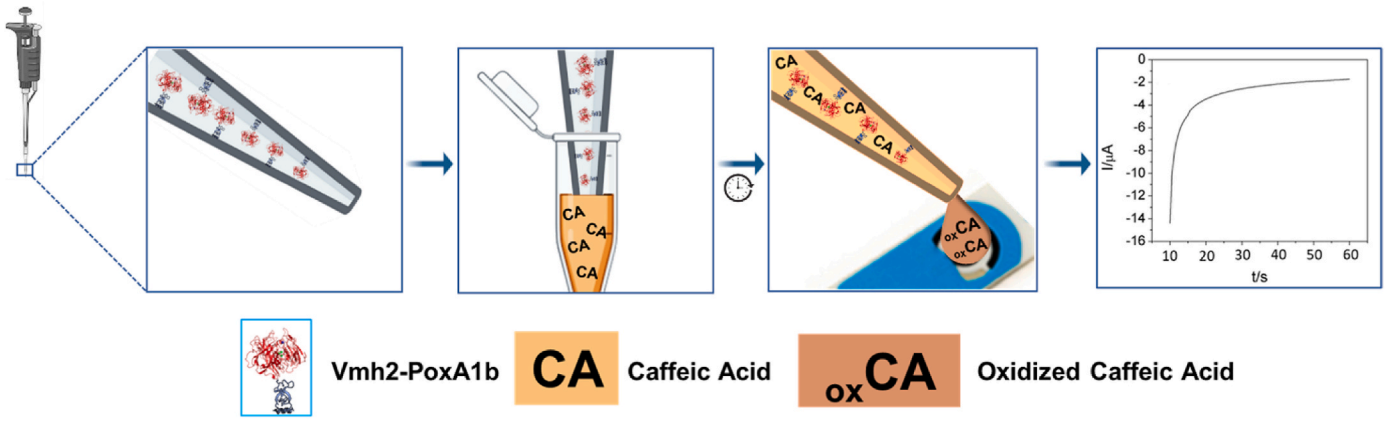

Fig. 1. Schematic representation of the developed system.

spectrometric analysis (Al-Majdoub et al., 2013). In these cases, the analyte was not bound to the tip and was directly dispensed for further determination. Instead, recently, in a very innovative way, a low-cost paper-based platform has been combined with functionalized micropipette tips to diagnose celiac disease (González-López et al., 2019). Here, immobilization of transglutaminase enzyme into commercial tips converted them into the scaffold in which an immunoassay took place (González-López et al., 2019). This paves the way for their employment in the fabrication of analytical devices to detect a plethora of analytes only by changing the biomolecules immobilized into the tips.

Enzymes are bioreceptors widely exploited in biosensors (Bollella, 2021) due to their capability to specifically react with an analyte. In the case of electrochemical biosensors, redox enzymes, such as peroxidases, glucose oxidases and laccases, have been widely used since they are directly involved in electron transfer reactions. Thus, they have been largely applied in many fields ranging from medical and clinical diagnostics to environmental monitoring (Hosu et al., 2017).

Laccases (*p*-diphenol-dioxygenoxidoreductases) are oxidoreductases produced by fungi, plants, and bacteria, that catalyze the oxidation of a wide range of phenolic compounds together with the simultaneous reduction of molecular oxygen to water. The presence of four copper ions in their active site are responsible for the electron transfer. These unique characteristics allow their use in several application fields such as agricultural, industrial, or environmental. In many cases, the electrochemical detection of phenolic and aromatic compounds can be performed by laccase-based bioplatforms (Albayati et al., 2019). However, one of the critical steps in the development of biosensors based on the use of enzymes is related to their immobilization (Tortolini et al., 2012). This process should ensure a good stability of the enzyme and, at the same time, provide a good accessibility of substrates to the active site. Until now, laccase immobilization has been performed through physical adsorption (Cabaj et al., 2010), covalent binding after chemical derivatization of the surfaces (Fernandes et al., 2007; Klis et al., n.d.), or entrapment into a polymer matrix (Zhang et al., 2022).

To further enhance laccase immobilization and to develop a more biocompatible and less time-consuming strategy, we have recently designed a chimeric protein in which the laccase PoxA1b from the fungus *P. ostreatus* has been genetically fused to the class I hydrophobin Vmh2 produced by the same microorganism. The amphiphilic nature of the hydrophobin and its ability to self-assemble at hydrophobic-hydrophilic interfaces have been exploited to allow laccase immobilization on several surfaces. Among them, polystyrene (Sorrentino et al., 2019a), few-layer graphene (Sorrentino et al., 2020) and carbon nanotubes (Sorrentino et al., 2021) have been functionalized with the fusion protein to develop optical and electrochemical biosensors. However, the research of new and innovative surfaces for laccase immobilization is still in progress. Herein, for the first time, the adhesive capability, and the enzymatic activity of Vmh2-PoxA1b fusion protein have been exploited to immobilize the chimeric protein into common polypropylene tips to develop an innovative and easy-to-use electroanalytical system. Then, taking advantage of the enzymatic oxidation of caffeic acid (CA) and the subsequent electrochemical reduction of the product, the developed system has been used, as a proof of concept, to detect the antioxidant molecule in tea samples, with high content of phenolic compounds (Fig. 1).

CA, a powerful antioxidant present in tea leaves, was chosen as model analyte to be determined in real samples. CA is the most abundant hydroxycinnamic acid especially in fruits (Guo et al., 2017), alcoholic and non-alcoholic beverages (Nehru et al., 2020), tea, chocolate (Bhagat et al., 2019) and so on. As a polyphenol, it is used as anti-inflammatory and antibacterial agent in medical applications (Kang et al., 2009; Nasr Bouzaiene et al., 2015), while in food industry it is widely employed to maintain the color stability and to protect beverages from oxidative deterioration (García-Guzmán et al., 2019; Oliveira-Neto et al., 2016). Nonetheless, an excessive usage of CA can cause opposite effects on human health (Thangavelu et al., 2017) with carcinogenic and genotoxic effects. Thus, the development of simple methods for its decentralized detection is of great interest.

2. Experimental section

2.1. Recombinant production of the chimeric protein Vmh2-PoxA1b

The recombinant production and secretion system of the *Pichia pastoris* yeast was exploited to express the fusion protein Vmh2-PoxA1b in which both proteins were linked with a thrombin cleavage site. As already described (Sorrentino et al., 2019), the gene sequence was optimized according to *P. pastoris* codon usage and synthesized by Thermo Fischer Scientific (Waltham, Massachusetts, USA). Then, it was cloned into the pJGG_αkR vector, in frame with the sequence for the α factor (signal peptide) under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGG_Vmh2-POXA1b. Thus, the recombinant clones were inoculated from solid culture in 20 mL of BMMY medium (13 $g{\cdot}L^{-1}$ yeast nitrogen base with ammonium sulfate without amino acids; 10 $g{\cdot}L^{-1}$ yeast extract; 20 $g{\cdot}L^{-1}$ peptone; 100 mM potassium phosphate, pH 6.0; $4{\cdot}10^{-4}$ $g{\cdot}L^{-1}$ biotin; 0.5% methanol) in a 100 mL shaken flask. These precultures were grown for 3 days at 28°C on a rotary shaker (220 rpm). Then, in 250 mL baffled shaken flasks containing 50 mL of BMMY medium, with addition of 0.6 mM $CuSO_4$, a volume of suspension sufficient to reach a final value of 0.5 OD600 mL^{-1} was inoculated. After 7 days, the fusion protein was recovered in the culture broth following a centrifugation step to remove the cells. Then, proteins were dialyzed towards 50 mM Tris-HCl buffer, pH 8.0, using Centricon Centrifugal Filter Units (Vivaspin, Sartorius Lab Instruments, Germany) with a 10 kDa Polyethersulfone (PES) membrane. According to the protocol of Pierce 660 Protein assay (Thermo Fischer Scientific,

Waltham, Massachusetts, USA), the total protein concentration was determined using bovine serum albumin (BSA) as standard protein.

2.2. Laccase activity

The enzyme activity was assayed monitoring the oxidation of 2 mM ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (VWR International, Radnor, Pennsylvania, USA) at 420 nm ($\varepsilon_{420nm} = 3.6{\cdot}10^4$ $M^{-1}{\cdot}cm^{-1}$), dissolving the substrate powder in 50 mM sodium citrate buffer pH 5.0.

2.3. Immobilization of Vmh2-PoxA1b in polypropylene tips

The optimized protocol for the Vmh2-PoxA1b immobilization consists in the aspiration of 40 μL of 2.5 $U{\cdot}mL^{-1}$ of chimera solution (100 mU) into polypropylene tips (2–200 μL volume range). Then, the lower end of the tip was capped with Parafilm® and samples were left to dry at 37°C. As a control, the same experiment was performed with free laccase PoxA1b. Then, after dried, the tips were subjected to three washes with 50 mM Tris-HCl buffer, pH 8.0 and the immobilization yield inside each tip was calculated as the difference between the deposited units (mU) and those released after the three washes.

2.4. Electrochemical measurements of caffeic acid solutions

A 100 mM acetate buffer solution pH 5.0 was used to prepare caffeic acid (CA) standards. For the pH study, Britton-Robinson (BR) buffer solutions were used but without adding boric acid (i.e., prepared by mixing 0.04 M H_3PO_4 and 0.04 M CH_3COOH solutions and taking to the desired pH with 0.2 M NaOH). The use of boric acid was avoided due to the formation of catechol–boric acid complexes that can modify the analyte redox behavior (Rafiee & Nematollahi, 2008).

With the aim of defining the right potential to perform chronoamperometric measurements, the electrochemical behavior of the CA was studied by cyclic voltammetry (CV). A volume of 40 μL (García-González et al., 2015; Rama et al., 2012) of the antioxidant solutions (1.0, 2.5 and $5.0{\cdot}10^{-3}$ mM) was carefully deposited on a DRP-110 screen printed electrode (SPE) cell (Methrohm DropSens, Oviedo, Spain) covering completely the surface of the three electrodes. Voltammograms were recorded in the potential range comprised from −0.4 to +1.3 V (Bounegru & Apetrei, 2020) with a scan rate of 0.05 $V{\cdot}s^{-1}$. For the determination of CA, 40 μL of solutions with different concentrations of CA were aspirated into tips functionalized with Vmh2-PoxA1b and let react for a fixed time to perform the enzymatic oxidation of CA. Then, the solution containing the reaction product was carefully deposited on a SPE, covering completely the surface of the three electrodes, and a chronoamperometric measurement (i-t curve) was performed at −0.25 V for 60 s to record the reduction of the oxidized form of CA. The analytical signal was the average of the current recorded in the range of 45–50 s. To compare the performance of the Vmh2-PoxA1b chimera and the free enzyme, the same experiments were carried out with PoxA1b functionalized tips.

2.5. Preparation of tea samples

CA was determined in two different commercial tea samples (black and green). The sample treatment was adapted from reported protocols (David et al., 2015; Pradela-Filho et al., 2020): 1.5 g of tea leaf samples were infused in 25 mL of boiled 100 mM acetate buffer at pH 5.0 for 20 min and after cooling, they were diluted up to 50 mL with the same buffer. Then, tea samples (30 μL of black tea or 12 μL of the green one) were further diluted to a final volume of 5 mL with 100 mM acetate buffer at pH 5.0.

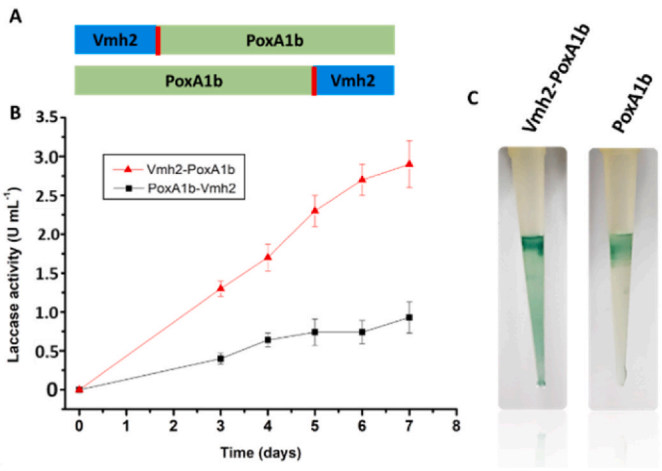

Fig. 2. (A) Schematic representation of gene constructs (in blue the hydrophobin, in red the thrombin cleavage site, in green the laccase); (B) Comparison of the activity curve of Vmh2-PoXa1b (black dot) and PoxA1b-Vmh2 (red triangle); (C) Photograph of tips functionalized with Vmh2-PoXa1b and PoxA1b after 1 min of reaction with 2 mM ABTS.

2.6. Determination of CA in real samples

The electrochemical determination of the antioxidant in both tea types was carried out by adding to the samples the necessary volume to obtain a CA concentration of 10^{-5} M. Also in this case, 40 μL of each sample were aspirated into the tips functionalized with the chimeric protein and, after 1 min of reaction, the solution was dropped on the SPE performing the chronoamperometric measurement as reported above. Three replicates were performed.

3. Results and discussion

3.1. Production and immobilization of Vmh2-PoxA1b

The intrinsic characteristics of the fusion protein allowed us to avoid any kind of purification step. Indeed, not only the enzymatic activity gave us the possibility to specifically detect the production of the chimera during the cell growth, furthermore the adhesive capability of the hydrophobin permitted its adhesion on several surfaces (Sorrentino et al., 2019b, 2020) and washing out of other proteins. The recombinant production of the chimeric protein Vmh2-PoxA1b was performed in the *P. pastoris* yeast exploiting its capability to easily secrete the proteins in the culture medium. This expression system was already used to produce the chimera PoxA1b-Vmh2 (Sorrentino et al., 2019) with the enzyme laccase at the N-terminus of the fusion protein (Fig. 2A).

The idea of designing a new protein with inverted moieties arose from previous experiments that showed a different production yield depending on the relative position of the hydrophobin (Puopolo et al., 2021; Stanzione et al., 2021), probably due to a different propensity to aggregation. Indeed, also in this case, the enzyme activity of Vmh2-PoxA1b assayed in the culture broth after 7 days was 2.8 ± 0.2 $U{\cdot}mL^{-1}$, about three-fold higher than that of PoxA1b-Vmh2 (0.8 ± 0.2 $U{\cdot}mL^{-1}$) (Fig. 2B).

Otherwise, the ability of the two chimeric proteins to adhere on polystyrene multiwell plates was very similar, obtaining comparable immobilization yields (85–90%). Nonetheless, to be sure that the adhesion of the chimera was related to the presence of the Vmh2 moiety, the free enzyme PoxA1b was used as a control, since its adhesion on hydrophobic surfaces has been previously observed (Sorrentino et al., 2019; Sorrentino et al., 2020) even if yielding a lower immobilization (35–40%). Thus, considering these results, we decided to use the Vmh2-PoxA1b chimera for further experiments, exploiting its higher

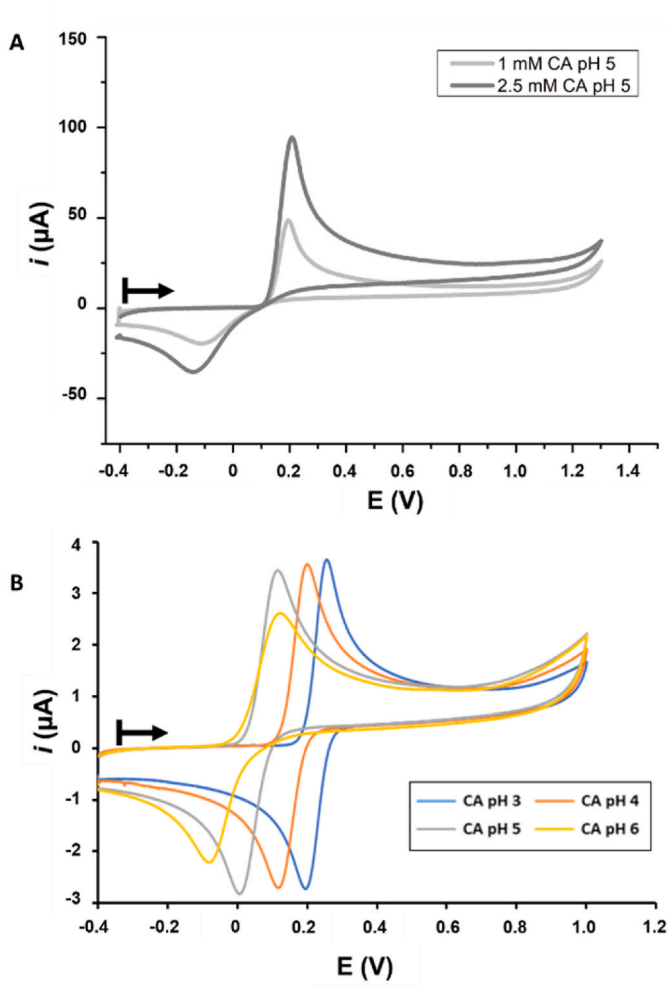

Fig. 3. (A) Cyclic voltammograms of CA solutions of different concentration in 100 mM acetate buffer pH 5.0. Scan rate: 100 mV·s^{-1}; (B) Cyclic voltammograms of 0.1 mM CA solutions (in BR buffer without boric acid) at different pH values. The black arrow indicates the direction of the scan.

production in the culture broth.

The employment of common polypropylene tips as surface for protein immobilization is based on a wide range of advantages related to their characteristics. Indeed, these tips are not only a very cheap material, commonly used in laboratories, but also the existence of different sizes (e.g., 0.5–10 μL, 20–200 μL or 200–1000 μL) can allow their use for many applications. This would permit changing, for example, the amount of immobilized protein. Thus, protein solutions were aspirated into the tips and let dry. After abundant washes, the obtained immobilization yields were 48 ± 2% for Vmh2-PoxA1b and 21 ± 2% for the free enzyme, which can be qualitatively observed by the naked-eye (Fig. 2C). As expected, PoxA1b adhered on polypropylene surface, although with a lower value of immobilization yield.

3.2. Electrochemical detection of caffeic acid with functionalized tips

With the aim of developing an electrochemical platform for detection of CA, the electrochemical behavior of the molecule was studied. Cyclic voltammograms of CA solutions (10^{-3} and $2.5{\cdot}10^{-3}$ M) at pH 5.0 were recorded, after depositing a drop on a SPE, in a wide potential range. As shown in Fig. 3A, the voltammogram recorded for 1 mM CA solution in acetate buffer pH 5.0 presents two processes (anodic and cathodic at +0.19 V and −0.10 V respectively), which were related to the oxidation of caffeic acid and further reduction of the product generated at the

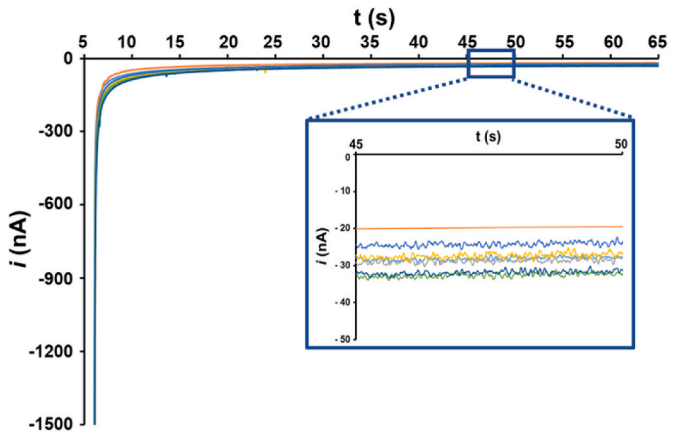

Fig. 4. Chronoamperograms (n = 7) recorded at −0.25 V in 10^{-5} M CA solutions after 1 min of enzymatic interaction in tips functionalized with the chimeric protein to study the precision. The blue framework indicates the current sampling period.

surface of the working electrode. It is well-known that CA, and its derivatives, are oxidized to the corresponding quinones, via the semiquinone derivative, through a two-electron redox process which also involves two protons (Uranga et al., 2016) (Fig. S1). The separation between anodic and cathodic peak potentials is 0.29 V, with a ratio of anodic/cathodic peak currents of 1.9, clearly far different from 0.059/n V (being *n* the number of electrons) and 1, respectively, indicating the irreversibility (slow electron transfer) of the process. Moreover, as seen also in Fig. 3A, an increase of the current intensity for increasing substrate concentration was obtained.

Since protons are involved in the CA redox process (deprotonation occurs during the oxidation), this is affected by pH (Fig. 3B) (Hotta et al., 2002): the anodic peak shifts to less positive potentials with increasing pH up to pH 5. The redox process becomes slightly more irreversible (the difference between anodic and cathodic peak potentials goes from 0.06 V at pH 3.0 to 0.11 V at pH 5.0, while the ratios of anodic/cathodic peak currents are 1.15 and 1.10, respectively), probably due to the participation of the quinone in an irreversible chemical reaction (Hotta et al., 2002). Due to the difference in the background electrolyte, the reduction peak of CA at pH 5 occurs at a slightly different potential in acetate buffer (Fig. 3A) and in Britton-Robinson buffer without boric acid (Fig. 3B). There was not too much difference in the peak current between pH 3.0 and pH 5.0. However, this intensity decreased notoriously for higher pH, with the process becoming more irreversible (0.20 V of difference between anodic and cathodic peak potentials at pH 6.0). Thus, we have chosen pH 5 as a good compromise between the redox behavior of CA and the stability of the laccase enzyme (Giardina et al., 1999). Laccase oxidase is a multi-copper enzyme able to catalyze the oxidation of *ortho*- and *para*-diphenol groups including mono-, di-, and polyphenols (Rodríguez-Delgado et al., 2015). Many of these phenols (e.g., hydroquinone, catechol, ferulic acid, vanillic acid) show similar redox behavior as CA. Although enzymes are commonly used as bio-recognition elements to provide sensors with selectivity to just one species, it has been reported the use of laccase to oxidize polyphenols and to determine an antioxidant (polyphenol) index using the combination of enzymatic oxidation and further reduction measurement of the oxidized substrates (Rodríguez-Delgado et al., 2015).

In this case, as also reported (David et al., 2015), the electrochemical behavior of CA and its higher stability compared to other antioxidants (Turner et al., 2011) allow its use as a reference compound to assess the polyphenol content in a sample. Thus, in this work, the determination of CA index was performed by CA enzymatic oxidation with Vmh2-PoxA1b and then, chronoamperometric measurement of the current due to the reduction of the oxidized substrate.

The selection of an adequate reduction potential to later carry out a

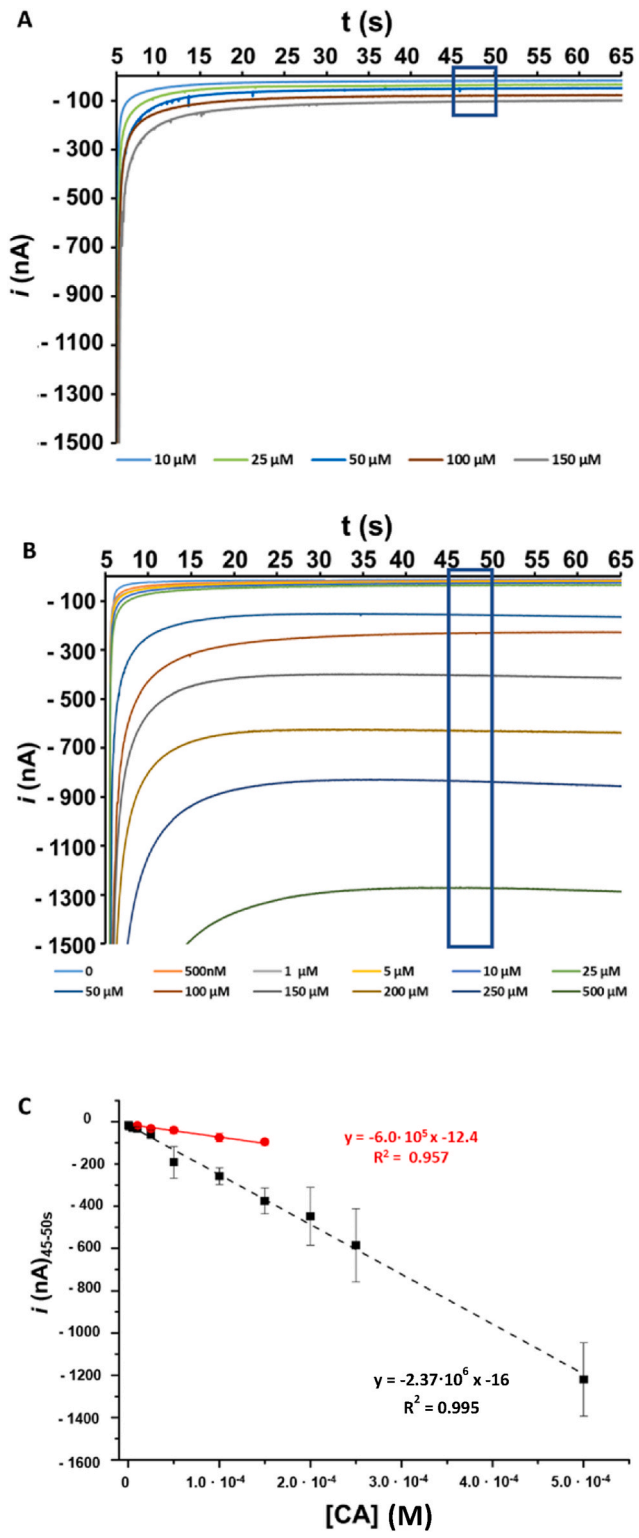

Fig. 5. (A) Chronoamperograms recorded at −0.25 V in CA solutions of increasing concentration (ranging from $5{\cdot}10^{-5}$ to $1.5{\cdot}10^{-4}$ M) performed with the immobilized wild-type protein. (B) Chronoamperograms recorded at −0.25 V in CA solutions of increasing concentration (ranging from $5{\cdot}10^{-7}$ to $5{\cdot}10^{-4}$ M) performed with the immobilized chimeric protein. The blue framework indicates the current sampling period. (C) Calibration curve of CA in the range from $5{\cdot}10^{-7}$ to $5{\cdot}10^{-4}$ M using Vmh2-PoxA1b (black square) functionalized tips. The curve corresponding to tips functionalized with PoxA1b (red dot) is presented for comparison. Error bars correspond to the standard deviation of five measurements.

chronoamperometric measurement was made according to the voltammograms in Fig. 3A. The reduction of the oxidized substrate was recorded applying a potential of −0.25 V for 60 s. Before performing a calibration curve, the enzymatic CA oxidation time was optimized by testing 1 and 3 min and using a 10^{-5} M CA solution. As shown in Fig. S2, signals obtained after 1 and 3 min were comparable, therefore reactions were carried out for 1 min to decrease analysis time. Furthermore, using the same CA concentration, the precision of the tips functionalized with the chimeric protein was tested (Fig. 4).

Indeed, these chronoamperometric measurements were carried out by using 7 functionalized tips and 7 SPEs and a good reproducibility was observed, with an average value of the analytical signal of - 27 ± 3 nA.

Then, tips biofunctionalized with the enzyme were used to obtain a calibration curve in concentrations of caffeic acid ranging from $5{\cdot}10^{-7}$ to $5{\cdot}10^{-4}$ M. As it can be seen in Fig. 5, the analytical signals obtained when laccase was immobilized in the tips through the hydrophobin moiety (Fig. 5B) were much higher than those obtained when free laccase was used (Fig. 5A). In this case the calibration curve displayed a linear trend in a wide range (three orders of magnitude) with a good correlation coefficient (0.995) (Fig. 5C). As a comparison, a slightly lower correlation coefficient (0.957) in a very short range of concentrations was obtained with PoxA1b. Moreover, the sensitivity (slope of the calibration curve) increased around one order of magnitude. The electrochemical methodology based on the tips functionalized with fusion protein reached a limit of detection (LOD), calculated as three times the standard deviation of the blank (n = 9, Fig. S3) divided by the slope, of $1.4{\cdot}10^{-6}$ M, while for the PoxA1b functionalized tips the LOD value was $7.3{\cdot}10^{-6}$ M. These values are in the range of those reported recently with other analytical systems (Araújo et al., 2020; Pradela-Filho et al., 2020). These results confirmed, once again, that for the development of this innovative electrochemical system the use of chimeric protein, instead of free enzyme has many advantages, an important increase in the sensitivity with decrease in the limit of detection among them.

To characterize the herein developed ready-to-use functionalized tips, their stability and reusability were studied. Thus, Vmh2-PoxA1b functionalized tips were stored a 4°C and their stability was verified through chronoamperometric measurements performed in triplicate in a 10^{-5} M CA solution. As shown in Fig. S4A, the ability of the functionalized tips to detect CA gradually decreased during time, up to a value of 2.1 ± 0.3 nA (69% of the value at t_0) after 20 days. However, if commercial purposes were aimed, a more thorough study of the stability should be made. As regards to the reusability, the current intensity reached the blank value after the first measurement indicating that the functionalized tips are of single use (Fig. S4B).

Considering all the results, the innovation of our method is represented by a fast and easy functionalization of the tips with an advantageous chimeric protein (no previous surface derivatization or electrode preparation is needed) as well as low analysis time (1 min enzymatic reaction before a fast measurement is performed).

3.3. Electrochemical determination of CA in real samples

The developed biosystem was used to determine CA in two types of tea (black and green). Since laccase can oxidize a plethora of phenolic compounds, CA can be used as indicator of the total level of polyphenolic content in the sample (Pradela-Filho et al., 2020). Tea is one of the beverages that often contains a high amount of CA, thus, as already reported in other studies (Araújo et al., 2020; Pradela-Filho et al., 2020), samples were subjected to a strong dilution to make the sample CA concentration fit in the calibration range of the method. After measuring chronoamperometrically the tea samples, measurements were recorded in samples spiked with CA to calculate the recovery and test the performance of the developed system.

Considering that the polyphenol content of tea heavily depends on the geographical place where the plants were grown (Dias et al., 2014),

Table 1
CA recovery in tea samples.

Tea samples	CA added (μM)	CA found (μM)	CA expected (μM)	Recovery (%)
Black	0	12.7 ± 0.3		
	10	22 ± 2	23	92 ± 8
Green	0	3.3 ± 0.5		
	10	13 ± 1	13	102 ± 7

it can be considered that the results obtained, summarized in Table 1, are in concordance with those obtained with previously reported electroanalytical platforms (David et al., 2015). In almost all the cases the recovery of the antioxidant is higher than 80% with low RSD values, which indicates good precision in the analysis. Species such as phenolic acids, flavonoids and ascorbic acid are potential interferences for electrochemical determination of CA (Tyszczuk et al., 2011). However, in our electrochemical platform, the use of laccase as biorecognition element is combined with the use of a low detection potential, which may reduce the effect of possible interferences, as demonstrated by the good recovery values obtained. As a summary, the obtained results indicate that the developed tip-based platform is appropriate for CA determination and can be convenient for other applications.

4. Conclusions

In the development of low cost, highly sensitive and fast bioanalytical devices, micropipette polypropylene tips represent a very cheap material to immobilize different biomolecules. In this work we have developed an electrochemical bioplatform in which tips were functionalized with the chimeric protein Vmh2-PoxA1b. Thus, the biofunctionalized surfaces were used as "enzymatic reactors" in which the phenolic compounds can be oxidized by the activity of laccase and then detected chronoamperometrically on SPEs. Even if the developed biosystem has not an outstanding LOD, the innovation of this analytical method is mainly due to the immobilization of the enzyme laccase in the tips without chemical derivatization and the use, for the first time, of these tips to electrochemically detect phenolic compounds. Herein, the developed system was used, as a proof of concept, to evaluate CA content in tea samples. Nevertheless, only by changing the enzyme, our system can be applied to other fields. Thus, the obtained results pave the way to the development of advanced biosystems that can include, for example, tips biofunctionalized with different enzymes to determine several analytes, all at once. Furthermore, the easier progress could be represented by the in-tip integration of the different electrodes required for the electrochemical measurement (González-López et al., 2019) to develop a real tip-based biosensor.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Author statement

Ilaria Stanzione: methodology, formal analysis, investigation, visualization, writing: original draft, review, and editing. **Anna Pennacchio:** methodology, writing: review and editing. **Alessandra Piscitelli:** methodology, writing: review and editing. **Paola Giardina:** conceptualization, resources, funding acquisition. **Estefanía Costa-Rama:** conceptualization, methodology, resources, visualization, writing: review and editing, supervision, funding acquisition. **M. Teresa Fernández-Abedul:** conceptualization, methodology, visualization, supervision, resources, writing: review and editing.

Declaration of competing interest

The authors declare that they have not known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102426.

Abbreviations

Screen-Printed Electrode (SPE)
Caffeic Acid (CA)
Bovine Serum Albunim (BSA)
Polyethersulfone (PES)
2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)
Cyclic Voltammetry (CV)
Relative Standard Deviation (RSD)
Limit Of Detection (LOD)

References

Al-Majdoub, M., Opuni, K. F. M., Koy, C., & Glocker, M. O. (2013). Facile fabrication and instant application of miniaturized antibody-decorated affinity columns for higher-order structure and functional characterization of TRIM21 epitope peptides. *Analytical Chemistry, 85*(21), 10479–10487. https://doi.org/10.1021/ac402559m

Albayati, S. A. R., Kashanian, S., Nazari, M., & Rezaei, S. (2019). Novel fabrication of a laccase biosensor to detect phenolic compounds using a carboxylated multiwalled carbon nanotube on the electropolymerized support. *Bulletin of Materials Science, 42*(4), 1–8. https://doi.org/10.1007/s12034-019-1850-0

Araújo, D. A. G., Camargo, J. R., Pradela-Filho, L. A., Lima, A. P., Muñoz, R. A. A., Takeuchi, R. M., Janegitz, B. C., & Santos, A. L. (2020). A lab-made screen-printed electrode as a platform to study the effect of the size and functionalization of carbon nanotubes on the voltammetric determination of caffeic acid. *Microchemical Journal, 158*(July). https://doi.org/10.1016/j.microc.2020.105297

Bhagat, A. R., Delgado, A. M., Issaoui, M., Chammem, N., Fiorino, M., Pellerito, A., & Natalello, S. (2019). Review of the role of fluid dairy in delivery of polyphenolic compounds in the diet: Chocolate milk, coffee beverages, matcha green tea, and beyond. *Journal of AOAC INTERNATIONAL, 102*(5), 1365–1372. https://doi.org/10.1093/jaoac/102.5.1365

Bollella, P. (2021). *Biosensors – recent advances and future challenges. Biosensors – recent Advances and future challenges*. https://doi.org/10.3390/books978-3-03943-888-4

Bordbar, M. M., Sheini, A., Hashemi, P., Hajian, A., & Bagheri, H. (2021). Disposable paper-based biosensors for the point-of-care detection of hazardous contaminations—a review. *Biosensors, 11*(9), 1–51. https://doi.org/10.3390/bios11090316

Bounegru, A. V., & Apetrei, C. (2020). Voltammetric sensors based on nanomaterials for detection of caffeic acid in food supplements. *Chemosensors, 8*(2). https://doi.org/10.3390/CHEMOSENSORS8020041

Cabaj, J., Sołoducho, J., & Nowakowska-Oleksy, A. (2010). Langmuir-Blodgett film based biosensor for estimation of phenol derivatives. *Sensors and Actuators, B: Chemical, 143*(2), 508–515. https://doi.org/10.1016/j.snb.2009.09.047

Chen, S., & Shamsi, M. H. (2017). Biosensors-on-chip: A topical review. *Journal of Micromechanics and Microengineering, 27*(8). https://doi.org/10.1088/1361-6439/aa7117

David, I. G., Bizgan, A. M. C., Popa, D. E., Buleandra, M., Moldovan, Z., Badea, I. A., Tekiner, T. A., Basaga, H., & Ciucu, A. A. (2015). Rapid determination of total polyphenolic content in tea samples based on caffeic acid voltammetric behaviour on a disposable graphite electrode. *Food Chemistry, 173*, 1059–1065. https://doi.org/10.1016/j.foodchem.2014.10.139

Dias, P. M., Changarath, J., Damodaran, A., & Joshi, M. K. (2014). Compositional variation among black tea across geographies and their potential influence on endothelial nitric oxide and antioxidant activity. *Journal of Agricultural and Food Chemistry, 62*(28), 6655–6668. https://doi.org/10.1021/jf501611w

Fernandes, S. C., de Oliveira, I. R. W. Z., & Vieira, I. C. (2007). A green bean homogenate immobilized on chemically crosslinked chitin for determination of caffeic acid in white wine. *Enzyme and Microbial Technology, 40*(4), 661–668. https://doi.org/10.1016/j.enzmictec.2006.05.023

García-González, R., Costa-García, A., & Fernández-Abedul, M. T. (2015). Enzymatic amplification-free nucleic acid hybridisation sensing on nanostructured thick-film electrodes by using covalently attached methylene blue. Talanta, 142, 11–19. https://doi.org/https://doi.org/10.1016/j.talanta.2015.03.028.

García-Guzmán, J. J., López-Iglesias, D., Cubillana-Aguilera, L., Lete, C., Lupu, S., Palacios-Santander, J. M., & Bellido-Milla, D. (2019). Assessment of the polyphenol indices and antioxidant capacity for beers and wines using a tyrosinase-based biosensor prepared by sinusoidal current method. *Sensors (Switzerland), 19*(1). https://doi.org/10.3390/s19010066

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., & Sannia, G. (1999). Protein and gene structure of a blue laccase from Pleurotus ostreatus. *Biochemical Journal, 341*(3), 655–663. https://doi.org/10.1042/0264-6021:3410655

González-López, A., Blanco-López, M. C., & Fernández-Abedul, M. T. (2019). Micropipette tip-based immunoassay with electrochemical detection of antitissue transglutaminase to diagnose celiac disease using staples and a paper-based platform. *ACS Sensors, 4*(10), 2679–2687. https://doi.org/10.1021/acssensors.9b01096

Guo, J., Yuan, Y., Dou, P., & Yue, T. (2017). Multivariate statistical analysis of the polyphenolic constituents in kiwifruit juices to trace fruit varieties and geographical origins. *Food Chemistry, 232*, 552–559. https://doi.org/10.1016/j.foodchem.2017.04.037

Hosu, O., Simona, M., Sandulescu, R., & Cristea, C. (2017). Minireview: Smart tattoo, microneedle, point-of-care, and phone-based biosensors for medical screening, diagnosis, and monitoring. *Analytical Letters, 52*. https://doi.org/10.1080/00032719.2017.1391826

Hotta, H., Ueda, M., Nagano, S., Tsujino, Y., Koyama, J., & Osakai, T. (2002). Mechanistic study of the oxidation of caffeic acid by digital simulation of cyclic voltammograms. *Analytical Biochemistry, 303*(1), 66–72. https://doi.org/10.1006/abio.2002.5577

Kang, N. J., Lee, K. W., Shin, B. J., Jung, S. K., Hwang, M. K., Bode, A. M., Heo, Y. S., Lee, H. J., & Dong, Z. (2009). Caffeic acid, a phenolic phytochemical in coffee, directly inhibits Fyn kinase activity and UVB-induced COX-2 expression. *Carcinogenesis, 30*(2), 321–330. https://doi.org/10.1093/carcin/bgn282

Klis, M., Maicka, E., Michota, A., Bukowska, J., Sek, S., Rogalski, J., & Bilewicz, R. (n.d.). Electroreduction of laccase covalently bound to organothiol monolayers on gold electrodes. Electrochimica Acta, 52(18), 5591–5598. https://doi.org/DOI:101016/jelectacta200702008.

Nasr Bouzaiene, N., Kilani Jaziri, S., Kovacic, H., Chekir-Ghedira, L., Ghedira, K., & Luis, J. (2015). The effects of caffeic, coumaric and ferulic acids on proliferation, superoxide production, adhesion and migration of human tumor cells in vitro. *European Journal of Pharmacology, 766*, 99–105. https://doi.org/10.1016/j.ejphar.2015.09.044

Nehru, R., Hsu, Y.-F., & Wang, S.-F. (2020). Electrochemical determination of caffeic acid in antioxidant beverages samples via a facile synthesis of carbon/iron-based active electrocatalyst. *Analytica Chimica Acta, 1122*, 76–88. https://doi.org/10.1016/j.aca.2020.05.001

Oliveira-Neto, J. R., Rezende, S. G., De Fátima Reis, C., Benjamin, S. R., Rocha, M. L., & De Souza Gil, E. (2016). Electrochemical behavior and determination of major phenolic antioxidants in selected coffee samples. *Food Chemistry, 190*, 506–512. https://doi.org/10.1016/j.foodchem.2015.05.104

Pradela-Filho, L. A., Noviana, E., Araújo, D. A. G., Takeuchi, R. M., Santos, A. L., & Henry, C. S. (2020). Rapid analysis in continuous-flow electrochemical paper-based analytical devices. *ACS Sensors, 5*(1), 274–281. https://doi.org/10.1021/acssensors.9b02298

Puopolo, R., Sorrentino, I., Gallo, G., Piscitelli, A., Giardina, P., Le Goff, A., & Fiorentino, G. (2021). Self-assembling thermostable chimeras as new platform for arsenic biosensing. *Scientific Reports, 11*(1), 2991. https://doi.org/10.1038/s41598-021-82648-9

Rafiee, M., & Nematollahi, D. (2008). Electrochemical study of catechol–boric acid complexes. Electrochimica Acta, 53(6), 2751–2756. https://doi.org/"https://doi.org/10.1016/j.electacta.2007.10.055" \o "https://doi.org/10.1016/j.electacta.2007.10.055" https://doi.org/10.1016/j.electacta.2007.10.055.

Rodríguez-Delgado, M. M., Alemán-Nava, G. S., Rodríguez-Delgado, J. M., Dieck-Assad, G., Martínez-Chapa, S. O., Barceló, D., & Parra, R. (2015). Laccase-based biosensors for detection of phenolic compounds. *TrAC - Trends in Analytical Chemistry, 74*, 21–45. https://doi.org/10.1016/j.trac.2015.05.008

Sharma, A., Jain, K. K., Jain, A., Kidwai, M., & Kuhad, R. C. (2018). Bifunctional in vivo role of laccase exploited in multiple biotechnological applications. *Applied Microbiology and Biotechnology, 102*(24), 10327–10343. https://doi.org/10.1007/s00253-018-9404-8

da Silva, R. A. B., Rabelo, A. C., Munoz, R. A. A., & Richter, E. M. (2010). Three-electrode-integrated sensor into a micropipette tip. *Electroanalysis, 22*(19), 2167–2171. https://doi.org/10.1002/elan.201000244

Sorrentino, I., Giardina, P., & Piscitelli, A. (2019a). Development of a biosensing platform based on a laccase-hydrophobin chimera. *Applied Microbiology and Biotechnology, 103*(7), 3061–3071. https://doi.org/10.1007/s00253-019-09678-2

Sorrentino, I., Giardina, P., & Piscitelli, A. (2019b). Development of a biosensing platform based on a laccase-hydrophobin chimera. *Applied Microbiology and Biotechnology, 103*(7), 3061–3071. https://doi.org/10.1007/s00253-019-09678-2

Sorrentino, I., Stanzione, I., Nedellec, Y., Piscitelli, A., Giardina, P., & Le Goff, A. (2020). From graphite to laccase biofunctionalized few-layer graphene: A “one pot” approach using a chimeric enzyme. *International Journal of Molecular Sciences, 21*(11). https://doi.org/10.3390/ijms21113741

Sorrentino, I., Stanzione, I., Piscitelli, A., Giardina, P., & Goff, A. Le (2021). Carbon-nanotube-supported POXA1b laccase and its hydrophobin chimera for oxygen reduction and picomolar phenol biosensing. *Biosensors and Bioelectronics:, X*(June), Article 100074. https://doi.org/10.1016/j.biosx.2021.100074, 8.

Stanzione, I., Izquierdo-Bote, D., González García, M. B., Giardina, P., & Piscitelli, A. (2021). Immobilization of antibodies by genetic fusion to a fungal self-assembling adhesive protein. *Frontiers in Molecular Biosciences, 8*(October), 1–8. https://doi.org/10.3389/fmolb.2021.725697

Thangavelu, K., Raja, N., Chen, S. M., & Liao, W. C. (2017). Nanomolar electrochemical detection of caffeic acid in fortified wine samples based on gold/palladium nanoparticles decorated graphene flakes. *Journal of Colloid and Interface Science, 501*, 77–85. https://doi.org/10.1016/j.jcis.2017.04.042

Tortolini, C., Rea, S., Carota, E., Cannistraro, S., & Mazzei, F. (2012). Influence of the immobilization procedures on the electroanalytical performances of Trametes versicolor laccase based bioelectrode. *Microchemical Journal, 100*(1), 8–13. https://doi.org/10.1016/j.microc.2011.08.001

Turner, S., Cogoi, L., Isolabella, S., Filip, R., & Anesini, C. (2011). Evaluation of the antioxidant activity and polyphenols content of Ilex paraguariensis (mate) during industrialization. *Advance Journal of Food Science and Technology, 3*(1), 23–30. https://www.scopus.com/inward/record.uri?eid=2-s2.0-79951932208&partnerID=40&md5=c88223f9ab2e89d936e7b3ba7eb687f0.

Tyszczuk, K., Skalska-Kamińska, A., & Wozniak, A. (2011). Voltammetric method using a lead film electrode for the determination of caffeic acid in a plant material. *Food Chemistry, 125*, 1498–1503. https://doi.org/10.1016/j.foodchem.2010.10.075.

Uranga, J., Podio, N., Wunderlin, D., & Santiago, A. (2016). Theoretical and experimental study of the antioxidant behaviors of 5-O-caffeoylquinic, quinic and caffeic acids based on electronic and structural properties. *ChemistrySelect, 1*, 4113–4120. https://doi.org/10.1002/slct.201600582

Weese, M. E., Krevh, R. A., Li, Y., Alvarez, N. T., & Ross, A. E. (2019). Defect sites modulate fouling resistance on carbon-nanotube fiber electrodes. *ACS Sensors, 4*(4), 1001–1007. https://doi.org/10.1021/acssensors.9b00161

Zhang, M., Zhang, Y., Yang, C., Ma, C., Zhang, Y., & Tang, J. (2022). Synthesis of three-dimensional laccase-Cu3(PO4)2·3H2O microflowers via biomineralization for UV–vis epinephrine biosensing. *Microchemical Journal, 172*, Article 106911. https://doi.org/10.1016/j.microc.2021.106911

Rama, E. C., Biscay, J., González García, M. B., Reviejo, A. J., Pingarrón Carrazón, J. M., & Costa García, A. (2012). Comparative study of different alcohol sensors based on Screen-Printed Carbon Electrodes. *Analytica Chimica Acta, 728*, 69–76. https://doi.org/10.1016/j.aca.2012.03.039