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Amperometric biosensor based on Laccase immobilized onto a screen-printed electrode by Matrix Assisted Pulsed Laser Evaporation

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Abstract

A Laccase-based biosensor for the determination of phenolic compounds was developed by using Matrix Assisted Pulsed Laser Evaporation as an innovative enzyme immobilization technique. and the deriving biosensor was characterized and applied for the first time. Laccase was immobilized onto different substrates including screen printed carbon electrodes and spectroscopic, morphologic and electrochemical characterizations were carried out. A linear range from 1 to 60 μM was achieved working at 5.5 pH and -0.2 V detection potential vs Ag pseudoreference. The limits of detection and quantification were found to be 1 and 5 μM, respectively. A good fabrication reproducibility, stability of response and selectivity toward interferents were also found The potential of the developed biosensor was tested in the determination of total polyphenol content in real matrices (tea infusion, ethanolic extract from *Muscari comosum* bulbs and aqueous solution of a food supplement from black radish root and artichoke leaves) and the results were compared with those obtained by using the Folin-Ciocalteu method.

Keywords: Biosensor, Matrix Assisted Pulsed Laser Evaporation, Screen Printed Electrode, Laccase, Polyphenols Assessment

1. Introduction

The development of new biosensors for analytical purposes is still a topical issue, and the attention is focused on the fabrication of flexible, reliable, and handy devices, suitable for miniaturization and mass scale production with very good characteristics in terms of reproducibility, selectivity, sensitivity and stability. Screen printed technology has become an alternative method for mass production of disposable electrodes at low cost, easy to manage, with peculiar configuration characteristics, widely exploited in the development of miniaturized biosensors [1]. Indeed, screen-printed electrodes (SPEs), comprising the three electrodes (working, reference and counter), can be considered as "disposable electrochemical cells" when a sample droplet, covering the electrodes, is used for the measurement [2]. Typically, amperometric biosensors with enzymatic recognition element are a powerful analytical tool, since the characteristics of the biocomponent specificity and the high sensitivity, simplicity of operation and low cost of the transducer are fully integrated [3,4]. Enzyme immobilization on the transducer is a fundamental and critical step in a biosensor design. The strategy of immobilization of biological components, greatly influences the properties of the biocatalyst, in terms of activity, life-time, stability of response and cost of the resulting device [5]. Several methods have been developed for enzyme immobilization, mainly based on physical and chemical mechanisms, such as entrapment in carbon paste [6] or in electro-synthesized polymeric films [7,8], crosslinking with glutaraldehyde [9] or other bifunctional agents, and covalent immobilization [5]. Beside to the above immobilization techniques, new deposition and/or immobilization technologies have been applied aiming at the development of enzyme thin films. In particular, the printing technologies (e.g. inkjet printing, screen printing, microcontact printing) and the laser printing technologies (e.g. Laser Induced Forward Transfer) can be mentioned [10-12]. These advanced techniques are promoting the miniaturization and the mass production of biosensors and bio-devices [10]. In this context, also laser deposition techniques, such as Pulsed Laser Deposition (PLD) [13-15] and the Matrix Assisted Pulsed Laser Evaporation (MAPLE) [16-20], can be considered useful alternative approaches for enzyme immobilization. Indeed, these techniques have been established as simple, cheap and versatile methods for the deposition of thin films of a very wide range of materials [21,22]. Nevertheless, MAPLE is considered the most suitable laser deposition technique for obtaining biological thin films [11,23].

In MAPLE a pulsed laser beam, focused inside a vacuum chamber, impinges on the surface of a rotating target (Fig. 1). The target is a frozen solution of the material to be deposited, previously dissolved in an appropriate solvent. Thus, when the laser beam impacts the target, the laser pulsed energy is mainly absorbed by the solvent and converted to thermal energy, allowing the solvent to vaporize and the molecules of interest to receive enough kinetic energy to be transferred and

deposited as a thin film on proper substrate [23]. The MAPLE ability of producing active enzyme thin film makes it exploitable for biosensor development [17,22,24].

In a previous work [16], we explored this technique for the production of Laccase thin films and we concluded that MAPLE, working under the investigated conditions and using water as the solvent, was able to deposit Laccase partially active. Therefore, further investigations about MAPLE aiming at the Laccase-based biosensor development needed to be carried out.

Laccase (E.C. 1.10.3.2, *p*-benzenediol:oxygen oxidoreductase), a copper-containing enzyme belonging to the group of the blue oxidases, is able to catalyse the oxidation of various polyphenol compounds to the corresponding quinone, with the concomitant reduction of oxygen to water [25]. Polyphenols, a secondary metabolite class widespread in the plant kingdom, are of great interest for both food and environmental field [9,26]. Indeed, although they play an important role for their numerous properties, such as those organoleptic and beneficial for the human health, they can also be considered a serious potential hazard for human health and aquatic life when they are released into the environment as industry wastewaters [9,27].

In this work the use of MAPLE for the production of Laccase-based biosensors, using screen printed carbon electrodes (SPCEs) as the transducer, has been implemented and, for the first time, the performances of the biosensor obtained were investigated.

Laccase was immobilized by MAPLE using benzene as the solvent for the target preparation. Laccase thin films, investigated for their functionality, were also characterized with respect to the molecular structure and the morphology. MAPLE deposition conditions were investigated paying particular attention to the reproducibility of biosensor preparation, considering the relevance of this feature for the development of biosensors on a mass-scale. The biosensor fabricated was characterized in terms of sensitivity, linearity, limits of detection (LOD) and quantification (LOQ), as well as selectivity and stability of response using catechol as the phenolic substrate and pH and applied potential values selected. Moreover, the total polyphenols content of the investigated samples (tea infusion, ethanolic extract from *Muscari comosum* bulbs and aqueous food supplement from black radish root and artichoke leaves) assessed by the biosensor were compared with those obtained by using the routine Folin-Ciocalteu (F-C) method.

2. Materials and Methods

2.1. Chemicals and Materials

Two Laccase from *Trametes versicolor* (Sigma Aldrich cod. 38429 and 51639), with an estimated activity of 0.5 U/mg and 13.6 U/mg solid were used in this study. Syringaldazine and catechol, used as the enzyme substrates, potassium phosphate monobasic anhydrous, sodium acetate anhydrous,

potassium hydroxide (KOH) and benzene were purchased from Sigma Aldrich (Germany). Sodium carbonate, Folin-Ciocalteu reagent, acetic acid, methanol, ethanol and water plus were purchased from Carlo Erba reagents (Italy). All chemicals were of analytical grade and used as received. The solvents used were of spectral grade.

The composition of buffers was the following: phosphate buffer (PB) at pH 6.5, 100 mM potassium phosphate adjusted by using KOH 1M; acetate buffer (ABa) at pH 4.5 and 5.5, 100 mM sodium acetate adjusted by using 10% acetic acid.

Glass, silicon and SPCE were used as deposition substrates. SPCEs (cod. DS 150), purchased from DropSens (Oviedo, Spain), consist of a platinum counter electrode, a silver pseudo-reference electrode, and a carbon working electrode with an area of 0.1257 cm^2. SPCEs were used as received, hence no treatments were carried out before enzyme deposition by MAPLE.

A proper stainless steel mask, having a length and a width equal to those of SPCE and a 0.166 cm^2 circular hole at the working electrode, was realized ad-hoc and assembled with deposition substrates by using the laboratory film (Parafilm M®) in order to deposit Laccase layers with an area equal to the SPCE working electrode one.

2.2. Real samples

Three different real samples, namely commercial black tea (English Breakfast Tea, TWININGS of London, Jasin-Swarzedz, Poland) infusion (BT), ethanolic extract of tassel hyacinth [*Muscari comosum* (L.) Mill.] bulbs (Mc) and commercial food supplement (CFS) mainly consisting of black radish root (*Raphanus sativus*) and artichoke leaves (*Cynaras colymus*) aqueous extracts (LEVANAT PLUS, NATURHOUSE, Ferrara, Italy), were analysed for the total phenolic content (TPC) by both the biosensor and the F-C method. BT was prepared by adding 100 mL of boiling distilled water to 1g of tea. The BT was kept in infusion for 300 seconds, then filtered first through a strainer and then through a 0.45 μm cellulose filter. The Mc ethanolic extract was prepared by adding 5 mL of ethanol to 0.5 g of dried and ground bulbs. The mixture was stirred for 24 hours at 4° C and then centrifuged for 3600 seconds at 10000 RCF. The BT, Mc extracts and the commercial food supplement (CFS) were properly diluted prior to analysis.

2.3. *Instruments and experimental conditions*

Laccase thin films were deposited by MAPLE onto proper substrates (silicon, glass and SPE) in order to both carry out a characterization study of enzyme layers and develop the biosensor.

2.3.1. MAPLE

MAPLE depositions were performed inside a homemade stainless steel vacuum chamber by using a Nd-YAG laser working at 266 nm and 10 Hz pulse repetition rate. The target irradiation was performed at the laser fluency of 0.25 J/cm^2, with 60000 pulses. Laser beam impinged on the rotating target at 45° with respect to the surface. A 0.35% enzyme solution in benzene was freshly prepared and kept under stirring for one hour, then it was flash frozen and used as the target material; during the laser irradiation it was maintained frozen by the cryogenic target holder system. Moreover, both target and substrate holder were in rotation during the deposition process, which was performed with a rastering laser beam to avoid hole-drilling in the ice. The distance target-substrate was of 3.5 cm and the size of the laser spot reaching the target was about 0.003 cm^2. The pressure in the chamber during the deposition was about 10^{-2} Pa.

2.3.2. Laccase thin film spectroscopic characterization

The molecular structure of Laccase deposited onto silicon was investigated by Fourier Transform InfraRed (FTIR) spectroscopy. FTIR spectra were recorded using a 6300 FTIR instrument (Jasco, Cremella, Italy) in the range 400-4000 cm^{-1}, at a 4 cm^{-1} resolution. An Attenuated Total Reflectance (ATR) and a transmission module were used for recording the spectrum of Laccase powder and films, respectively.

2.3.3. Laccase thin film microscopic characterization

The morphology and thickness of the Laccase thin layers deposited onto glass by MAPLE were evaluate by Atomic Force Microscopy (AFM) and using a"XE-100" setup (Park Systems, Suwon, Korea).

2.3.4. Laccase thin film spectrophotometric characterization

Laccase thin films, deposited onto glass, were also spectrophotometrically analyzed to test the enzyme activity, as described by Ride [28] with some modifications. The assay is based on the oxidation of syringaldazine, used as the enzymatic substrate, determining the absorbance increase at 530 nm. Laccase deposited onto glass was dissolved in 900 μL of ABa, and then 100 μL of a 0.216 mM syringaldazine solution in methanol were added. The kinetic reaction was monitored for 600 seconds. Laccase standard solutions, in the concentration range 0.34-1.36 μg mL^{-1}, were used to construct a calibration curve plotting enzyme amounts versus the relative absorbance values at 210 seconds. A blank, containing 900 μL of buffer and 100 μL of syringaldazine solution, was used for

the instrument zeroing. An Ultrospec 4000 UV–vis spectrophotometer by Amersham Pharmacia Biotech (Milan, Italy) was used for this analysis.

2.3.5. Chronoamperometric measurements

In order to develop the Laccase-based biosensor, the enzyme was immobilized by MAPLE onto working electrode of SPCEs used as the transducers. Electrochemical measurements were performed by using a PalmSens^3 electrochemical analyser and the PSTrace software (Ver. 4.22) (AW Utrecht, The Netherlands). Electrochemical experiments were carried out at room temperature by a single-drop chronoamperometry analysis with an applied potential of -200 mV vs pseudo-reference silver electrode. Briefly, the background response of the modified electrodes was measured by casting 100 μL of buffer solution onto the surface, ensuring that all three electrodes were coated by the solution, and the signal was recorded every 0.1 seconds for the duration of 300 seconds. Buffer solution was then drained from the surface and a 100 μL drop of sample (catechol solutions at different concentration used for calibration curves or real samples diluted properly) was cast coating again all the three electrodes. The amperometric responses, still recorded every 0.1 seconds for the duration of 300 seconds, was due to the electrochemical reduction of the quinones produced by the Laccase-catalized oxidation reaction of phenols. The biosensor response was expressed as a difference between current signals deriving from analyte and background, considering the average values of current recorded in the last 50 s.

2.3.6. Determination of polyphenols content by using the Folin-Ciocateu method

The total phenolic content was determined by the F-C micro-method according to Cicco *et al.* [29] by using catechol, as the phenolic standard at concentrations in the range 10-50 μM. Catechol solutions were freshly prepared from a 20 mM stock solution containing 10% ethanol by proper dilution with ABa. The reaction mixtures were analysed spectrophotometrically at 740 nm. The total phenolic content was expressed as mg of catechol equivalents per litre or kilogram of sample. The measurements of the total phenolic content were carried out in triplicate for each investigated sample. The Ultrospec 4000 UV–vis spectrophotometer, mentioned above, was used for this analysis.

3. Results and discussion

This research activity was aimed at the development of a Laccase-based biosensor by using MAPLE as an innovative immobilization technique. The results from previous experiments showed

the ability of MAPLE technique to deposit Laccase thin films [16]. Nevertheless, such films were considered unsuitable for the development of a biosensor owing to the low activity showed. Taking in account that the laser-matter interaction may cause a photochemical damage of solute molecules being into MAPLE target [23], in this work the first investigations were carried out in order to reduce the bio-component degradation as much as possible to deposit an enough amount of active Laccase suitable for the biosensor application. Moreover, the attention was also focused to make the biosensor fabrication process reproducible. In this perspective, a particular attention has been devoted to some MAPLE deposition conditions, such as kind of solvent, fluency, enzyme concentration, and substrate position and orientation.

3.1. MAPLE solvent

The solvent is a very important key to the successful enzyme transfer by MAPLE since it can affect the deposit quality [30]. In previous experiments, aimed at the deposition of Laccase thin films by MAPLE [16], water was used as the solvent since it is considered the most suitable solvent for biological material due to its bio-compatibility [11,18]. Nevertheless, Laccase thin films obtained by MAPLE using water resulted not adequately active for biosensor application. In this study, benzene was tested as the MAPLE solvent by depositing the same Laccase (0.5 U/mg) of the previous work [16] in order to carry out a better comparison in terms of film activity. The higher volatility of benzene (boiling point 80.1°C) with respect to water, allowed the use of a lower pulse energy, thus preserving the enzyme activity. Moreover, no negative effects were observed on activity of Laccase starting powder dissolved in benzene, that was proved by using the syringaldazine spectrophotometric assay. Furthermore, benzene allowed the obtainment of a good frozen target (no defects, bubbles or cracks) in an easier way because of his melting point (5.53 °C). As a result, when benzene was used as the solvent in place of water, it was possible to reduce the fluency up to 0.25 J/cm^2 obtaining Laccase thin films with a more than double activity, evaluated by spectrophotometric and chronoamperometric analyses.

3.2. Target preparation and substrate arrangement

MAPLE process reproducibility is related to both own beam quality (e.g. pulse-to-pulse stability) and working conditions which can be managed by operator. In this study, particular attention was focused both on the enzyme concentration, aimed at the target preparation, and the position of substrates, as well as their orientation to make the deposition process reproducible. Laccase with a higher activity (13.6 U/mg) was used in these experiments in order to fabricate biosensors with an better sensitivity. With respect to the effect of the enzyme amount on the target, different Laccase

concentrations in benzene were prepared starting from 1% (w/w) as the maximum concentration. As a result, the concentration of 0.35% (w/w) was the highest possible to achieve a homogeneous suspension, in other words a uniform target and consequently a reproducible enzyme deposition.

Regarding the position and orientation of the substrates, they were carefully arranged on the rotating circular holder, allocating three SPCEs (named A, B and C) with the working electrodes on the vertexes of an equilateral triangle inscribed into a circumference drawn concentric to the holder (dotted rectangle in Fig. 1). Therefore, three biosensors were simultaneously obtained from each deposition and subsequently analyzed by chronoamperomery.

Intra-, inter- and overall deposition reproducibility of MAPLE were evaluated by calculating the percentage relative standard deviation (RSD%) of the sensitivity. The better intra-deposition reproducibility was 3.5% evaluated by testing three biosensors deriving from the same deposition process. The inter-deposition reproducibility was 16.9% evaluated by testing four biosensors derived from different deposition processes and collocated in the same position on the substrate holder (C position in insert of Figure 1). The overall deposition reproducibility, evaluated by testing 11 biosensors from five deposition processes including those considered for the intra- and inter-reproducibility, was 12.9%. Considering that these results were obtained using a homemade system, the fabrication reproducibility can be considered good, suggesting that MAPLE can represent a suitable technique to obtain Laccase-based biosensors on a mass-scale.

3.3. Laccase thin film characterization

Laccase thin films were deposited by MAPLE, under the above mentioned conditions, onto proper substrates necessary to carry out a characterization study by AFM and FTIR spectroscopy. AFM analysis of three different Laccase films, obtained from the same deposition process, showed that the surfaces of the Laccase layers are very similar, being uniform and very compact, so confirming the good reproducibility in the MAPLE intra-deposition of Laccase thin films. Moreover, it was evidenced a high surface roughness (Fig. 2) which is common in MAPLE depositions, due to the ejection and deposition of droplets generated in the process, as reported by other authors [23,31]. Nevertheless, the film thicknesses obtained were very similar with an average value of about 20 μm. The enzyme film uniformity and compactness, showed by the AFM analysis, evidenced that the biosensor active area could correspond to the geometric surface of the SPCE working electrode, also considering that a proper mask was used in the Laccase deposition by MAPLE.

FTIR analyses were carried out to check the Laccase molecular structure. The FTIR spectra of both Laccase from MAPLE and the enzyme powder (Fig. 3) showed no differences in terms of typical absorption peaks of Laccase at 3401, 2923 and 1649 cm^{-1} [32].

FTIR analysis demonstrated that Laccase deposited using benzene as the solvent underwent no substantial modifications. However, it should be noted that enzyme modifications cannot be ruled out, being the IR spectroscopy able to provide only information on primary and secondary structures of the enzyme [23,33]. Therefore, the determination of the enzymatic activity was necessary.

The spectrophotometric assay facilitated the control of MAPLE process especially during the preliminary phase of investigations to evaluate the presence or the absence of enzyme activity. Moreover, it allowed to quantify the active Laccase, deposited under the chosen working conditions, by interpolating the absorbance value from Laccase films on the enzyme calibration curve. Laccase with a higher activity was used for this purpose and for the biosensor development. The estimated active amount was equal to 0.46 ± 0.05μg (n=5).

3.4. Choice of the experimental conditions for the biosensor application

Besides the enzyme activity, other factors, such as working pH and applied potential, influence the biosensor response in the chronoamperometric measurements. Since the enzyme activity is strongly dependent on the pH of the buffer solution, in particular when the biocomponent is immobilized [34], the biosensor responses to 30μM catechol were evaluated at pH values of 4.5, 5.5 and 6.5, starting from 4.5, considering that the optimum pH of the enzyme in solution is 4.6 [35]. The 5.5 pH resulted the best (Fig. 4a) in terms of biosensor response, and it was used in the next experiments. Moreover, it is known that chloride ions are generally added to the buffer solution (buffer saline) in order to guarantee a stable and known potential value of the silver based pseudo-reference of the SPE [36,37]. Nevertheless, this halide has an inhibitor effect on Laccase [38]. Therefore, the electrochemical analyses were accomplished in the absence of chloride, and the detection potential was optimized in terms of response to catechol and background current. Fig. 4b shows the normalized responses of the biosensor to 30 μM catechol and blank as a function of the applied potential in the range -0.4 / 0.0 V. The best response in terms of plateau current and background was observed at −0.2 V, which was chosen as the detection potential.

3.5. Biosensor characterization

The Laccase biosensor was characterized by the evaluation of performance parameters, such as linear range, limits of detection (LOD) and quantification (LOQ), and response stability since these parameters strictly depend on the immobilization method [8] as well as the biosensor architecture [39,40]. In particular, sensitivity and detection limit are also affected by the diffusion constrains of substrate and cofactor [39] due to the presence of biocomponent-entrapping polymer films and/or the biocomponent layer itself. In the biosensor preparation it is very important to obtain a uniform coverage of the electrode surface, as the MAPLE process has allowed. In fact, a partial electrode surface coverage determines different diffusion pathways that can affect adversely the biosensor response repeatability and the rejection capability of the interferents. Fig. 5 reports the chronoamperograms of some catechol standard solutions (range 5–1000 μM). The current transients are related to the electrochemical reactions occurring at the working electrode, also involving Laccase that catalyzes the substrate (i.e. catechol) oxidation to ortho-quinones with the concomitant reduction of oxygen to water [41]. Applying an appropriate potential, quinones are electrochemically reduced to catechol, originating a current proportional to the substrate concentration . while The inset of Fig. 5 displays a typical calibration curve. The correlation between the delta measurement of current responses and the catechol concentrations clearly indicate that the response trend follows the Michaelis–Menten law [$y=213.23x/(71.80+x)$; $R^2=0.998$].

The biosensor response was linear in the range 5 - 60 μM with a determination coefficient of 0.994 ± 0.002, and a mean sensitivity (S) of 13.4 ± 1.8 nA μM^{-1} cm^{-2} (n=9). LOD and LOQ, calculated as 3.3*SD/S and 10*SD/S where SD is the standard deviation of the blank responses and S expressed as nA μM^{-1}, were 1.0 μM and 5.0 μM, respectively.

Regarding the biosensor stability under repeatability conditions, it was found that the current responses to 30 μM catechol did not decrease significantly up to 5 measurements. Afterwards, the current signal showed a slight decrease of about 5% up to the eighth measurement and up to 20% at the thirtieth (Fig. 6). The signal decrease observed after the first 5 measurements can depend on different factors. Among them, the most probable is the enzyme leaching out of the carbon electrode, since the immobilization process by MAPLE could involve a combination of weak bonds, such as Van der Walls and hydrogen bonds and/or electrostatic interactions, that bind the enzyme to the electrode surface not in a strong manner. These interactions are similar to those occurring in the immobilization by physical-chemical adsorption [42]. However, a slight decrease of the enzyme activity during measurements cannot be ruled out.

The biosensor lifetime (storage stability under reproducibility condition) was also investigated by testing the biosensor response after 7, 14, 30 and 100 days of storage at 4°C under dry conditions.

The sensitivity RSD% was found to be 11.6% that, being within the intra- and inter-deposition variability, allows asserting that the biosensor possesses a very good lifetime.

The performance of the proposed biosensor has been compared with that reported in the literature for other devices developed on SPE and using Laccase from *Trametes versicolor* and catechol as bio-mediator and substrate, respectively (Table 1). The data show that by using MAPLE is possible to obtain biosensors having a good linear range and a sensitivity four folds higher in comparison with the biosensor produced with a laser printing technique. A lower sensitivity is shown with respect to biosensors obtained with conventional immobilization methods, such as entrapment that, however, may show a worse linear range or have not been tested for the selectivity in the presence of interferents.

3.6. Effect of interferences

Despite of the enzyme specificity, Laccase biosensors could suffer from interferents and/or electroactive species because most of them are present in complex media as foodstuffs [44]. Among the possible interferents present in real matrices, glucose (Glu), cysteine (Cys) and ascorbic acid (AA) were considered in this work. Furthermore, they are also known to interfere in the determination of polyphenols when the F-C method is used [44,45]. Moreover, AA was also considered because it is a substrate for Laccase [46]. Since the content of polyphenols in food matrices is very high a sample dilution at least of 50 times including the extraction step is required prior to analysis. As a consequence and taking into account the typical concentration of the possible interferents in foodstuffs, the latter were tested at micromolar levels. Glu and Cys at 2.5 μM concentration gave no significant current responses with respect to the blank signal. On the contrary, AA tested at the same concentration gave an absolute bias of 0.9 μM that was negligible with respect to the polyphenol concentration in the tested real samples.

3.7. Analyses of real samples

Biosensor performances were tested by the analyses of total phenol content in three different real samples by using the standard additions method and the calibration curve from catechol standard solutions. Results obtained by the two methods were not significantly different (α=0.05), indicating the absence of any matrix effect and a satisfactory accuracy of the developed biosensor. The total phenol content of BT, Mc, and CFS , were 6111 ± 60 mg kg^{-1}, 1462 ± 15 mg kg^{-1} and 596 ± 84 mg

L^{-1}, respectively, while the analysis on the same samples carried out by the F-C method gave the following concentrations: 18091 ± 320 mg kg^{-1}, 1754 ± 32 mg kg^{-1} and 1653 ± 55 mg L^{-1}.

The results showed that the TPC of BT and CFS determined by the biosensor method is about three times lower than that assessed by using the F-C method. This evidence can be explained taking into account the presence in the above-mentioned samples of other natural compounds (e.g. reducing sugars) that can affect the spectrophotometric response because of the well-known non-selective action of the F-C reagent [45]. Similar results of overestimation for TPC in tea infusions were found by Ibarra-Escutia *et al*. when they used F-C method with respect to their Laccase-based biosensor [43]. In this context, the slight difference in the TPC value of the Mc extract, assessed by using the two methods, can be explained considering that the extraction solvent (ethanol) can enhance the selective recovery of the polyphenols with respect to the F-C typical interferents.

Conclusions

In this study we characterized for the first time a Laccase-based biosensor developed by MAPLE. The biosensor showed an adequate linear range, good sensitivity, stability, life-time and a very good selectivity as well as suitability for the assessment of TPC in plant extracts (tea infusion, ethanolic extract from *Muscari comosum* bulbs and aqueous food supplement from black radish root and artichoke leaves). Moreover, the kind of transducer (SPEs) used to develop the Laccase biosensor and the easy and handy chronoamperometric single-drop set up can allow the use of this device for in-situ analyses.

Laccase-based biosensors can be expected to be developed on a mass-scale by MAPLE considering the promising reproducibility of the fabrication process obtained by using a homemade system, and the real possibility to further improve the MAPLE set up. Moreover, MAPLE can be considered as an advantageous technique since it allows a high degree of control on film thickness due to its pulsed nature that can support the biosensor production reproducibility. Furthermore, the use of MAPLE for the biosensor realization resulted advantageous also due to the very simple one-step immobilization procedure. As a perspective, MAPLE will be explored for the deposition of other enzymes, lonely or simultaneously to cofactors and/or mediators.

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Table 1 Comparison of the performances of Laccase based amperometric biosensors for catechol detection

Immobilization Method	Screen-printed Electrode	Sensitivity ($nA\ \mu M^{-1}\ cm^{-2}$)	LOD (μM)	Linear Range (μM)	Interferents	Real Samples	Reference
MAPLE	Carbon (DS150)	13.4	1	5-60	AA, Cys, Glu	BT, CFS, Mc	Present work
Laser printing	Carbon (DS110)	3.4	0.15	0.15-0.75	n.d.	n.d.	[12]
Entrapment in PVA-AWP	Carbon (home-made)	156.9	0.56	0.5-175	n.d.	Tea	[43]
Entrapment in PAP	MWCNT (DS)	825.5	0.18	0.63-20.7	n.d.	Red and white wine	[41]

Abbreviations: AA=Ascorbic Acid; Cys=Cysteine; Glu=Glucose; BT=Black Tea Infusion; CFS=Liquid Commercial Food Supplement; Mc=*Muscari comosum* extract; PVA-AWP= polyvinyl alcohol photopolymer(azide-unit pendant water-soluble photopolymer); PAP= polyazetidine prepolymer; MWCNT=Multi-Walled Carbon Nanotubes; n.d=not determined.

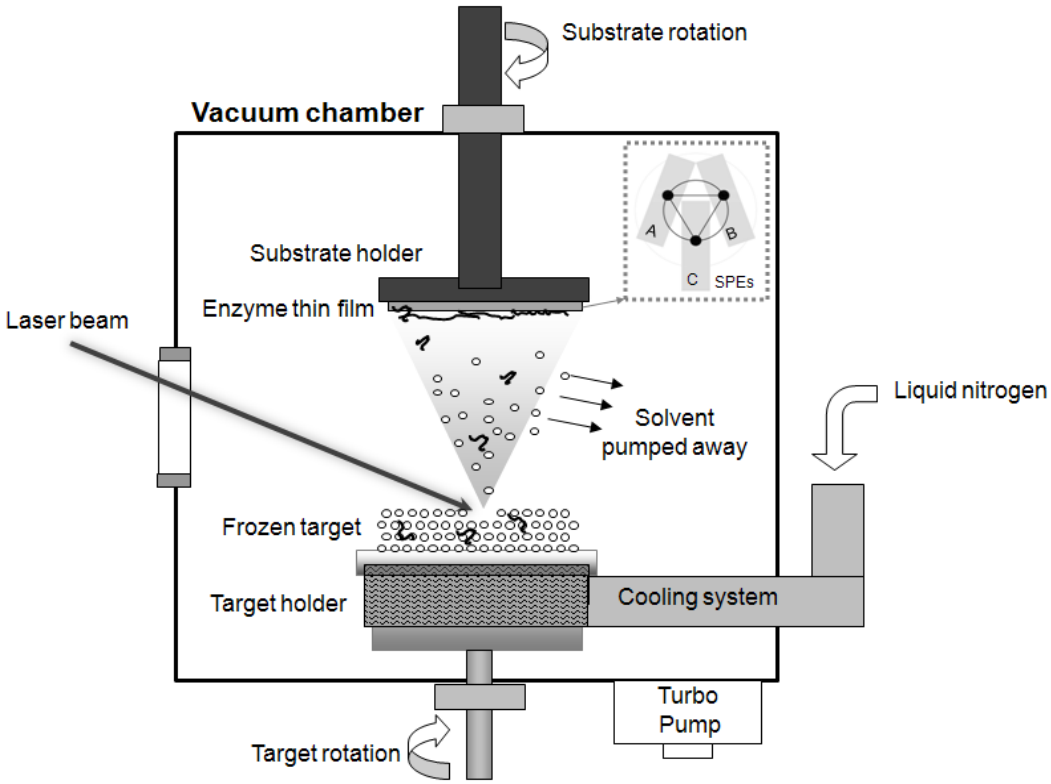


Fig. 1 MAPLE experimental set up. The position of the Screen Printed Electrodes (SPE) onto the substrate-holder is highlighted in the dotted rectangle

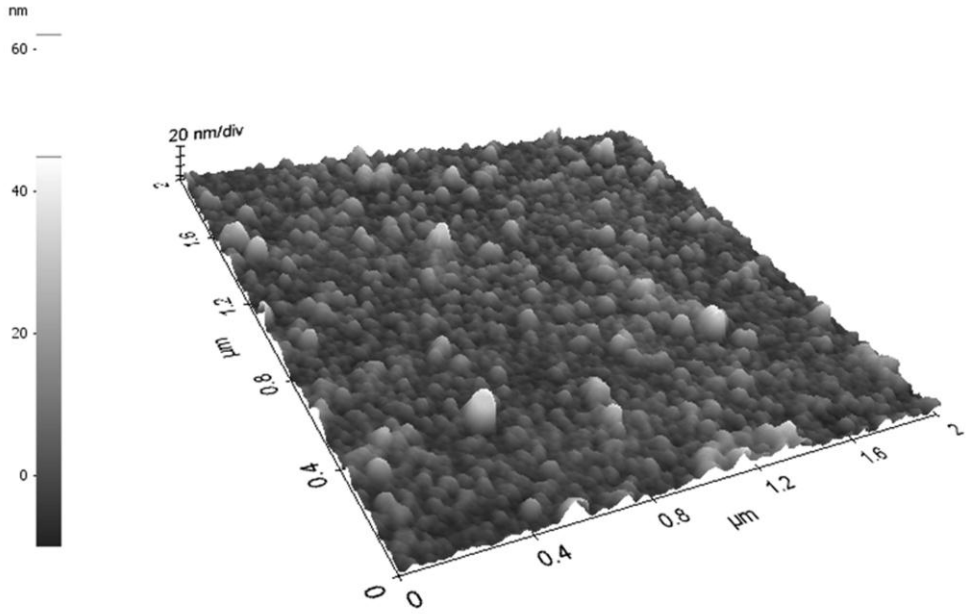


Fig. 2 AFM image of Laccase deposited on glass by MAPLE

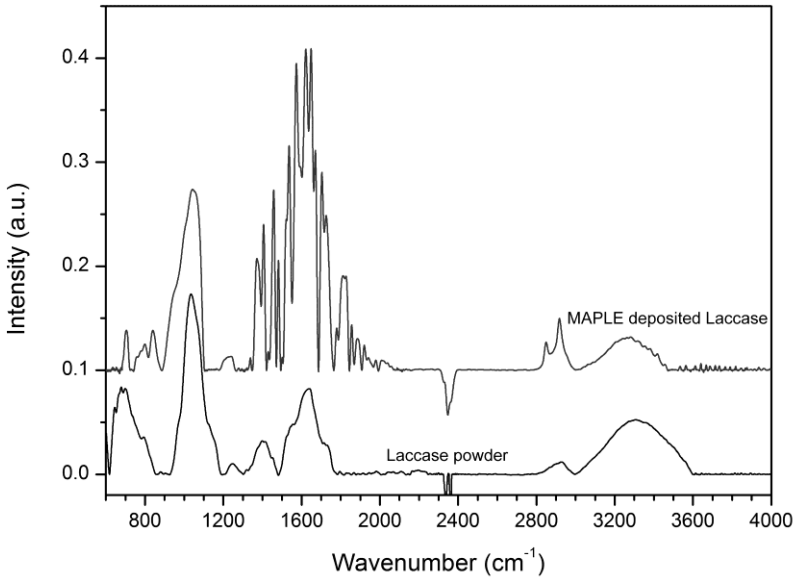


Fig. 3 FTIR spectra of Laccase deposited on silicon by MAPLE and Laccase powder

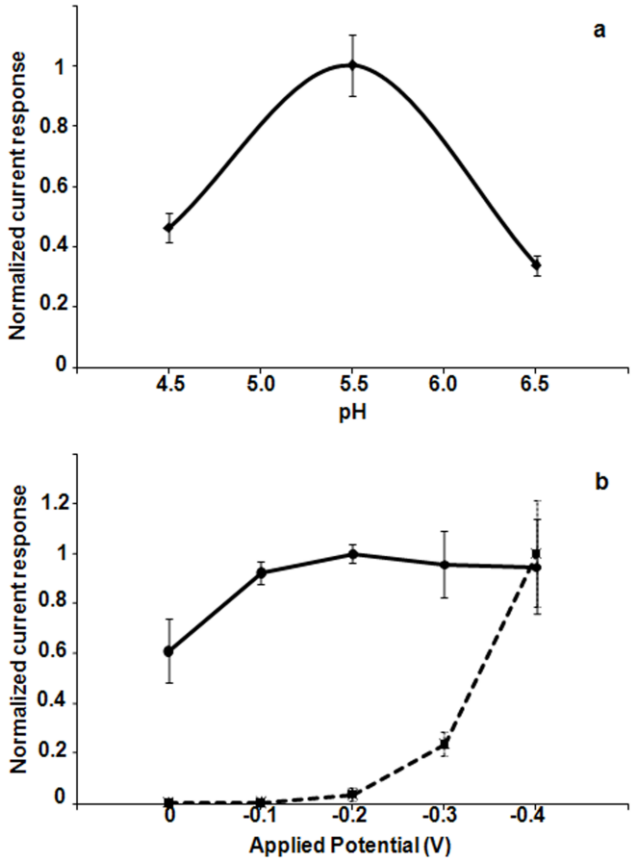


Fig. 4 Normalized amperometric responses of the Laccase biosensor to 30 μM catechol as a function of pH (applied potential: -0.2 V vs Ag pseudoreference) (a), and as a function of applied potential in the presence (●) and absence (background current) (■) of the catechol solution at pH 5.5 (b).

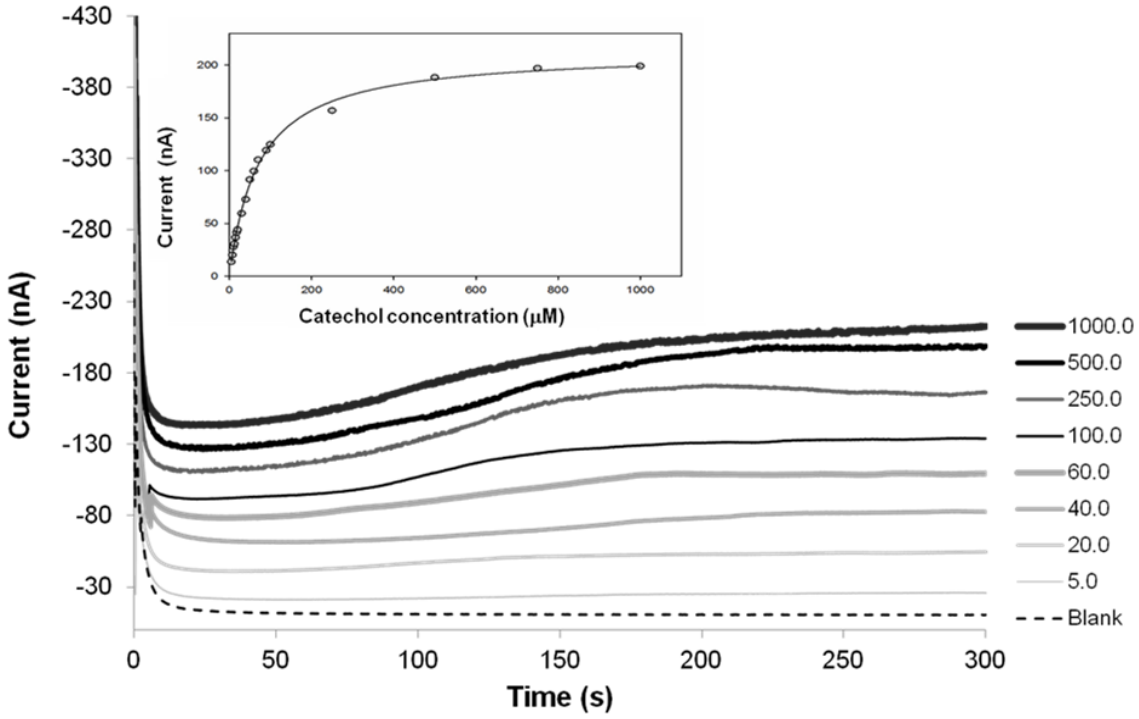


Fig. 5 Chronoamperometric responses at a Laccase biosensor for analysis of catechol and a typical calibration curve (inset) obtained in a 100mM acetate buffer at pH 5.5. Applied potential: -0.2 V vs Ag pseudoreference

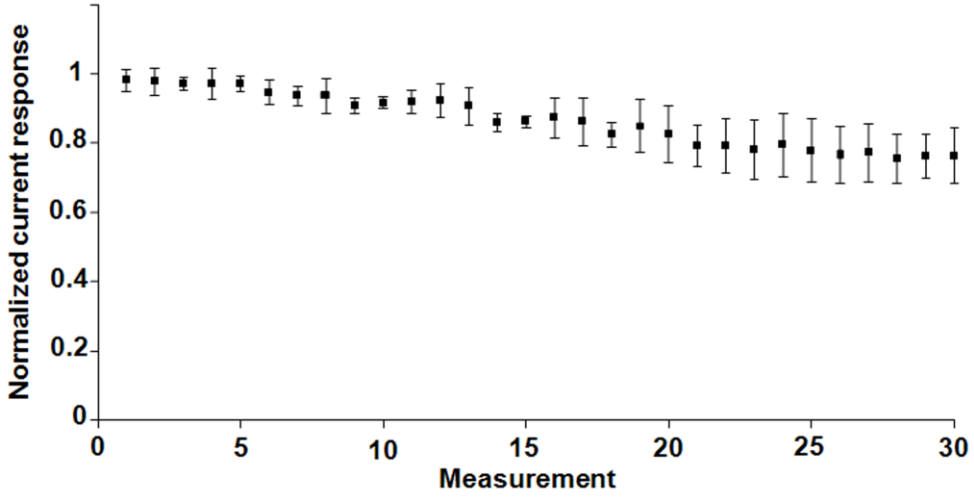


Fig. 6 Intra-day operational stability evaluated as normalized amperometric responses of the Laccase biosensor from repetitive analyses of a 30 μM catechol solution at pH 5.5. Applied potential: -0.2 V vs Ag pseudoreference

Highlights

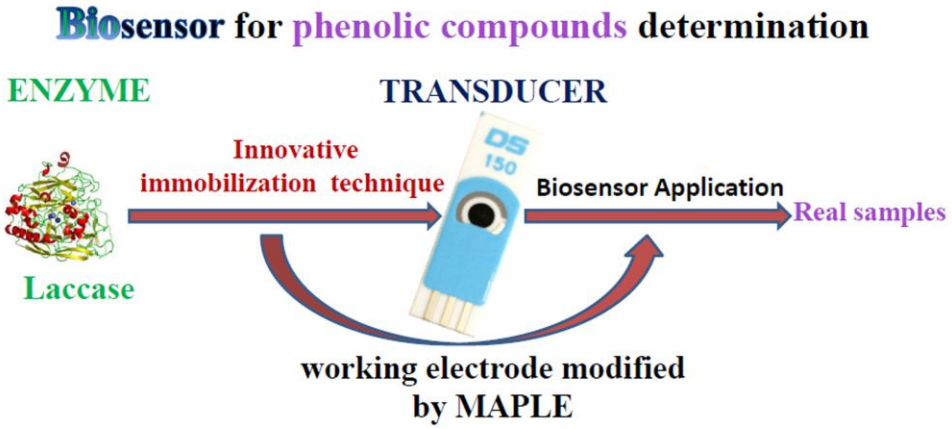
A innovative disposable biosensor based on Laccase was developed by MAPLE

The carbon screen printed working electrode was used as the deposition substrate

The biosensor showed good sensitivity, linearity, selectivity, stability and lifetime

The potential of the developed biosensor was tested in real samples

Laccase-based biosensor production on a mass-scale can be foreseen by using MAPLE



Graphical Abstract