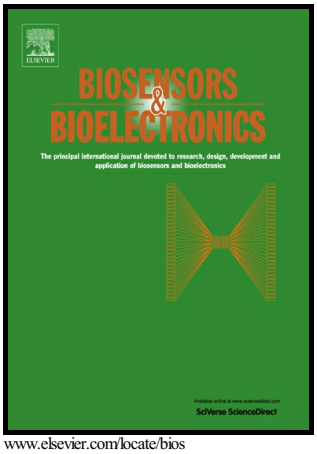

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Electrochemical sensing platform amplified with a nanobiocomposite of L-phenylalanine ammonia-lyase enzyme for the detection of capsaicin

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Abstract

The present study involves the development of a sensitive electrochemical biosensor for the determination of capsaicin extracted from chilli fruits, based on a novel signal amplification strategy using enzyme technology. For the first time, platinum electrode modified with multiwalled carbon nanotubes where phenylalanine ammonia-lyase enzyme was immobilized using nafion was characterized by attenuated total reflectance infrared spectroscopy, transmittance electron microscopy and thermo-gravimetric analysis supported by computational methods. Cyclic and differential pulse voltammetry measurements were performed to better understand the redox mechanism of capsaicin. The performance of the developed electrochemical biosensor was tested using spiked samples with recoveries ranging from 98.9 - 99.6 %. The comparison of the results obtained from bare and modified platinum electrodes revealed the sensitivity of the developed biosensor, having a detection limit (S/N=3) of 0.1863 µg mL^{-1} and electron transfer rate constant (*ks*) of 3.02 s^{-1}. Furthermore, adsorption and ligand-enzyme docking studies were carried out to better understand the redox mechanisms supported by density functional theory calculations. These results revealed that capsaicin forms hydrogen bonds with GLU355, GLU541, GLU586, ARG and other amino

acids of the hydrophobic channel of the binding sites thereby facilitating the redox reaction for the detection of capsaicin.

Keywords: Capsaicin; Phenylalanine ammonia lyase; Differential pulse voltammetry; Electrochemical biosensor; Food samples; Molecular docking.

1. Introduction

Capsaicin (8-methyl-*N*-vanillyl-trans-6-nonenamide), is an alkaloid compound found mainly in hot chilli peppers and fruits (capsicum annum and capsicum frutescence) (Reilly et al., 2001; Srinivasan, 2015). Together with its derivative, dihydrocapsaicin, they have a strongest burning effects that is believed to be evolved as a plant protection against herbivores (Supalkova et al., 2007). Capsaicinoids have been reported to have high antioxidant activity (Henderson et al., 1999), anti-tumoral (Sanchez et al., 2006), anti-bacterial (Satyanarayana, 2006) and anti-carcinogenic properties (Huynh and Teel, 2005). Capsaicin has been used for different applications in the past; for example: manufacturing of spices, chilli sauces, pain-inducing defensive pepper sprays (Pershing et al., 2006) and creams for the treatment of painful conditions such as psoriasis, rheumatoid arthritis, diabetic neuropathy, cluster headache and reflex sympathetic dystrophy (Hautkappe et al., 1998). Sanchez and co-workers have reported capsaicin as a promising anti-tumor agent in hormone-refractory prostate cancer (Sanchez et al., 2006). It has also been reported that the metabolism of capsaicinoids by enzymes such as P450 can produce reactive electrophiles capable of modifying biological macromolecules (Reilly and Yost, 2006).

Literature studies revealed that carbon nanotubes (CNTs), due to their unique sensing properties have received considerable attention in the field of electrochemical sensing. Some of their unique properties includes excellent conductivity, large surface area and good biocompatibility (Bathinapatla et al., 2015, 2016; Dresselhaus et al., 1988; Hu and Hu, 2009; Wang and Dai, 2015). For this purpose, they are widely used in electronic, biomedical, pharmaceutical, catalytic, analytical and material fields. Additionally, their special

nanostructural properties are attributed to their overwhelming advantages in fabricating electrochemical sensors.

The CNT-based electrochemical transducers offer substantial improvements in the performance of amperometric enzyme electrodes, immunosensors and biosensors (Kachoosangi et al., 2008; Lyons and Keeley, 2008; Wang, 2005). Specifically, phenylalanine ammonia-lyase received much attention for studies involving the regulation of phenolic biosynthesis. The phenolic portion of capsaicinoids is formed from phenylalanine as a product in the phenylpropanoid pathway (Reyes-Escogido et al., 2011; Sutoh et al., 2006). This enzyme possesses primary features of an electron mediator, because of its stability and good catalytic activity. Electron transfer in biological systems is one of the key reasons for considerable interest in the direct electron transfer between redox enzyme and electrode surfaces (Kuznetsov and Ulstrup, 1999).

In the past, several methods have been reported for the determination of capsaicin, namely: Scoville organoleptic test (Kachoosangi et al., 2008), high performance liquid chromatography (Othman et al., 2011; Supalkova et al., 2007), thin layer chromatography (Spanyar and Blazovich, 1969), UV-Vis spectroscopy (Othman et al., 2011), optical biosensor (Mohammad et al., 2014) and electrochemistry (Kachoosangi et al., 2008; Manaia et al., 2012; Mohammad et al., 2013; Randviir et al., 2013; Ya et al., 2012; Yardım and Şentürk, 2013).

However the classical method, 'Scoville Organoleptic Test' suffers from few drawbacks such as cost factor, lack of proximity and poor sensitivity. On the other hand, HPLC methods have some limitations like elaborate sample preparation, high cost of instrument, and sample preparation for the detection of capsaicinoids. Recently, Xue and co-workers reported an electrochemical sensor for the detection of capsaicin using mesoporous cellular foams (Xue et al., 2015). To the best of our knowledge, this is the first

electrochemical biosensor using PAL/Nafion/MWCNTs/Pt-E reported for the detection of capsaicin. The developed biosensor offers advantages such as reproducibility, repeatability, precision, accuracy and objectivity over the classical Scoville and HPLC methods. The PAL/Nafion/MWCNTs/Pt-E biosensor is simple, cost effective and sensitive compared to existing chromatographic methods.

Capsaicin is a hydrophobic molecule, characterized into 3 functional groups; the aromatic head group with hydrogen bond potentiality, the dipolar amide bond region and the hydrophobic tail (see supplementary data, Fig. S1). The approach used in this work took advantage of the characteristic feature of this molecule probing its ability to undergo redox process through enzymatic reactions. Accordingly, this work reports on the performance of a platinum electrode (Pt-E) modified with multiwalled carbon nanotubes (MWCNTs) coated with phenylalanine ammonia-lyase (PAL) enzyme infused with nafion. The PAL enzyme was chosen for the electrochemical redox reaction of capsaicin and its related compounds due to its good catalytic activity. In addition, adsorption of the enzyme on the MWCNTs along with the interaction between the enzyme and capsaicin molecule were performed using molecular docking studies. Moreover, the electrochemical methods employed in this study demonstrated the proof-of-concept that this approach can easily be incorporated into a biosensing device that is relatively simple and less expensive, in contrast to the existing Scoville test and HPLC methods used in the food industry.

2. Experimental

2.1. Instrumentation

Voltammetric measurements were carried out using a three electrode system in an electrochemical cell (Metrohm, Herisau, Switzerland) consisting of a 3 mm diameter disc-working electrode (Pt-E); Ag/AgCl as a reference (saturated AgCl, 3 M KCl) electrode, and the platinum wire as a counter electrode using a 797 VA Computrace instrument. A 781

pH/ion meter coupled with an 801 stirrer (Metrohm, Herisau, Switzerland) was used to adjust the pH of the buffer solutions at room temperature. All working solutions including the buffer were prepared with deionized water from a water purification system, Aqua MaxTM Basic 360 (Trilab, South Africa (SA)). Since MWCNTs are insoluble in most solvents, sonication in DMF was employed using an Ultra-sonic 50194 (Labcon, SA) for effective dispersion, prior to immobilization on Pt-E. The Scientific oven Series 2000 was used to evaporate DMF. The attenuated total reflectance (ATR) spectra were obtained using Perkin-Elmer FTIR, Midrand, South Africa. Thermal analysis studies were carried out on the TGA/DSC, 1 SF/1346 model operating on a STAR^{e} Software version 9.20 supplied by Mettler Toledo (Johannesburg, SA). The samples were placed in a 10 µL alumina sample holder for thermal analysis at a heating rate of 10 °C min^{-1}.

2.2. Reagents and chemicals

All chemicals were of analytical grade and used as received without any further purification. Capsaicin-360376-IG (cas no. 404-86-4) and 20-30% MWCNT basis, O.D. x L 7-12 nm x 0.5-10 µm (cas number 308068-56-6) were purchased from Sigma Aldrich (Durban, SA). *N*,*N*- dimethylformamide (DMF) (cas no. 68-12-2), glacial acetic acid (cas no. 64-19-7) and sodium acetate anhydrous were supplied by Associated Chemical Enterprises (Johannesburg, SA). Nitrogen gas (99.9 % purity) was obtained from AFROX (Durban, SA). Ethanol (absolute, 99.9 %) used for extraction of samples and nafion (cas no. 31175-20-9), were supplied by Capital Lab Supplies (Durban, SA). Phenylalanine ammonia lyase-*Rhodotorula glutanis* (PAL) (101M8617) was purchased from Sigma-Aldrich, USA.

2.3. Preparation of working solutions

Sodium acetate buffer solution of pH 4.0 was prepared by mixing sodium acetate and acetic acid (0.1 M each) in a ratio of 15:85 respectively. This solution was then stored at 4 °C until used. The solution of PAL enzyme was prepared by adding approximately 3.0 mg into 1

mL of 67 mM phosphate buffer solution (pH 7.4). The standard solution of capsaicin was prepared by dissolving approximately 10 mg capsaicin standard powder in 100 mL of absolute ethanol (99.9 % purity) to produce 100 mg L^{-1} standard solution and refrigerated at 4 °C.

2.4. Preparation of PAL/Nafion/MWCNTs/Pt-E

The bare Pt-E was prepared manually by polishing on a mirror like surface with an alumina slurry (≤ 3 μm) and then rinsed with distilled water. Then, the MWCNTs previously dispersed in DMF (5 mg in 1 mL) and sonicated for 5 min were immobilized onto the surface of the Pt-E by dropping 10 μL aliquot and oven-dried at 50 °C. Later, ~10 μL of 5 % nafion solution was casted to form a film on MWCNTs/Pt-E. The PAL enzyme was physically adsorbed on the Nafion/MWCNTs/Pt-E surface by dropping 10 μL and allowing the solvent to dry at room temperature for 2 h. The modified PAL/Nafion/MWCNTs/Pt-E electrode was then rinsed with the buffer solution prior to the electrochemical determinations. The optimized electrochemical detection conditions were as follows: 10 mL of 0.1 M acetate buffer solution pH 4.0 used as an electrolyte, 0.2 mL chilli extract and scan rate of 0.01 V s^{-1}.

2.5. Sample preparation

Capsaicin was extracted from chilli fruits as reported in the Methods section (Mpanza et al., 2014; Othman et al., 2011). Briefly, 100 g of the crushed chilli pepper fruits were transferred into a 500 mL round bottom flask containing 350 mL of absolute ethanol. The mixture was refluxed for 2 h and the solid residue was removed by filtration through a Whatman filter paper of 0.45 μm pore size and discarded. Furthermore, the reddish-brown filtrate was distilled to remove the excess ethanol from the extract. The remaining chilli extracts were refrigerated at 4 °C for subsequent electrochemical studies.

2.6. Computational methodology

2.6.1. Procedure for interaction of PAL with capsaicin using molecular docking

The crystal structure of the PAL enzyme was obtained from the RCSB Protein Data Bank (ID1T6J) (Calabrese et al., 2004) in a complex form. The whole enzyme was selected and hydrogen atoms were added. Then the water and the residue molecules present in the enzyme structure were removed. Furthermore, the structure was cleaned to have a desirable confirmation. The CHARMm forcefield was applied to enzyme while the protonation occurred at pH 7.4, corresponding to an ionic strength of 0.145. The ionization and residue *pKa* demonstrated that the enzyme possessed a zero charge at pH 7.7 and electrostatic energy of -36.00 kJ mol^{-1}.

Capsaicin ligand obtained from pubchem (CID: 1548943) database was optimized with Gaussian 09 (Frisch et al., 2009) and thereafter the conformation with the lowest energy was used for the docking simulation using Discovery Studio 4.0 (Wu et al., 2003). Docking studies were performed using the CDOCKER module in Discovery studio 4.0, whereby the PAL enzyme was held rigid while the capsaicin ligands were flexible. The CHARMm forcefield was used as an energy grid forcefield for docking and scoring function calculations. The conformation with the highest docking score obtained by CDOCKER energy was used for the binding energy calculations.

3. Results and discussion

3.1. Characterization of PAL/Nafion/MWCNTs/Pt-E

The TEM image obtained for PAL/Nafion/MWCNTs nanobiocomposite was shown in Fig. 1A. There was no sputtering done onto samples prior to microscopic studies as the resolution of the nanotubes was adequate. The PAL/Nafion/MWCNTs nanobiocomposite exhibited a more dense structural morphology with a hollow wired structure of the regular pure MWCNTs. The enzyme immobilization, on the surface was not attached in a unique fashion. The ATR-IR spectrum in Fig. 1B was recorded in the range 4000 to 500 cm^{-1}. The spectrum of pure MWCNTs showed a peak at 1740 cm^{-1}, assigned to the stretching mode of

C=C bonds that formed the carbon nanotubes side wall framework. The peak observed at 2962 cm^{-1} corresponds to C-H asymmetric and symmetric stretching modes. However, a less intense and broader peak observed at 3312 cm^{-1} corresponds to the O-H group of the carboxylic acid. In the case of PAL/Nafion/MWCNTs spectrum, a broader peak at 3312 cm^{-1} is attributed to the O-H stretching, whereas peaks observed at 2962 cm^{-1} corresponds to C-H stretching bands. The peak observed at 1638 cm^{-1} is assigned to the C=C bond stretch while the 1037 cm^{-1} peak attributed to an aromatic C-H bend. The pure nafion spectrum showed C-O-C, S-O and CF_2 stretching vibrations at 871 cm^{-1}, 1045 cm^{-1} and 1077 cm^{-1} respectively. The spectrum also showed a broader O-H stretching at 3312 cm^{-1} and C-H alkyl group stretching at 2963 cm^{-1}. The PAL enzyme spectrum has an amide bond peak observed at 1639 cm^{-1} that remains prominent in the final composite used for Pt-E modification.

The electrode composite was further studied for thermal stability and quantification of the composition by thermal gravimetric analysis (TGA) as illustrated in Fig. 1C. This approach was very informative for understanding the thickness of the Pt-E surface corresponding to the modification of each layer. The MWCNTs curve showed a change in mass (decomposition temperature) at 350 °C, another typical gradient drop in mass is noticed at about 750 °C, due to the oxidation of carbon (Bom et al., 2002). The MWCNTs modified with PAL enzyme showed a TGA profile with two shoulders observed at 56 °C and 248 °C, with a mass loss of 30.91 % and 48.50 %, respectively. The first mass loss phase in PAL/MWCNTs curve can be attributed to the irreversible thermal denaturation of the enzyme, normal dehydration process of PAL and the loss of DMF which was used as a dispersion medium. Beyond 248 °C a further drop in mass percentage is observed due to the melting and decomposition processes of PAL around 300 °C. The PAL/Nafion/MWCNTs curve showed similar trends to that of PAL/MWCNTs, but with a higher loss of 58.75 % in the first step followed by a 36.07 % loss. Interestingly, there is an additional mass loss step of

2.32 % between 390 ^{o}C and 400 ^{o}C, beyond that 3.32 % is left behind with a slightly greater than 2.02 % that is retained from PAL/Nafion/MWCNTs. This conforms that the addition of PAL/Nafion does improve the thermal stability of the nanobiocomposite and most likely the ionic polymer, nafion, improve the interfacial adhesion of PAL on the MWCNTs.

3.2. Electrochemical characterization of PAL/Nafion/MWCNTs/Pt-E

Electrochemical determination of capsaicin was explored by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) signals. The analyses were conducted using bare and modified Pt-E with PAL/Nafion/MWCNTs as depicted in Scheme 1. The Pt-E has been widely used for several decades because of its excellent corrosion resistance and good electrical conductivity.

The bare Pt-E was mechanically polished as described in previous studies (Bathinapatla et al., 2015, 2016; Mpanza et al., 2014) and thereafter modified to obtain the CV and DPV scans are illustrated in Fig. 2A-B. In both the scans it is evident that a biosensor exhibited higher capsaicin peak currents than the bare and PAL/Nafion/MWCNTs/Pt-E respectively.

In Fig. 2A, the CV scans of a bare Pt-E showed only prominent oxidation peak (*pa1*) at 0.58 V with 9.55 µA and reduction peak (*pc2*) at 0.32 V. The non-existence of the second peak (*pa2*) around 0.35±0.03 V is noticed which raises concern about the sensitivity of the bare Pt-E. When MWCNTs were introduced, the second oxidation peak (*pa2*) was detected at 0.38 V. This is a good reflection of the improvement in the sensitivity of the electrode upon addition of MWCNTs to the bare Pt-E. Moreover, the presence of MWCNTs changed the detection capability of the electrode completely as the current of *pa1* increased to 51.75 µA and therefore, there is a significant difference in the current signal of both CV and DPV in contrast to the bare Pt-E. The PAL enzyme further enhanced sensitivity of the modified electrode to 59.37 µA; this is attributed to a better surface area and electrical conductivity of

the nanobiocomposite, resulting in an additional reduction peak (*pc1*) observed at 0.57 V. The DPV scans in Fig. 2B also followed the same pattern as CV scans (Fig. 2A), where the introduction of MWCNTs drastically increased the sensitivity of the Pt-E. This confirmed that the electrochemical oxidation of capsaicin at the PAL/Nafion/MWCNTs electrode was a diffusion-controlled process described by the following equation for *pa1* and *pa2*:

$$E_p - E_{p/2} = \frac{1.857RT}{\alpha F} = \frac{47.7}{\alpha} mV \quad (1)$$

Where, E_p and $E_{p/2}$ represent the peak potential and the half-height, α is the electron transfer coefficient, *R* and *F* represent the usual significance (Li et al., 2015). The charge transfer coefficient (α) was calculated to be 0.25 and of 0.17 for *pa2* and *pa1*, respectively. Thereafter, the electron transfer rate constant (*ks*) was calculated using Laviron's equation at modified electrode for a charge transfer coefficient (α) of 0.42 at 100 mV s^{-1} on a quasi-reversible reduction peak at 0.1 V (versus Ag/AgCl) (Laviron, 1979a, b, c).

$$logks = \alpha \log (1-\alpha) + (1-\alpha) \log \alpha - \log \frac{RT}{nFv} - \frac{(1-\alpha)\alpha nF\Delta E_p}{2.3RT} \quad (2)$$

Where, *R* is the gas constant (8.314 J K^{-1} mol^{-1}), *T* is the temperature (298 K), *F* is the Faraday constant (96,485 C mol^{-1}), Δ*E*p represents the peak-to-peak separation (Δ*E*p = (E_{pa}+E_{pc})/2), *v* is the scan rate in V s^{-1}; *n* is the number of electrons transferred. The *ks* value of the PAL/Nafion/MWCNTs/Pt-E was calculated to be 3.02 s^{-1}. One can deduce that this is probably due to larger surface area of the Nafion/MWCNTs/Pt-E in comparison to the bare Pt-E, and due to the catalytic activity of PAL enzyme on the modified Pt-E. Furthermore, higher the peak current for PAL/Nafion/MWCNTs/Pt-E and MWCNTs/Pt-E signals compared to bare Pt-E, stronger the adsorption effect on modified Pt-E than on the bare Pt-E. It was interesting to notice that the oxidation and reduction peak heights are significantly increased in the order of bare Pt-E < MWCNTs/Pt-E < PAL/Nafion/MWCNTs/Pt-E. The PAL/Nafion/MWCNTs/Pt-E superiority over bare Pt-E is based on it catalytic activity,

surface area and significant diffusion process of capsaicin on a modified Pt-E based electrochemical biosensor.

3.3. Optimization of pH and scan rate based on peak currents

Using the nanobiocomposite PAL/Nafion/MWCNTs/Pt-E, the influence of the pH ranging from 4.0 to 6.5 on the detection of capsaicin was evaluated. Fig. 2C shows almost all the peaks shifted linearly towards a lower potential with an increase in pH. However, the corresponding current density dropped significantly in the case of *pa2* and *pc2*, indicating that the redox mechanisms corresponding to the catechol and 1, 2 benzoquinone rings were not favoured at higher pHs.

The effect of pH on both the current and potential of *pa1* and *pa2* are illustrated in Fig. 2D. Similar behaviour were observed for the scan rates between 0.1 to 1.0 V s^{-1} for capsaicin in 0.1 M acetate buffer of pH 4.0. The relationship between E_{pa} and ln v (see supplementary data, Fig. S2) of the scan rates followed the equations: $E_{pa2} = 0.102 \ln v + 0.5252$ ($R^2 = 0.9888$) and $E_{pa1} = 0.0937 \ln v + 0.7843$ ($R^2 = 0.9608$). The redox reaction in the last step (III) of the mechanism (Scheme 2) was displayed in CV as *pc2* and *pa1*. At an optimum scan rate of 100 mV s^{-1}, PAL/Nafion/MWCNTs/Pt-E showed 79.37 µA of the peak current which was 8-fold compared to peak current measured at bare Pt-E (9.55 µA) indicated a fair catalytic activity of the nanobiocomposite materials.

3.4. Redox mechanism of capsaicin

From the mechanism for the electrochemical oxidation/reduction of capsaicin (Kachoosangi et al., 2008) shown in Scheme 2, illustrates that the chemical structure in "I" has a guaiacol ring, a benzene with –OH and OCH_3 substituents. It losses H^+ from the alcohol group forming the intermediate structure "II" which further breaks the ethyl group through hydrolysis in acidic medium to become benzoquinone. The benzoquinone is much more stable than the guaiacol ring and therefore, one reversible peak is observed in the CV due to

the formation of the catechol with the gain of $2e^-$ and $2H^+$ ions. Overall, it is significant that the conversion of capsaicin by losing H^+ ions (guaiacol to benzophenone) is a forward reaction attributed to *pa1* in Fig. 2A. The reaction between benzoquinone and catechol is reversible as represented by *pc1* and *pc2*, respectively in Fig. 2A (Kachoosangi et al., 2008; Xue et al., 2015).

3.5. Reproducibility, repeatability and stability of PAL/Nafion/MWCNTs/Pt-E

The reproducibility and repeatability of the developed electrochemical biosensor were evaluated at 0.25 µg mL^{-1} of capsaicin. The obtained current variation measurements using the PAL/Nafion/MWCNTs/Pt-E suggests acceptable repeatability with RSD values ranging from 4.56 - 7.31 % (n = 6). The reproducibility measurements were obtained using the different electrodes for same capsaicin concentration resulting in promising RSD values of 2.10 - 4.93 % (n = 6). The obtained RSD values indicates that the results were reproducible using PAL/Nafion/MWCNTs/Pt-E. The stability of the developed electrochemical biosensor was studied by determining the current response in 0.25 µg mL^{-1} of capsaicin for six replicate measurements (n = 6) over three successive days using the same coating. The electrochemical biosensor maintained 91 % of its original response by the third day, hence its activity was retained to a large extent. The data obtained by PAL/Nafion/MWCNTs/Pt-E for the detection of capsaicin was compared with the already reported methods in the literature as shown in Table 1.

3.6. Effect of interferences on detection of capsaicin

The selectivity of the present electrochemical biosensor towards capsaicin was evaluated by the simultaneous addition of some common organic and inorganic compounds. The interferents such as catechol (25 µg mL^{-1}) and their derivatives (urushiol, catecholamine, catechin) (25 µg mL^{-1}), 4-chlorophenol (25 µg mL^{-1}), dichlorophenol (25 µg mL^{-1}), trichlorophenol (25 µg mL^{-1}), pentachlorophenol (25 µg mL^{-1}), Na^+ (25 µg mL^{-1}), Mg^{2+} (25

µg mL^{-1}), and K^+ (25 µg mL^{-1}) were added to 0. 45 µg mL^{-1} of standard capsaicin solution and then current responses were measured. The obtained results revealed that there were no changes in the current response. The applied tolerance limit for the interference species at the maximum concentration resulted in a relative error of ±5%. However, 50-fold catechol and their derivatives and 10-fold 4-cholorophenol, dichlorophenol, trichlorophenol, pentachlorophenol, Na^+, Mg^{2+} and K^+ had no effect on the detection of capsaicin. Therefore, our results indicates that the developed electrochemical biosensor, PAL/Nafion/MWCNTs/Pt-E has an excellent selectivity for the detection of capsaicin.

3.7. Real sample analysis

In order to convert an electrochemical sensor into a biosensor, the PAL enzyme was immobilized on the Nafion/MWCNTs/Pt-E. The developed electrochemical biosensor contributed to a better performance and higher sensitivity for the detection of capsaicin in real samples. The capsaicin peaks observed in Fig. 3A, with a correlation coefficient of R^2 = 0.9987, slope of 0.189 ± 0.003 and the intercept of 10.781±0.213 µA.

The PAL enzyme improved the interfacial adhesion through carboxylation with MWCNTs under acidic conditions and anchored onto the MWCNTs exhibiting catalytic activity and promoting the direct electron transfer for the detection of capsaicin which enhances the sensitivity of electrochemical biosensor. In 0.1 M acetate buffer solution (pH 4.0), the DPV response of the PAL/Nafion/MWCNTs/Pt-E sensor to capsaicin increased about 8-fold as compared with the Nafion/MWCNTs/Pt-E sensor, with a detection limit of (S/N =3) 0.1863 µg mL^{-1}. The capsaicinoid equivalent extracted from the chilli pepper fruits showed a mass percentage of 67.08 % and 68.30 % (m/m) with a relative standard deviations of 2.32 (n=6) and 1.92 (n=6) using electrochemical biosensor and HPLC, respectively. Clearly, the poor standard deviation is attributed to inconsistencies with the regeneration of exactly the same size of the nanobiocomposite onto the electrode surface. However, the

percentage recoveries ranging from 98.9 - 99.6 % (n =6) obtained from this study, suggests that the PAL/Nafion/MWCNTs/Pt-E biosensor is a superior electrochemical biosensor for the detection of capsaicin.

3.8. HOMO-LUMO calculations and molecular docking analysis

The electrons transferred from the highest occupied molecular orbitals (HOMO) in a molecule are related to its ability to undergo redox reactions. During the reduction process, the electrons move to the lowest unoccupied molecular orbital (LUMO). Therefore, using the density functional theory (DFT) calculations, the density of the electrons in the molecule can be located thereby predicting the active site of the ligand/molecule based on the electron density maps. The ability of the molecule to undergo redox reactions is dependent on the functional groups or atoms identified through DFT calculations. This should be in agreement with the redox mechanisms and the extent of HOMO-LUMO band gaps of the molecule (Bathinapatla et al., 2016). In this study, the geometry of the molecule was fully optimized at the DFT level using the 6-31+G(d) basis set. The loosely bound electrons in the HOMO shown in Fig. 3B are located in the carbonyl group of the guaiacol ring, in contrast to those displayed in the LOMO.

The redox mechanism (see Scheme 2) for capsaicin is a quasi-reversible reduction reaction between the -OH and R-O-R groups of the guaiacol ring and the residues of the enzyme resulting in the reversible catechol ring. Therefore, the electron density is in agreement with redox mechanism as shown in Scheme 2. Docking studies were used to assess the chemical interaction between the capsaicin and the PAL enzyme.

In order to ensure that the prepared ligand mimicked the experimental conditions used, it was allowed to ionize over a pH ranging from 6.5 to 8.5, whilst the tautomers were enumerated. Thereafter, full minimization was performed using a smart minimizer algorithm coupled with the CHARMm forcefield (Wu et al., 2003) using Discovery Studio 4.0. From

the same ligand, a total of ten poses were generated with Monte-Carlo ligand conformation generation, and docked using a LigandFit shape filter into an active site of the PAL enzyme with 573.15 $Å^3$ that was derived based on the PDB site record (Venkatachalam et al., 2003). All ten poses were included in the calculation of the binding energies during which the ligand flexible receptor atom properties were created by In Situ Ligand Minimization that enabled the ligand optimization in the binding pocket of the PAL enzyme (Tirado-Rives and Jorgensen, 2006). The energy minimization involved 1000 steps of steepest descent with a RMS gradient tolerance of three followed by conjugate gradient minimization performed with a smart minimizer algorithm. Further analysis revealed that the three π-alkyl interactions with average distances of 4.75, 4.67, 5.03, and 4.51 Å were involved between PAL enzyme and capsaicin. Interestingly these interactions occurred on the hydrophobic end of capsaicin (Fig. 3C).

The hydrophobic map of the PAL enzyme revealed no hydrophobicity in the site and this led to the penetration with hydrogen bonds and hydrophilic interactions. The analysis of ten ligand poses (see supplementary data Table S1) revealed that ARG 596 residue was most favourable because of a stronger hydrogen bonding followed by ARG 450 with the highest hydrophobic counts. However, the conformation with the highest Docking Score (see supplementary data, Table S2 and Figs S3-5) had a seven hydrogen bonds (O---H) with the shortest distance of 1.61 Å between LYS545 and O1 of the carbonyl. Among these bonds, four of them involved oxygen atoms of the GLU residues as an acceptor for protons from the capsaicin molecule. Among the surrounding residues, GLU355 formed three hydrogen bonds, as the most interactive residue. Further analysis suggested only two hydrophobic-alkyl bonds for residues PRO 453 and ILE 450 with 4.61 Å and 4.42 Å. The carbonyls of the capsaicin molecule appeared to be the focal point of interaction, due to the participation of three

hydrogen bonds. These results confirm that the PAL enzyme facilitated the electron transfer from the capsaicin ligand, hence improving the biosensing response.

4. Conclusions

In this paper, a nanocomposite based electrochemical biosensor was developed for the detection of capsaicin, characterized by TEM, TGA, CV and DPV techniques and supported by computational studies. The results revealed that the present electrochemical biosensor showed excellent reproducibility, high sensitivity, steady coating and higher exchange current density for the detection of capsaicin from chilli extracts. Additionally, their magnitudes of the voltammetric signals ranged in the order of PAL/Nafion/MWCNTs/Pt-E > MWCNTs/Pt-E > bare Pt-E, suggesting the role of the PAL enzyme in the signal amplification. Interestingly, our studies confirm that the interaction of the PAL enzyme with the phenolic component of capsaicin is key to the improvement in terms of sensitivity of the electrochemical biosensors. Moreover, HOMO-LUMO calculations obtained at the DFT level, were helpful in the prediction of the most appropriate functional groups or atoms undergoing oxidation or reduction reactions. Molecular docking analysis on the other hand, further revealed that capsaicin showed a stronger tendency to bind with the PAL enzyme *via* hydrogen bonding and hydrophobic interactions. These results confirmed that phenylalanine ammonia enzyme facilitated the electron transfer from the capsaicin ligand, in accordance with the 8-fold signal amplification observed by differential pulse voltammetry. The superior performance of the PAL/Nafion/MWCNTs/Pt-E provided a promising alternative in routine sensing applications of capsaicin in the food industry.

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Fig. 1 TEM image of (**A**) PAL/Nafion/MWCNTs nanobiocomposite (**B**) ATR-IR spectra of MWCNTs, PAL enzyme, PAL/MWCNTs, Nafion and PAL/Nafion/MWCNTs (**C**) TGA curves of (i) MWCNTs, (ii) PAL/MWCNTs and (iii) PAL/Nafion/MWCNTs.

Fig. 2 Voltammograms for (**A**) Cyclic and (**B**) Differential pulse voltammetric responses of capsaicin at 0.1 V (vs Ag/AgCl) with the bare Pt-E (curve a), MWCNTs/Pt-E (curve b) and PAL/Nafion/MWCNTs/Pt-E (curve c). (**C-D**) Effect of pH on peak potential (V) and peak current (I_p) for electrochemical detection of capsaicin between 4.0 and 6.5. Optimized conditions: Deposition potential = 0.5 V; Deposition time = 60 s; Scan rate = 0.1 V s^{-1} for 5 cycles.

Fig. 3 (A) Differential pulse voltammogram of capsaicin sample (0.2 mL) from chilli pepper fruit extract in 0.1 M acetate buffer solution of pH 4.0; scan rate of 0.013 V s^{-1}; deposition time of 60 s using PAL/Nafion/MWCNTs/Pt-E. **(B)** (i) Highest occupied molecular orbitals (HOMO) and [ii] lowest occupied molecular orbitals (LUMO) of capsaicin and [iii] is the Alpha molecular orbitals of capsaicin obtained with DFT level 6-31+G(d) basis set **(C)** Molecular docking complex of the capsaicin into PAL enzyme binding site.

Scheme 1. Pt-E modification with PAL/Nafion/MWCNTs for the detection of capsaicin.

Scheme 2. Mechanism for the electrochemical oxidation/reduction of capsaicin. Pa and Pc refers to the anodic and cathodic peaks respectively.

Table 1 Comparison of present electrochemical biosensor with the reported analytical methods for the detection of capsaicin.

Analytical Method	Electrode Modification	Analytical Parameters		Merits/Demerits	Citation
		R^2	LOD's		
LSV/SWV/ DPV/ Amperometry	MCFs/CPE	0.9990	0.08 µM	Good Linearity, low detection limits, excellent performance, but less stable	(Xue et al., 2015)
Amperometry	HRP/Ferrocene	0.998	1.94 µM	Biosensor is simple but less stable and sensitive	(Mohammad et al., 2013)
AdsSV	Boron-Doped Diamond Electrode	0.986	0.034 µM	The detection limits were satisfactory, but the electrode is highly expensive	(Yardım, 2011)
SPME-GC-MS	-	0.9970	0.014 µg mL^{-1}	Low detection limits were achieved, but very expensive instrumentation	(Peña-Alvarez et al., 2009)
AdsSV	MWCNT/SPE	0.996	0.31 µM	Fair performance, but the detection limits were high and life time of the electrode is less	(Kachoosangi et al., 2008)
UV-Visible	MBTH/Sol-gel/Butyl acrylate/HRP/Chitosan	0.986	51.92 ppm	The technique is cost effective, but LOD's were very high. The electrode modification is complicated involves too many components with poor performance	(Mohammad et al., 2014)
CE	-	0.9994	0.66 µg mL^{-1}	High detection limits	(Liu et al., 2010)
HPLC	-	0.998	0.09 µg g^{-}	Expensive technique and	(Othman et al.,

			^{1}	elaborate sampling procedure is required	2011)
AdsSV	Pencil Graphite Electrode	0.997	1.12 ng mL^{-1}	Cost Effective electrode modification with excellent detection limit	(Yardım and Şentürk, 2013)
EIS-CV	MWCNT-SPE	0.99	0.45 µM	The electrode modification is cost effective, but achieved high detection limits	(Randviir et al., 2013)
LSV	MS-NH_2-FMS-CPE	0.9965	0.020 µmol L^{-1}	Good Recoveries with low detection limit	(Ya et al., 2012)
DPV	PAL/Nafion/MWCNTs/Pt-E	0.9987	0.1863 µg mL^{-1}	Good reproducibility, repeatability, stability of the electrode material. Fair recoveries were achieved. PAL/Nafion/MWCNTs/Pt-E is an anti-interferent material for capsaicin detection	This work

SPME: Solid Phase Micro Extraction, GC-MS: Gas Chromatography-Mass Spectroscopy, HPLC: High Performance Liquid Chromatography, CE: Capillary Electrophoresis, EIS: Electrochemical Impedance Spectroscopy, LSV: Linear Sweep Voltammetry, SWV: Squarewave Voltammetry, DPV: Differential Pulse Voltammetry, AdsSV: Adsorptive Stripping Voltammetry, CV: Cyclic Voltammetry, CPE: Carbon Paste Electrode, SPE: Screen Printing Electrode, MCFs: Mesoporous Cellular Foams, HRP: Horseradish Peroxidase, MWCNTs: Multiwalled Carbon Nanotubes, MBTH: 3-methyl-2-benzothiazolinone hydrazine, MS: Mesoporous Silica, NH_2-FMS: Amino-functionalized mesoporous silica, PAL: Phenylalanine Ammonia Lyase

Highlights

- Novel nanocomposite of L-phenylalanine ammonia-lyase enzyme for the detection of capsaicin
- PAL/Nafion/MWCNTs/Pt-E promote the electron transfer and provide enhanced performance.
- Ultrasensitivity and selectivity for capsaicin determination.

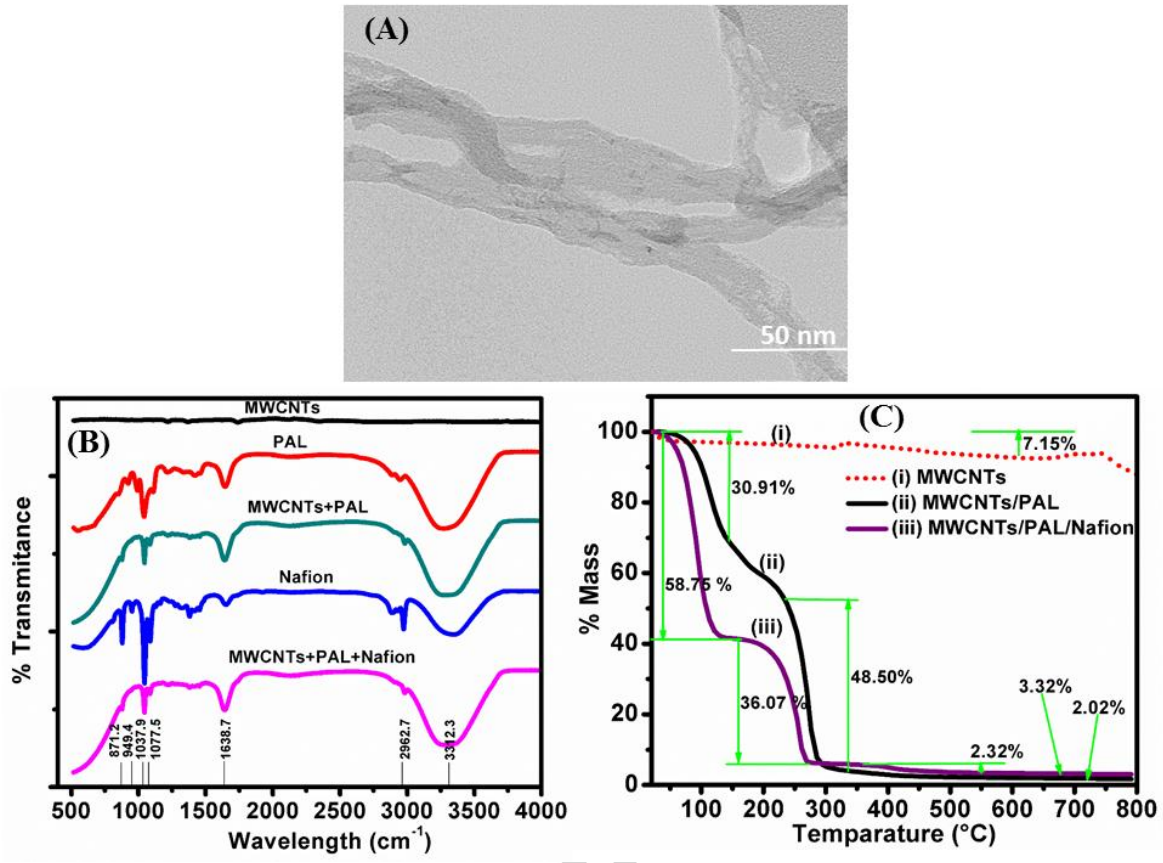
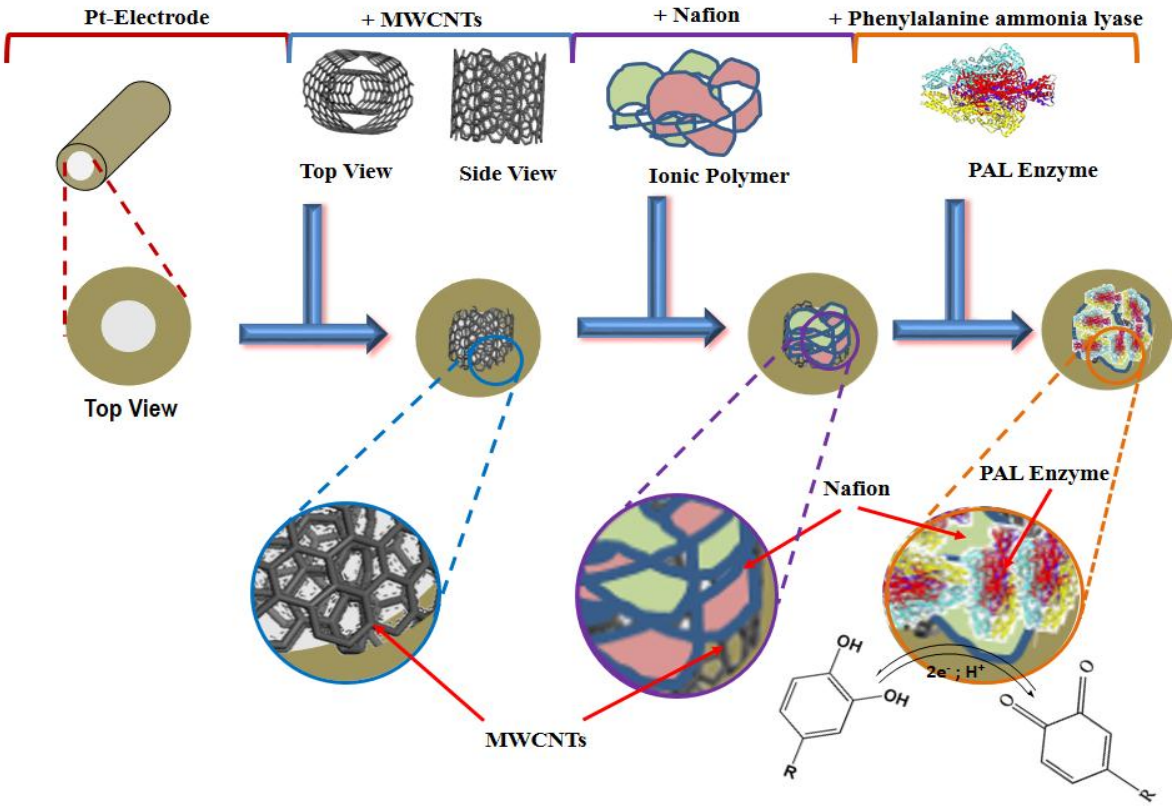


Fig. 1



Scheme 1

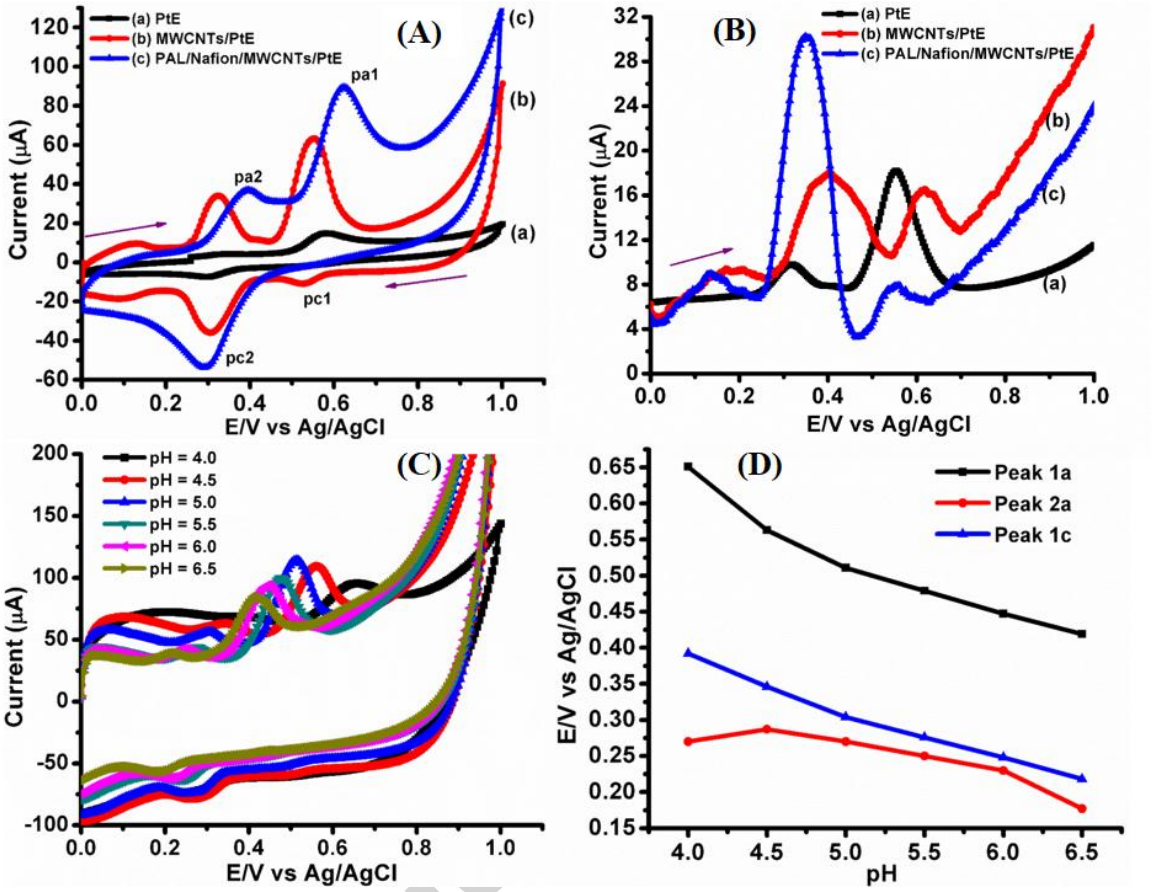
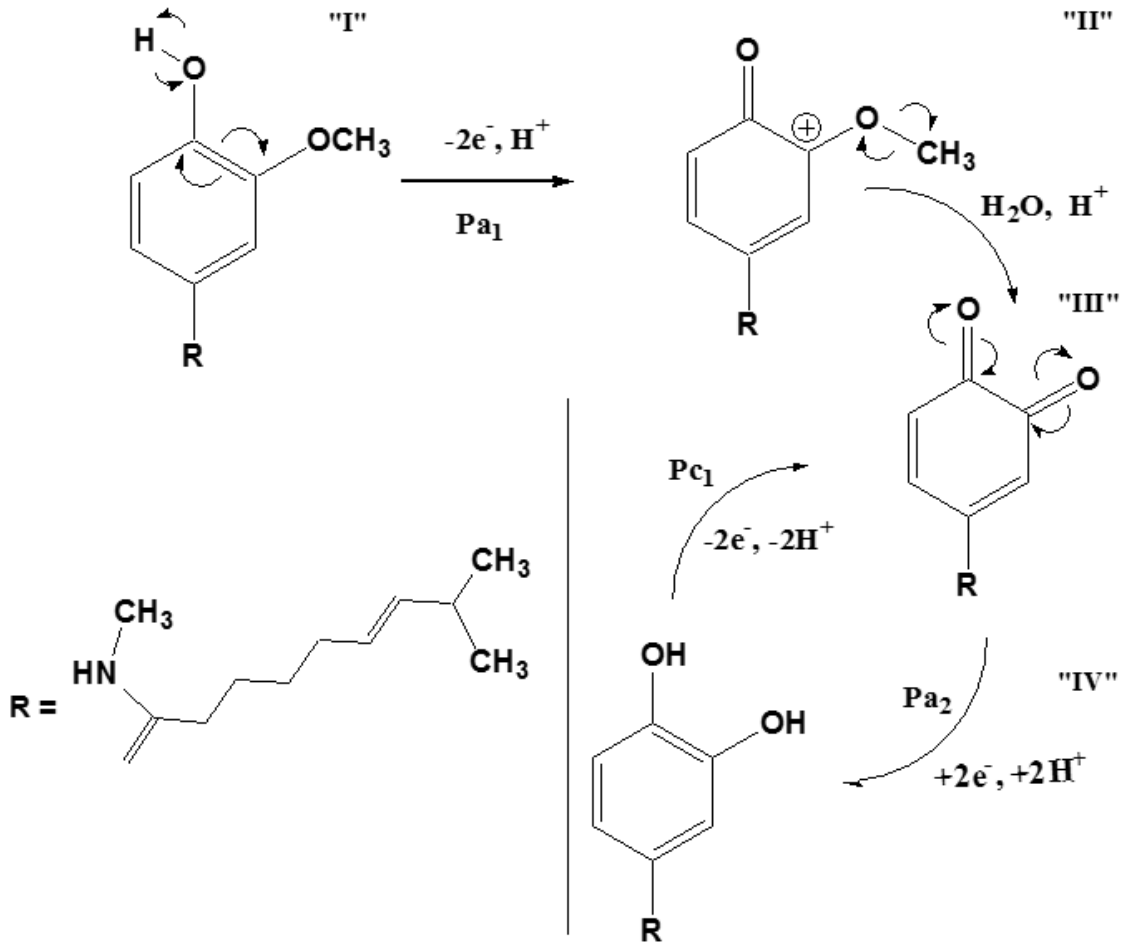


Fig. 2



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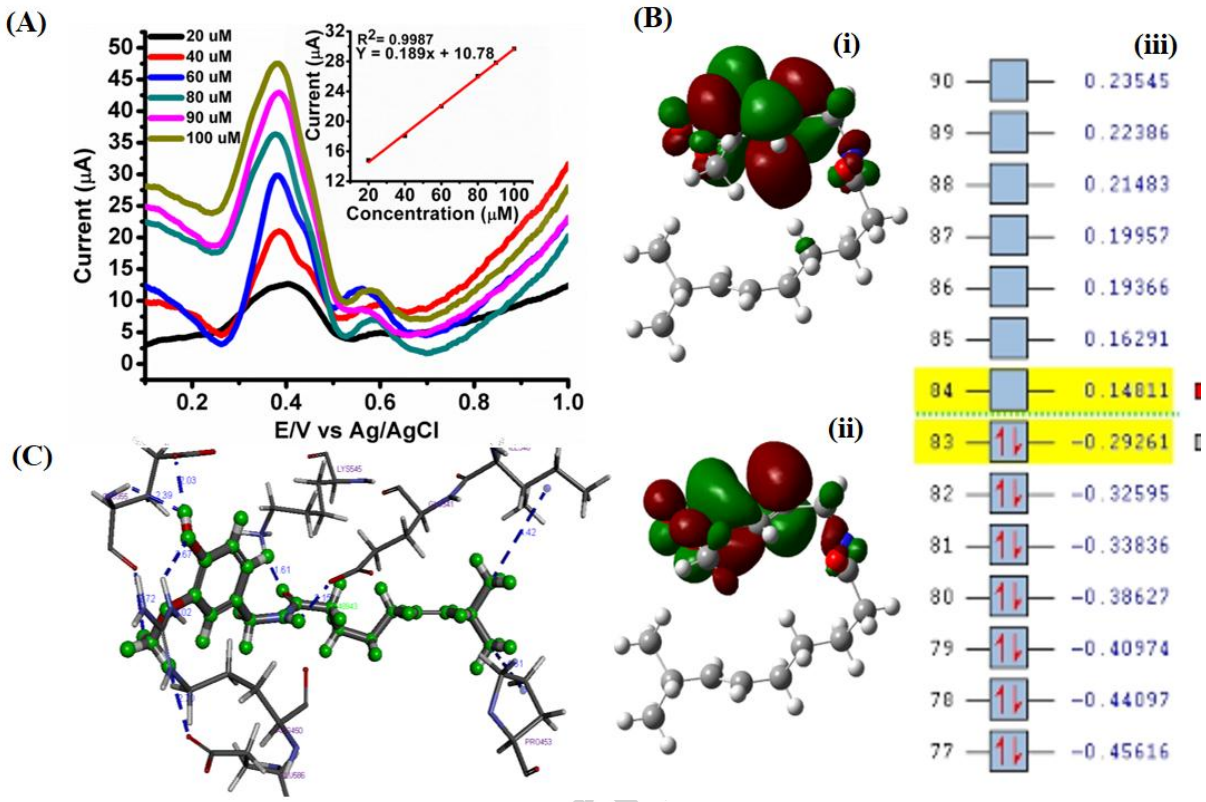

Fig. 3