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Study of a new solid carbon paste tyrosinase-modified amperometric biosensor for the determination of catecholamines by high-performance liquid chromatography

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Abstract

The performance of a tyrosinase-modified "solid carbon paste" electrode (SCPE) as electrochemical detector has been studied in comparison with a glassy carbon electrode detector in high-performance liquid chromatography for the simultaneous determination of dopamine, 3,4-dihydroxyphenylacetic acid, norepinephrine and homovanillic acid. The influence of pH, flow-rate and amount of organic solvent in the mobile phase on the biosensor response was investigated. The stability and selectivity of the detector were significantly affected by the mobile phase pH. No effect of 2% isopropanol in the mobile phase was observed. The biosensor response was fast, reproducible, highly sensitive and linear over the concentration range 0.09 μM–1 mM (detection limit of ca. 290 pg of neurotransmitter injected).

Keywords: Biosensors; Electrochemical detection; Detectors, LC; Solid carbon paste electrode; Tyrosine-modified electrode; Enzyme-immobilized electrodes; Catecholamines; Dopamine; 3,4-Dihydrophenylacetic acid; Norepinephrine; Homovanillic acid

1. Introduction

The application of amperometric biosensors in the food industry, environmental control [1], medicine [2,3], biotechnology, etc., is gaining interest. With respect to automation, biosensors showing fast response rates may be successfully applied in hydrodynamic systems, e.g. in flow injection analysis (FIA) [4] and in HPLC [5–12]. Relatively few enzyme-immobilized electrodes have been successfully applied as electrochemical detectors in HPLC mainly because of poor enzyme and electrode matrix stability and due to both the low stability and low response rates under hydrodynamic conditions. Mus-

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hroom tyrosinase is a polyphenol oxidase which contains a $Cu^{1+/2+}$ redox couple and which, by the use of labeled oxygen, has been shown to catalyse the *o*-hydroxylation and subsequent oxidation of (di-)phenolic compounds as follows [13]:

$$PhOH + O_2^* + 2H^+ \xrightarrow{\text{Tyrosinase}} Ph(OH)(O^*H) + H_2O^* \quad (1)$$

$$2Ph(OH)_2 + O_2^* \xrightarrow{\text{Tyrosinase}} 2PhO_2 + 2H_2O^* \quad (2)$$

PhOH, $Ph(OH)_2$, PhO_2 and O* are phenol, catechol, quinone and isotopically labelled oxygen, respectively. Several tyrosinase-modified electrodes have already been developed which can be used to detect the quinones generated by the enzymatic reaction at the electrode surface polarised at −200 mV vs. Ag/AgCl [1,4,6,8,12–15,19] in accordance with Eq. 3:

$$PhO_2 + 2H^+ + 2e^- \rightarrow Ph(OH)_2 \quad (3)$$

Many compounds usually interfering in the electrochemistry of biosensors, such as ascorbate, urate, paracetamol, etc. [16–18], are not electroactive at this negative potential. Unfortunately, ascorbate can still interfere due to its reaction with the generated quinone molecules [19]. An electrode potential of −200 mV also protects the electrode surface from fouling by polyphenolic films generated during electrooxidation of phenols at positive potentials [18,20–22].

Immobilization of enzymes may be carried out chemically or physically. Chemical bonding is a very favourable procedure allowing protection of the enzyme from rapid degradation provided that its active site is not affected [10,11,23–25]. Physical entrapment is a more gentle procedure which keeps the enzyme in its native form. The activity is not much affected, but the biosensor signal may gradually decrease due to leaking of the biocatalyst into the sample solution [26–31]. The carbon paste electrode has been extensively investigated as a suitable matrix for enzyme entrapment [32]. The electrode usually consists of carbon powder (graphite, glassy carbon) and a pasting liquid (Nujol, paraffin, silicon oil, etc.). This electrode material can easily be modified for several different applications [32,33]. In this study, we investigated the performance of a new "solid carbon paste" electrode (SCPE) under well established HPLC conditions for the analysis of catecholamines [22]. This new enzyme electrode has been shown to be more stable and give better reproducibility compared to a carbon paste based on liquid paraffin [15,34]. From cyclic voltammetric experiments it has been found that carbon paste electrodes possess entrapped oxygen and that the amount of oxygen absorbed during the preparation of the SCPE was sufficient for the enzymatic reaction described above [34]. The tyrosinase enzyme-immobilized carbon electrodes have been extensively studied by Ortega and co-workers [4,6,12] but no detailed determination of catecholamines and their metabolites, including a performance comparison with the classical glassy carbon electrode detector, using a tyrosinase electrode in HPLC has been reported.

2. Experimental

2.1. Reagents

Tyrosinase (monophenol monooxygenase, polyphenol oxidase, EC 1.14.18.1, 4400 U/mg) obtained from Sigma (St. Louis, MO, USA) as a lyophilised powder was stored at a temperature below 0°C before use. Buffers contained 0.1 *M* sodium acetate (Merck, Darmstadt, Germany), 20 m*M* citric acid (Merck), 1 m*M* sodium 1-octanesulfonate (Sigma), 0.1 m*M* Na_2H_2EDTA (Merck) and 1 m*M* dibutylamine (Aldrich-Chemie, Steinheim, Germany). Sodium hydroxide (Merck) was used to adjust pH values to 4.0 and 5.0. The third buffer, of pH 6.5, was prepared as described above by replacing the citric acid with sodium dihydrogen orthophosphate (Merck).

All mobile phases contained the buffer with or without 1 or 2% (v/v) isopropanol (Merck). Stock solutions of dopamine (Sigma), norepinephrine (Fluka, Buchs, Switzerland), 3,4-dihydroxyphenylacetic acid (Janssen Chimica, Beerse, Belgium), homovanillic acid (Fluka) and ascorbic acid (Roche, Basle, Switzerland) were prepared at 1 m*M* in antioxidant solution to prevent compounds from air-oxidation. The antioxidant solution contained 0.01 *M* hydrochloric acid (Merck), 0.1% (w/v) sodium disulphite (Merck) and 0.01% (w/v)

Na_2H_2EDTA. All standard solutions were prepared daily by dilution of stock solutions with mobile phase in order to prevent baseline fluctuations caused by pH changes. The graphite powder (particle diameter 10–100 μm) was washed with acetone, activated with aqua regia, rinsed with water and dried at 400°C for 4 h, respectively. Solid paraffin (Pharmacopeia grade) with a melting point of 46–48°C was purchased from Merck. All chemicals were of analytical or HPLC grade and were used as obtained. Water was purified by a Seralpur Pro 90 CN system (Belgolabo, Brussels, Belgium) (ion-exchange, adsorption and reverse osmose filters: output conductivity 55 nS/cm), before use.

2.2. Apparatus

All experiments were carried out with a HPLC set-up consisting of a BAS PM-80 analytical pump (Bioanalytical Systems, IN, USA), a BAS LC-4C electrochemical detector with a BAS CC-5 thin-layer cell (gasket thickness 51 μm) in a three-electrode configuration comprising a Ag/AgCl (BAS MF-2021, 3M-NaCl) reference electrode, a stainless-steel block auxiliary electrode and a carbon paste (BAS MF-1000) or glassy carbon electrode (BAS MF-1004), both electrodes (3.2 mm I.D., area 8.04 mm^2) served as thin-layer working electrode. The detector was connected to a BAS x-t recorder (MF-8125). Separation was achieved on an analytical reversed-phase column (Ultrasphere ODS, particle size 5 μm, 250×4.6 mm I.D., Beckman, San Ramon, CA, USA). Sample solutions were injected by a 20-μl BAS sample loop.

2.3. Procedure

The solid carbon paste electrode was prepared with 5% (w/w) tyrosinase. The paraffin was melted in a mortar thermostatted in a water bath at ca. 48°C, then the mortar was removed from the bath, the tyrosinase was added and the paste homogenised. Finally, the graphite powder was inserted and properly homogenised. The ratio paraffin to graphite was 34:66 (w/w), the bulk composition was then 32.3 mg of graphite, 62.7 mg of paraffin and 5.0 mg of lyophilised enzyme powder. The tyrosinase-modified carbon paste was stored below 0°C before use. The paste was packed into the electrode body and smoothed to a mirror finish using a paper card. The resulting enzyme-SCPE was positioned into the thin-layer cell detector and was allowed to stabilise under the flow stream and applied working potential (−200 mV) for 1 h (as for GCE). All experiments were performed at room temperature (22±1°C).

The mobile phase was filtered (BAS MF-5621 filter, pore size 0.2 μm) and dissolved oxygen was removed by bubbling nitrogen gas through the solution for 15 min. Finally the solution was degassed in the ultrasonic bath for at least 30 min before use. Nitrogen was also admitted above the mobile-phase surface to prevent air diffusion during the experiment.

3. Results and discussion

3.1. Separation and detection

It is well-known that, because of their distinct pK_a values, the separation of ascorbic acid (AA), norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic (HVA) is highly affected by pH [35,36]. Non-dissociated forms are more retained on the column than their dissociated forms. This pH effect is pronounced for compounds with pK_a values around 4–6 (usually –COOH group), such as DOPAC and HVA. The latter appear closer to the injection peak when the pH is increased from 4 to 6.5, as shown in Fig. 1a, b and c, for chromatograms obtained using the GCE. A similar pH-dependence has been reported by Patthy et al. for several neurotransmitters and their metabolites [36]. In addition to the pH effect on retention time, the response of the tyrosinase-modified SCPE is also significantly affected due to the pH-dependency of the enzyme activity. The maximum of tyrosinase activity and also of tyrosinase-SCPE has been found to range between pH 6 and 8 [15,37]. As shown in Fig. 1d, the tyrosinase-SCPE baseline is unstable and exhibits a relatively high level of noise and the response for DOPAC and DA is low while no NE signal is detected at pH 4.0. When the pH value increases, the sensitivity for DOPAC, DA and NE raises dramatically (Fig. 1e and 1f). The distinct behaviour of DOPAC, DA, NE and HVA is likely

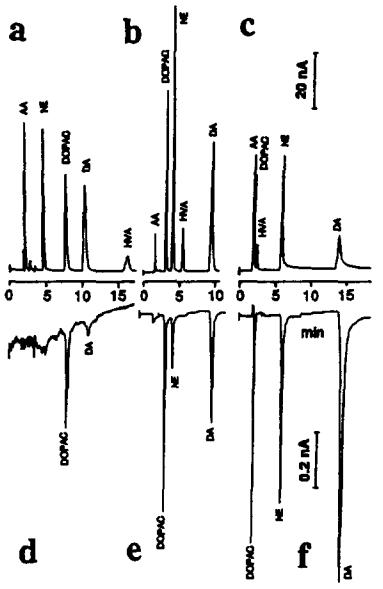

Fig. 1. Chromatograms obtained using the glassy carbon electrode (a, b, c) and tyrosinase-SCPE (d, e, f); pH 4.0 (a, d), pH 5.0 (b, e) and pH 6.5 (c, f). The GCE and tyrosinase-SCPE polarised at +700 mV and −200 mV vs. Ag/AgCl, respectively. Flow-rate: 1.0 ml/min, 2% isopropanol. Ascorbic acid (AA), norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) 10 μM injected.

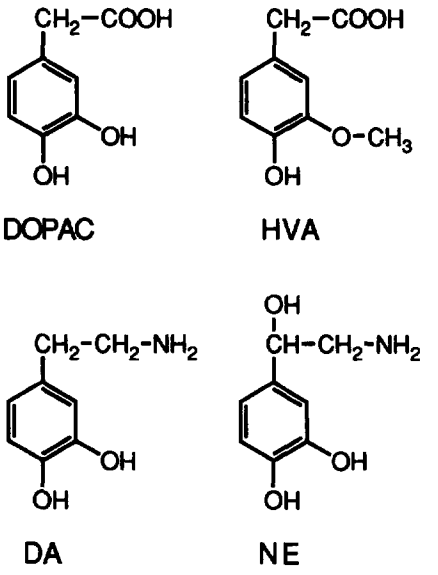

Fig. 2. Chemical structure of the compounds investigated.

related to different kinetic rate constants of the enzyme–substrate reaction. Note that HVA is not detected, because the *o*-oxidation is blocked by the methoxy group. Compared to DOPAC, only the 3-hydroxy group is free for oxidation (see Fig. 2). This also caused the lower sensitivity of HVA electrooxidation compared to that of DOPAC at the glassy carbon detector. This behaviour is similar to the distinct response of phenol and catechol (1,2-dihydroxybenzene) and their derivatives at a tyrosinase-modified liquid CPE in a flowing stream, as described earlier by Ortega et al. [6]. As expected, no peak of ascorbate is registered at the tyrosinase-SCPE, due to the low applied working potential. The separation of AA, DOPAC and HVA at pH 6.5 is poor (three closely spaced peaks are observed at the GCE), but nevertheless at the tyrosinase-SCPE the DOPAC peak is clearly detected, due to the selectivity of the enzyme (compare Fig. 1c and 1f).

Calibration parameters, such as sensitivity and detection limits of DA, DOPAC and NE, obtained at the GCE and tyrosinase-SCPE at different pH values, are presented in Table 1. Using the GCE it was found that pH 5.0 is the most favourable pH with respect to sensitivity and detection limit (LOD), which is close to the LOD obtained by Pastoris et al. [38]. The latter reported, using a GCE, a LOD of 7.5 nM for NE and 20 nM for DA at pH 3.2. In our study, the peaks are sharp, symmetric and well separated. The lowest detection limit and highest sensitivity at the tyrosinase-SCPE for those compounds were found at pH 6.5, as expected considering the pH for maximum tyrosinase activity.

The influence of the amount of organic solvent, i.e. isopropanol, on the separation, was also studied. The organic solvent may remove the water layer necessary for enzymatic activity [14,21] and destroy the electrode by dissolving the paraffin with subsequent instability of the system. Also the peak resolution may be influenced by the presence of an organic solvent. As shown in Fig. 3a, using the GCE electrode, DOPAC and NE are not separated in the absence of isopropanol. A good separation is obtained with 2% isopropanol in the mobile phase (Fig. 3c). No other effect on the biosensor response and separation has been observed.

Table 1
Calibration parameters, such as the detection limit and the sensitivity for dopamine, DOPAC and norepinephrine obtained on GCE and SCPE using mobile phases with pH values 4.0, 5.0 and 6.5, with 2% isopropanol

Electrode	pH	Compound					
		Dopamine		DOPAC		Norepinephrine	
		LOD (μM)[a]	Sensit. (nA/μM)[b]	LOD (μM)	Sensit. (nA/μM)	LOD (μM)	Sensit. (nA/μM)
Glassy carbon	4.0	0.025	3.0	0.020	3.4	0.015	5.0
	5.0	0.015	4.6	0.010	6.4	0.007	10.0
	6.5	0.050	2.8	–	3.3	0.036	4.1
Carbon paste	4.0	5.00	0.003	3.50	0.030	–	0.000
	5.0	1.04	0.035	0.75	0.089	1.32	0.017
	6.5	0.30	0.106	0.84	0.089	0.25	0.087

The GCE and the tyrosinase-modified SCPE were polarised at +700 and −200 mV, respectively; flow-rate was 1.0 ml/min.
[a] The detection limit (LOD) has been calculated as an injected concentration, where the signal was equal to 3× the noise.
[b] Sensitivity, the slope of the linear part of the calibration plot.

3.2. Working potential

The influence of the applied potential on the signal at the tyrosinase-SCPE is shown in Fig. 4. Hydrodynamic voltammograms show differences in sensitivity among the molecules in agreement with HPLC results (Fig. 4a). These distinct responses are likely related to differences in both enzyme–substrate (E–S) kinetics and in electrode reduction rates of the corresponding quinones. At potentials lower than −300 mV the sensitivity is increased, however with a simultaneous increase in noise and reduction of the entrapped oxygen. For analytical purposes the most significant parameter is not sensitivity, but the signal-to-noise (S/N) ratio. When this parameter is optimised, good analytical performances may be

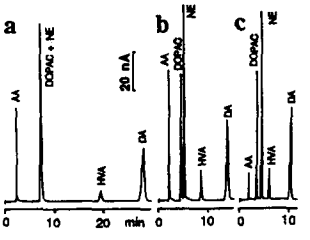

Fig. 3. Chromatograms obtained using the glassy carbon electrode as a function of isopropanol in the mobile phase: (a) 0%, (b) 1%, and (c) 2%. pH=5.0, E_{app}=+700 mV vs. Ag/AgCl. Other conditions as in Fig. 1.

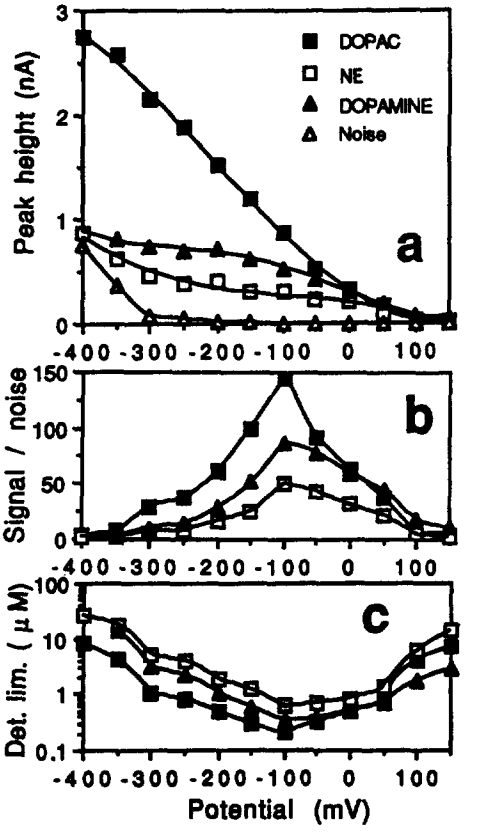

Fig. 4. (a) Hydrodynamic voltammogram in HPLC using the tyrosinase solid carbon paste electrode for DOPAC, DA and NE. (b) Signal-to-noise ratio. (c) The detection limit. All calculations are based on values taken from (a). pH 5.0, other conditions as in Fig. 1.

achieved [11]. The *S/N* ratio as a function of the working potential is shown in Fig. 4b. The maximum of *S/N* versus potential corresponds to the lowest value of the detection limit (Fig. 4c). The minimum of this plot at −100 mV corresponds to the best detection limit for DA, DOPAC and NE at pH 5. When operating at the optimum conditions for tyrosinase activity (pH 6.5) and for the *S/N* ratio (−100 mV), the detection limits for DA, DOPAC and NE were 0.085, 0.22 and 0.095 μM, respectively. Thus, compared with the most favourable conditions at the GCE (Table 1, pH 5), the biosensor responses are one order of magnitude lower.

3.3. Flow-rate

The dependence of peak height on the flow-rate of the mobile phase was investigated in the range 0.2–1.2 ml/min at the tyrosinase-SCPE and GCE. The effect of flow-rate (linear velocity) on plate height is well-known. At low rates, peaks are broad and retention times are high. Because the detector is also affected by the flow-rate, it was of interest to study the ratio of the signal from the tyrosinase-SCPE to the signal from the GCE in order to eliminate the chromatographic separation effect, and to point out the behaviour of the tyrosinase-SCPE. Fig. 5 shows a clear decrease in signal at the tyrosinase-SCPE with increasing flow-rate [12]. This is attributed to the E–S reaction rate, which is slower than the electroreduction of the quinone (SCPE), or the diphenol electrooxidation (GCE). Additionally, the DOPAC response is less affected by flow-rate than the NE response, suggesting a faster tyrosinase-DOPAC than tyrosinase-NE reaction. These results may likely be related to steric effects (see Fig. 2). Similar flow-rate effects for enzymatic electrodes have usually been observed in hydrodynamic systems [4,10,12].

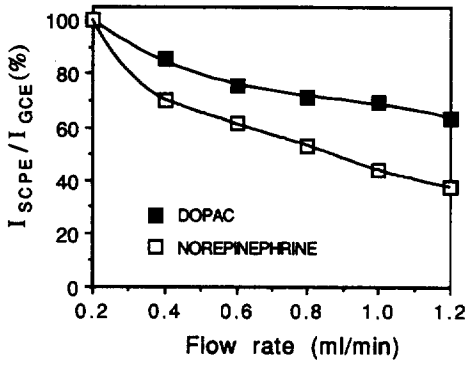

Fig. 5. Effect of flow-rate on the relative response ratio signal tyrosinase-SCPE to signal GCE; pH 5.0, other conditions as in Fig. 1.

3.4. Operational stability

The tyrosinase-SCPE stability has been investigated under different pH values of the mobile phase over a 24-h period. As shown in Fig. 6a, better stability is found at pH 6.5 corresponding to maximum tyrosinase activity. The detector lost 10 and 20% of its sensitivity after 8 and 24 h of use, respectively. When using the pH 4.0 mobile phase, the signal decreases rapidly, with only 13% of its initial activity remaining after 24 h. This loss in sensitivity could not be attributed to oxygen depletion, but rather to enzyme deactivation as we have shown earlier [34]. Indeed, under identical experimental conditions in flow injection analysis, no signal decrease was observed during the first 4 h by

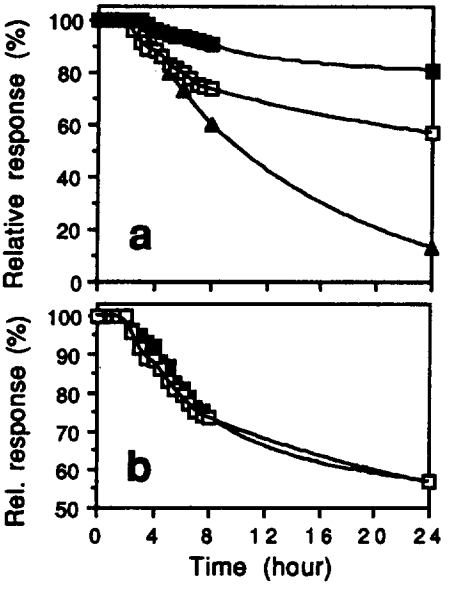

Fig. 6. Operational stability of the tyrosinase-SCPE operating in the mobile phase. (a) pH 4.0 (▲), pH 5.0 (□) and pH 6.5 (■), all with 2% isopropanol. (b) pH 5.0, without (■) and with 2% (□) isopropanol. $E_{app} = -200$ mV; flow-rate, 1.0 ml/min; 10 μM DOPAC injected.

multiple injections of high concentrations of dopamine and reoxygenation did not restore the response [34]. When the amount of isopropanol is varied between 0 and 2%, no significant changes in stability are observed (Fig. 6b). We may also point out that the baseline noise decreases with time as a result of enzyme electrode stabilisation. These trends suggest that tyrosinase-SCPE surface erosion (dissolution of paraffin) is not significant [15]. Peak repeatability calculated as a R.S.D. for $n=10$ and for 10 μM DOPAC injected was 5%. The signal after 24 h of use showed no significant decrease. Thus no fouling of the tyrosinase-SCPE or GCE electrode surface is observed, as has been shown for concentrations less than ca. 10 μM under the same experimental conditions by Sarre et al. [22]. Surface fouling phenomena may occur at high concentrations of generated quinones, as shown by Ortega et al. [12] for DA concentrations of 500 μM.

The possible use of an immobilized tyrosinase reactor (IMER) [9,11,12] was not investigated, despite the fact IMERs may contain higher amounts of enzyme than tyrosinase electrodes. Indeed higher sensitivities for DA were reported using the enzyme electrode (4769 nA/mM) instead of the IMER (790 nA/mM) [12]. These observations can be explained by a closer contact between the enzyme redox centre and the electrode surface, which makes the electroreduction of quinone faster than its polymerization. In addition, substrate recycling can favourably raise the response of the tyrosinase electrode due to the intimate contact between enzyme and electrode. However, the electrode response may decrease by fouling with polymers generated from quinones leaving the IMER.

4. Conclusions

The developed tyrosinase "solid carbon paste" electrode shows a broad specificity towards phenolic compounds, with a quite good stability and reproducibility under HPLC conditions. Compared to GCE, the detection limits are approx. 6×, 22× and 13× higher for DA, DOPAC and NE, respectively. The detection limits (approx. 0.1 μM) in this work are too high to be applicable for post vivo catecholamine determination in brain dialysates [22]. A further increase in sensitivity for catecholamines might be obtained by improving the carbon material or preparing a ruthenium dispersed solid carbon paste as recently shown by Wang et al. [39]. While the described tyrosinase electrode shows a limited sensitivity compared with that of the glassy carbon electrode, our study has demonstrated that this electrode allows improved selectivity and that enzyme-immobilized solid carbon paste electrodes may be successfully used in HPLC depending on the application and sensitivities required.

Acknowledgments

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