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Phenol oxidase-based biosensors as selective detection units in column liquid chromatography for the determination of phenolic compounds

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Abstract

Amperometric biosensors of two types based on the phenol oxidase tyrosinase (EC 1.18.14.1, monophenol monooxygenase) are presented. The enzyme was immobilised either on solid graphite electrodes or in carbon paste electrodes. The performance of the two biosensors was investigated with respect to immobilisation technique, pH, flow-rate and oxygen dependence. The use of detergents in the mobile phase was shown to greatly influence activity, selectivity, and operational stability of the biosensors.

One of the developed biosensors was further used as a selective and sensitive detector in a column liquid chromatographic system for the determination of phenolic compounds in a spiked wastewater sample.

1. Introduction

Due to the increased awareness of the multitude of problems caused by pollution around the world, environmental protection has taken a more important role in society and moved right into our everyday life and household. Environmental pollution is in many cases the cause of alterations in cellular activities in nature, resulting in a broad spectrum of effects, *e.g.* changes in membrane permeability, mutations of genetic materials and inhibition or acceleration of catalyzed reaction rates in *e.g.* respiration and photosynthesis [1]. A considerable number of organic pollutants, widely distributed throughout the environment, have a phenol-based structure. These phenols and substituted phenols are products of many industrial processes *e.g.* the manufacture of plastics, dyes, drugs, antioxidants and wastewaters from the pulp industry.

Various substituted phenols such as chloro- and nitrophenols are highly toxic to man and aquatic organisms. These two groups of substituted phenols are also known to be the main degradation products of organophosphorous and chlorinated phenoxyacid pesticides [2–4]. Even at low concentrations (<1 ppb) phenols affect

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the taste and odour of drinking water and fish [5]. As a consequence, the need to determine phenols and related aromatic compounds in the environment has increased over the years, and the screening for these compounds in complex environmental samples has stimulated the development of new detection principles in this area. This also recently led the European Economic Community (EEC) to initiate a research program, within the environmental programme, devoted to the development of biosensors for environmental control.

In the development of biosensors for these target compounds, the complex nature of environmental samples has to be considered [6,7]. In fact, selective detection systems have to be supplemented with adequate sample pretreatment techniques and liquid chromatography when a particular compound has to be monitored [8]. Consequently, the development of these detection units has to run in parallel to the considerations that dictate sample handling and chromatographic separations to make "on-line" systems possible [9].

Chemical derivatisation and reaction detection utilising immobilised enzymes are currently techniques in rapid development [10–12]. In the field of catalytic reaction detection in conjunction with column liquid chromatography (CLC) most studies have focused on the use of immobilised enzymes in solid-phase reactors (IMERs), used either in the pre- or post-column mode [13]. Only recently have enzyme-based amperometric biosensors been utilised as detection units in CLC [14]. We have previously developed IMER-based detection systems for the analysis of various groups of analytes in complex biotechnological samples [8,9,15,16].

We here report on the development of phenol-sensitive amperometric biosensors utilising immobilised tyrosinase (TYRase, EC 1.18.14.1, monophenol monooxygenase) based on both carbon paste (CP) and solid graphite electrodes. These sensors are developed with the intention to be used as selective and sensitive detection units in CLC systems for environmental applications.

2. Experimental

2.1. Carbon paste electrodes

TYRase from mushroom was purchased as a lyophilised powder [Sigma T-7755, 2100 U mg^{-1} solid (lot 71H9685), 4200 U mg^{-1} solid (lot 102H9585) and 8300 U mg^{-1} solid (lot 112H9580) measured as TYRase activity and 860 000 U mg^{-1}, $6 \cdot 10^6$ U mg^{-1} and $1.06 \cdot 10^6$ U mg^{-1}, measured as catecholase activity, respectively] and used without further purification.

Unmodified CP was prepared according to the following: 100 mg of graphite powder (Fluka 50870) and 40 μl of paraffin oil (Fluka 76235) were thoroughly mixed for 20 min in an agate mortar until a homogeneous paste was formed. The unmodified CP was then packed into plastic syringes (1-ml syringe, ONCE, ASIK, Denmark, I.D. 0.85 mm, surface area 0.023 cm^2); 3–4 mm of the syringe tip was left empty so that it could be filled with enzyme-modified graphite paste (see below). Electrical contact was obtained by immersing a silver wire (Aldrich 34, 878-3) into the unmodified CP.

TYRase was immobilised in CP in five different ways. (1) An amount of TYRase equivalent of 1900 U was first dissolved into 300 μl of 0.1 *M* phosphate buffer (pH 6.0) and then added to 100 mg of graphite powder. The enzyme–graphite suspension was mixed at 4°C for 90 min and then dried in a desiccator for 4–5 h at reduced pressure. A 40-μl volume of paraffin oil was then added to the dried enzyme–graphite powder and thoroughly mixed in an agate mortar for 20 min. Additionally six other equivalent TYRase-modified CPs were prepared in this way with the exception that the enzyme–graphite mixtures were exposed to different drying times (between 1.5 and 4.5 h) in the desiccator. (2) An equal amount as above (1900 U) of TYRase powder was taken but added in a dry state directly to the graphite powder and carefully mixed for 10 min in an agate mortar. A 40-μl volume of paraffin oil was then added and the final enzyme-modified carbon paste was prepared as above. (3) A 4.25-mg amount of water-soluble 1-ethyl-3-(3-

dimethylaminopropyl) carbodiimide hydrochloride ("carbodiimide", Sigma E-6383) was dissolved into 300 μl of 0.05 *M* acetate buffer (pH 4.8) and added to 100 mg of graphite. The mixture was stirred for 2 h at 4°C and then thoroughly washed with ultrapure water over a G4 filter. An amount of 1900 U of TYRase was then dissolved into 300 μl of 0.1 *M* phosphate buffer (pH 6.0) and added to the carbodiimide-activated graphite. The enzyme–graphite suspension was stirred for 2 h at ambient temperature and then dried in a desiccator for 4–5 h at reduced pressure. Finally, 40 μl of paraffin oil were added and the enzyme-modified carbon paste was prepared as described above. (4) An initial procedure was used as in (3) with carbodiimide activation of the graphite powder followed by addition of the enzyme dissolved in buffer but here also followed by the addition of glutaraldehyde (Sigma G-5882) to the enzyme carbodiimide-activated graphite. An amount of 1900 U of TYRase was dissolved into 300 μl of 0.1 *M* phosphate buffer (pH 6.0) and added to the carbodiimide-activated graphite followed by the addition of 10 μl of a 25% aqueous solution of glutaraldehyde. Prior to use, any polymerised aldehyde was removed by addition of activated carbon followed by centrifugation at 4°C and the supernatant was stored at −18°C. The mixture was stirred for 2 h at 4°C and then washed thoroughly with 0.1 *M* phosphate buffer (pH 6.0) over a G4 filter. The enzyme–graphite powder was dried for 4–5 h and the CP was prepared as described above. (5) Amounts of 2.0, 3.3 and 10.0 mg bovine serum albumin (BSA, Sigma A-6003) were each dissolved in 200 μl 0.1 *M* phosphate buffer (pH 6.0). The BSA solutions were added to each 100 mg of graphite powder. The BSA–graphite mixtures were stirred for 30 min after which they were dried for 70 min at reduced pressure. Lyophilised dry TYRase (4200 U 100 mg^{-1} graphite) and 40 μl paraffin oil were then added to each of the three different BSA-modified graphite preparations and the CPs were prepared as described above.

Aliquots of the enzyme-modified CPs were then packed into the empty tips of the syringes, prepared with unmodified CP as described above. The electrodes were then gently rubbed on a glass surface so that smooth electrode surfaces were obtained. When not in use the enzyme electrodes were stored in a dry state at 4°C.

2.2. Solid graphite electrodes

Rods of spectrographic graphite (RW 001, 3 mm O.D., Ringsdorff-Werke) were cut, polished on wet fine emery paper, thoroughly washed with deionized water, and allowed to dry at room temperature. They were then heated to 700°C for 90 s in a Muffle furnace. They were then cooled and stored in a desiccator until use.

Two different techniques were assayed for the immobilisation of the enzyme onto the surface of the solid electrodes: physical adsorption and covalent coupling via carbodiimide using glutaraldehyde as cross-linking agent. The covalent coupling was also studied with and without addition of BSA.

The adsorption coupling was made as follows: 12 μl of a TYRase solution (2.4 mg solid, 5040 U ml^{-1} in 0.1 *M* phosphate buffer pH 6) were added to polished ends of graphite rods. Evaporation was allowed to proceed at room temperature for 15 min. The covalent coupling with carbodiimide involves an activation step of the electrode surface described previously [17]. The enzyme was then coupled by dipping the activated electrodes into 0.5 ml of the TYRase solution (2.4 mg solid ml^{-1} in 0.1 *M* phosphate buffer pH 6) containing 1% glutaraldehyde. This binding between the activated carboxylic groups of the electrodes and the amino groups of the enzyme was allowed to proceed for 16 h at 4°C. The enzyme electrodes were then carefully rinsed with 0.1 *M* buffer solution pH 6 and kept at 4°C in the same buffer. When BSA was added, a final concentration of 4 mg BSA ml^{-1} was added in the solution containing the enzyme and glutaraldehyde.

When in use, the enzyme electrodes were press-fitted into a PTFE holder so that only the

flat circular end (0.0731 cm^2) was exposed to the flow [18].

2.3. Apparatus

The electrodes were inserted into a confined wall-jet flow through amperometric cell [18]. The cell was connected to a single-channel flow injection (FI) system consisting of a pneumatically operated injection valve (Cheminert SVA) with an injection volume of 25 μl, a peristaltic pump (Gilson Minipuls 3), and a potentiostat (Zäta Electronics, Lund, Sweden). The enzyme electrode was used as the working electrode, an Ag/AgCl electrode as the reference, and a platinum wire as the counter electrode. All the connections between the different parts of the FI system were made of PTFE tubings (0.5 mm I.D.) and Altex screw couplings. All measurements with the amperometric biosensors were performed at an applied potential of −50 mV *versus* SCE [17].

Phosphate buffers (0.1 *M*) of different pH values were carefully filtered through 0.4-μm pore diameter membranes (Millipore) and degassed and used as carriers in the FI system. Different flow-rates between 0.1 and 1.5 ml min^{-1} were used with 0.1 *M* phosphate buffer (pH 6.0) as the carrier. In some experiments two different detergents, polyoxyethylenesorbitan monolaurate (Tween 20, Sigma P-1379) and polyoxyethylenesorbitan monooleate (Tween 80, Sigma P-1754) were added to the carrier (0.05%, v/v) to investigate their influence on the biosensor activity and stability.

Stock solutions of 100 m*M* of catechol (Sigma C-9510) and phenol (Merck 206) were prepared by dissolving appropriate amounts in acetonitrile or methanol and stored at 4°C. Phosphate buffer was prepared from sodium dihydrogenphosphate and sodium hydroxide. Acetate buffer was prepared from acetic acid and sodium hydroxide. All chemicals used were of analytical grade. HPLC-grade water was produced in a Milli-Q system and was used throughout this work.

To investigate the oxygen dependence of the reactions taking place at the electrode surface, the following experiment was made. Phenol (0.2 m*M* or 2 μM) or 0.1 m*M* catechol solutions were prepared in 0.1 *M* phosphate buffer (pH 6.0) and saturated with air at 40°C for 20 min followed by cooling to room temperature. A steady-state current was obtained by continuously pumping this solution through the FI system also containing the TYRase biosensor. When a steady state was obtained the phenol-containing carrier was continuously deairated by flushing nitrogen through the solution.

Chromatographic analyses were performed using a Hewlett-Packard (HP) 1050 HPLC system with a 20-μl injection loop and a HP 1040 M photodiode array detector coupled to a HP 9000/300 personal computer and HP 9153 C disk drive. A stainless-steel column (250 × 4 mm I.D.) packed with Nucleosil 120 C_{18} material (particle size 5 μm) (Scharlau) was used. The mobile phase was pumped in the isocratic mode at 1 ml min^{-1} and consisted of acetonitrile–0.1 *M* acetate buffer pH 5.0 (20:80, v/v). Detection was effected at 270 nm. The effluent of the photodiode array detector was connected to the inlet of the amperometric cell containing a solid graphite TYRase electrode.

2.4. Sample handling of waste water

The waste water samples were used as obtained from a pulp industry in the south of Sweden. The samples were diluted 10-fold with distilled water, adjusted to pH 5.0, membrane filtrated, and finally subjected to clean-up by solid-phase extraction (SPE). The clean-up column, containing amine ($-NH_2$) functionalities (Sep-Pak, Waters, Milford, MA, USA), was conditioned by using 2 ml of methanol–water (80:20) to remove impurities from the cartridge, followed by 3 ml of water to remove this solvent mixture. The column was finally conditioned with 2 ml of 0.1 *M* phosphate buffer (pH 6.0) which then was removed by 3 ml of water. Next, 1.5 ml of the wastewater were eluted through the SPE column. The purified wastewater sample was then injected into the chromatographic systems.

3. Results and discussion

3.1. Enzymatic reactions

TYRase [19] is a phenol oxidase which preferably oxidises monophenols but is also active on certain diphenols preferably when the OH-groups are located in *ortho*-position. TYRase contains copper as the prosthetic group in the active site which takes part in the oxidation of molecular oxygen to water [20].

The oxidation reaction of monophenols proceeds in two separate consecutive steps, involving molecular oxygen. The first step is referred to the enzyme's hydroxylase activity where phenol is hydroxylated by the aid of molecular oxygen to produce catechol (*o*-hydroquinone). The second step is an oxidation step known as the enzyme's catecholase activity. The catechol formed in the first step is oxidised to an *o*-quinone, whereby the enzyme is oxidised by molecular oxygen to its native form under the production of water.

The entire reaction path, also including the electrochemical step, for the detection of various phenolic compounds at the TYRase-modified electrode is depicted in Fig. 1. The two enzymatic reactions are followed by electrochemical reduction of the enzymatically produced *o*-quinone forming catechol at the electrode surface. The coupling of enzymatic oxidation of catechol and electrochemical reduction of *o*-quinone thus forms a reaction cycle that results in an amplification of the signal response to phenolic compounds. However, quinones suffer from high instability in water and intermediate formation of radicals in both the enzymatic and electrochemical reactions may readily react and polymerise to polyaromatic compounds which have proved to inactivate the enzyme and to foul the electrode [21–24].

Two different electrode configurations were investigated in which the enzyme was immobilised on a solid graphite electrode or in a CP electrode. Both electrode configurations were incorporated into equal wall-jet electrochemical flow cells and their performances were investigated in an FI system, as described in the Experimental section.

3.2. Carbon paste electrodes

Considering the organic composition of a CP, a rather different catalytic behaviour of an enzyme can be predicted [25]. This opens up new possibilities for the detection of analytes, which may not be recognised by the enzyme in a complete aqueous phase, *i.e.*, when it is immobilised on the surface of a solid electrode or in an enzyme reactor [25]. Additionally, the fact that the enzyme is located in an organic phase imposes a distribution of substrates and products between aqueous and organic phases. The CP configuration allows to remove, by pushing down the paste, the outer catalytic layer once the response decreases by fouling and/or instability, giving a new and catalytically active layer. Finally, it has to be stressed that the CP configuration

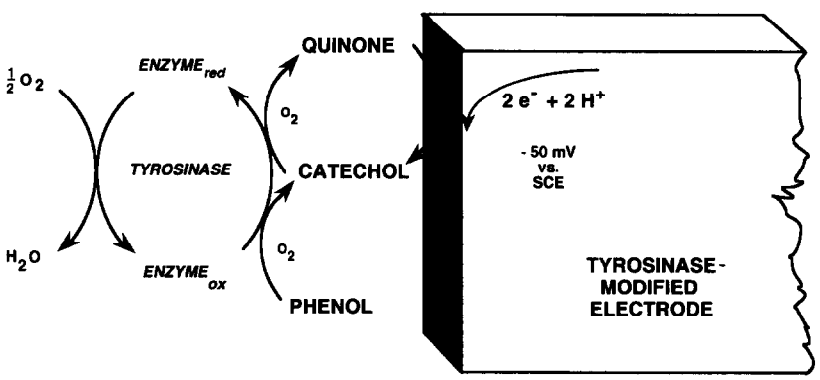

Fig. 1. The reaction sequence for a tyrosinase-modified biosensor, including both the enzymatic and the electrochemical steps.

seems to be a more versatile approach allowing so called bulk modification by the inclusion of various additives needed for an efficient catalysis and fast electron transfer, *e.g.* cofactors, mediators, activators and stabilisers.

In a first attempt when making TYRase-modified CP, the enzyme was physically adsorbed to the graphite powder surface before addition of the pasting liquid. This resulted in an electrode which gave a very low response for catechol (16 nA/m*M*) and a rapid decline of the signal response.

The rapid decline of the signal response with time, as seen in Fig. 2a, has been explained in the literature and it can be due to a number of factors. Bonakdar *et al.* [24] suggested that the rapid decline of the catalytic signal was due to polymerisation reactions followed by the deposition of the produced polyaromatic compounds at the electrode surface. The authors reported a more stable catalytic response of a TYRase-modified CP electrode, by adding the redox-mediator hexacyanoferrate(II) into the paste. The *o*-quinone produced by the enzymatic reaction was thus removed and indirectly monitored amperometrically by the redox-mediator. Wood and Ingraham [26] and Zachariah and Mottola [27] suggested that inactivation of immobilised TYRase was due to reaction inactivation by the substrate. Their explanation was that the nucleophilic lysine groups of the enzyme attack the quinone product, yielding a covalent adduct which blocks the active site of the enzyme. They found that working at concentrations below 10^{-5} *M* did not result in reaction inactivation. A more thorough investigation was therefore made in order to elucidate which factors influence the stability and activity of the TYRase-modified CP electrode.

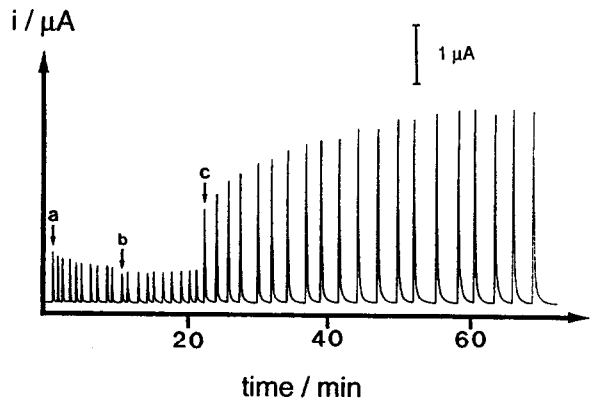

Fig. 2. Flow injection peaks of a 0.2 m*M* catechol solution when the carrier stream in the flow system contains (a) no additive, (b) 0.05% Tween 80 or (c) 0.05% Tween 20. Flow injection conditions as in Fig. 3.

Factors influencing stability and/or activity

It is generally agreed that covalent attachment of an enzyme results in a more stable enzyme preparation compared to immobilisation by pure adsorption. TYRase was therefore immobilised by way of adsorption, covalent coupling with carbodiimide, and covalent coupling with carbodiimide and subsequent cross-linking with glutaraldehyde. The highest catalytic activity was obtained when TYRase was covalently attached with carbodiimide and cross-linking with glutaraldehyde. However, better operational stability was not obtained by changing the immobilisation procedure from pure adsorption to either of the two ways of covalent attachment.

The low catalytic activities obtained in the above experiments were puzzling, since earlier experiments performed in our laboratory using exactly the same conditions gave much higher response for both catechol and phenol (data not shown). In the field of using enzymes in organic media the amount of water surrounding the enzymes in the organic environment is of utmost importance [28–32]. Depending on the type of enzyme, the polarity of the organic solvent, and the hydrophilicity/hydrophobicity of the support used, the necessary water for optimum activity differs. A similar water dependence was found when TYRase-modified CPs were exposed to different drying times in a desiccator (1–4.5 h). It was clearly seen that too much or too little water (short or long drying times, respectively) in the CP electrode resulted in low catalytic responses. Optimum activity was obtained when the enzyme-modified CP was dried for 2.5 h. However, when trying to reproduce some of the former experiments under optimum drying conditions, the catalytic responses of the resulting CP electrodes were extremely irreproducible.

The reason for this is probably that the necessary water content is changing depending on different practical conditions obtained from day to day in the desiccator. When the dry enzyme powder was added by direct admixing with the CP, the response to catechol and phenol increased drastically (data not shown). Apparently, the enzyme powder in its dry state contains the necessary water for full activity, which was indicated by fairly reproducible results obtained for several identical experiments. The immobilisations presented below in this paper were therefore performed by direct admixing of the dry enzyme powder with the CP.

When an enzyme is immobilized on solid supports with large active surfaces, the surface groups on the support can induce conformational changes on the enzyme and thereby blocking the active configuration. Wehtje *et al.* [33] showed that the surface coverage of adsorbed lipase on zeolite had great influence on the inactivation behaviour of the enzyme when used in different organic solvents. If a monolayer of lipase (2–3 mg m^{-2}) was immobilised, a stable enzyme preparation was obtained, whereas when smaller amounts were immobilised, inactivation was pronounced. The authors showed that the latter case could be counteracted by co-immobilising the lipase with an enzymatically inactive protein such as BSA, which thus protects lipase from the inactivating surface groups on the support. Based on these findings we were interested in investigating the role of a protein additive such as BSA on the stability of the CP electrode. The resulting currents for 0.2 m*M* catechol and phenol with different amounts of BSA pre-adsorbed to the CPs are shown in Table 1, columns 2 and 3. It can be seen that the catalytic current for catechol and phenol increases when the graphite is pre-treated with the protein barrier, BSA. The best response for both catechol and phenol was when the graphite was pre-treated with 3.3 mg of BSA. However, it has to be stressed that the stability of the sensor remained unsolved because it was only slightly improved by this pre-treatment. The following work was therefore focused on improving the stability of the CP configuration, particularly since the stability had not been a major problem in previous work with TYRase immobilized in different enzyme configurations [34].

Table 1
Current (i) intensities for 0.2 m*M* catechol and phenol with tyrosinase-modified carbon paste electrodes pre-treated with different amounts of BSA

BSA (mg)	$i_{catechol}$ (μA)	i_{phenol} (μA)	$i_{catechol}$ + Tween 20 (μA)	i_{phenol} + Tween 20 (μA)
0	0.43	–		
2	0.90	0.64	3.0	0.94
3.3	1.78	0.76	2.7	0.98
10	1.26	0.33	1.7	0.42

The flow injection conditions were as in Fig. 3 except that in columns 4 and 5 the carrier also contained 0.05% Tween 20.

Considering the organic nature of the TYRase–CP, a partitioning of substrates and products between an aqueous–oil (W/O) phase has to be considered. Addition of a surfactant could be beneficial for both the partition of substrates at the interface of the electrode and more so for the removal of water-insoluble catalytically produced polymer products from the oil phase of the electrode to the carrier stream. A non-ionic surfactant, Tween 20, was therefore added to the carrier at a concentration of 0.05% (w/w). The currents obtained for the different BSA pre-treated electrodes are presented in Table 1, columns 4 and 5. A further increase of the current in catalytic response was observed by the presence of Tween 20 and the signal remained stable (see below). A second non-ionic surfactant, Tween 80, was also studied and in Fig. 2, it can be observed for a non-BSA pre-treated TYRase–CP electrode, (a) a decline of the signal when no detergent was added to the carrier, (b) a stabilisation of the signal when 0.05% Tween 80 were added to the carrier and (c) a recovering and a pronounced increase of the signal, which eventually levels off to a stable signal, when 0.05% Tween 20 were added to the carrier. With both surfactants the signal remained stable. The reason for the increase in signal with Tween 20 and not with Tween 80, might be related to the difference in length of

the non-polar chains of the surfactants. Tween 20 contains monolaurate (C_{11}) groups and Tween 80 monooleate (C_{17}) groups. It is reasonable to think that a larger molecule, Tween 80, will occupy a larger surface on the electrode and the number of surfactant molecules at the interface will be presumably lower. A specific interaction of the surfactant and the enzyme is also a possible explanation. Ongoing work will contribute to a better knowledge and understanding of surfactants in CP electrodes.

pH dependence

The optimum pH of an enzyme electrode will be determined by the influence on both its catalytic activity and the electrochemical transduction. The pH profile obtained for phenol and catechol with a pH range from 4.0 to 8.0 in the carrier is presented in Fig. 3. The optimum pH for both substrates is 6.0, which was therefore the pH employed in the following experiments presented below.

Flow dependence

The flow dependence of the TYRase-modified CP electrode can be seen in Fig. 4 (left *y*-axis). The signal response for both catechol and phenol

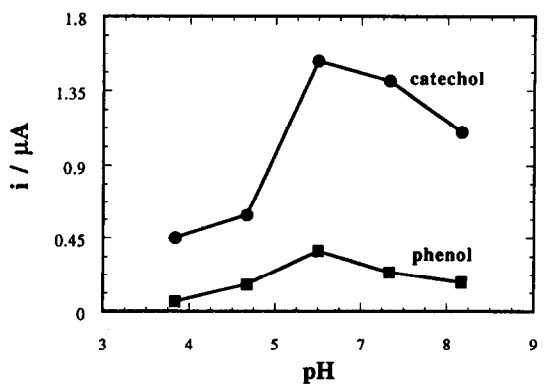

Fig. 3. Variation of the current intensity with pH for 0.2 m*M* catechol and phenol obtained for a tyrosinase-modified carbon paste electrode. A 25-µl volume of 0.2 m*M* catechol was injected into the carrier stream. The carrier was 0.1 *M* phosphate buffer (pH 6.0) and the flow-rate was 0.8 ml min^{-1}. The applied potential was −50 mV *vs.* Ag/AgCl.

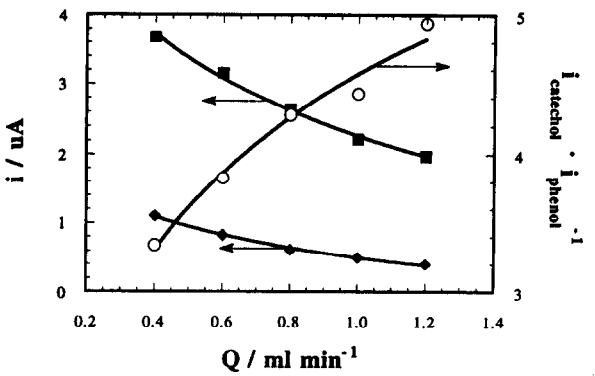

Fig. 4. Variation of the current intensity with the flow-rate (Q) for catechol (■) and phenol (◆) obtained with the tyrosinase-modified carbon paste electrode inserted in a flow injection system. Flow injection conditions as in Fig. 3.

decreases with increasing flow-rate. The response is dependent on the combined effect of the rate of mass transfer to the electrode surface, the rate of the enzymatic reaction, and the rate of the electrochemical reduction of the enzyme product. Since mass transfer increases with increasing flow-rate it can be realised that the decrease in signal response with the increase of flow-rate, is largely due to limitations by the enzymatic reaction. In Fig. 4 (right *y*-axis), this is verified by the increase of the current ratio (catechol · $phenol^{-1}$) with flow-rate. At low flow-rates a larger portion of phenol is converted to product than at high flow-rates, which indicates that the limitation for phenol conversion is mainly caused by the first step in the enzymatic reaction (phenol → catechol).

O_2 dependence

As for any oxidase, molecular oxygen is the natural electron acceptor responsible for the reoxidation of the reduced enzyme and its effect has to be considered in the design of the TYRase biosensor. However, when trying to determine the oxygen dependence of the TYRase–CP electrode, very contradictory results were obtained when the same experiment was performed several times. Both oxygen dependence and oxygen

independence for catechol and phenol were found when several identical experiments were performed. A conclusion concerning the oxygen dependence for a TYRase-modified CP electrode was difficult to make and a more thorough investigation is therefore needed.

3.3. Solid graphite electrodes

The solid electrode configuration allows the immobilisation of the enzyme on the porous surface of the spectrographic graphite. Physical and chemical coupling were assayed. Despite the limited surface area of the solid electrode (0.0731 cm^2), the porosity of this material and the preparation of the electrodes by polishing on fine emery paper gives an enhanced micro surface [35] ready to physically adsorb the enzyme molecules. Carbodiimide activation requires carboxylic functionalities from the graphite. It has been shown that an oxidative pre-treatment step of the carboneous material can increase the enzymatic loading and the specific activity of the preparation depending on the specific enzyme [36]. Heating of the electrodes at 700°C for 90 s was chosen as an oxidative cleaning procedure. Glutaraldehyde was added as a cross-linking agent to increase the rigidity of the enzyme molecules by covalent bonds between them. This covalent procedure was also assayed in the presence of BSA. Adsorption of the enzyme yields lower currents than when covalently coupled, but yields significantly higher currents than when covalently coupled in the presence of BSA. It has been previously discussed on the beneficial effect of the addition of BSA on the signal of the TYRase–CP electrode. From the results in Table 2, it seems obvious that BSA has a rather detrimental effect on the current intensity of the solid electrodes.

As stated in the Introduction, the peak shape of the biosensor response plays an important role in avoiding band broadening and loss of chromatographic resolution. The peak shapes of the different TYRase electrodes based on solid graphite are presented in Table 2 as peak width,

Table 2
Current intensities and peak shapes for solid graphite tyrosinase electrodes prepared with three different immobilisation techniques

	i (nA)	$t_{w1/10}$ (s)	$t_{w1/2}$ (s)
Adsorption			
Catechol (20 μM)	1040	33.50	6.25
Phenol (20 μM)	104	111.60	15.60
Covalent EDC + GA			
Catechol (20 μM)	2160	13.50	7.25
Phenol (20 μM)	900	32.10	10.80
Covalent EDC + GA + BSA			
Catechol (20 μM)	344	14.25	6.25
Phenol (20 μM)	80[a]	n.d.	n.d.

EDC = 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; GA = glutaraldehyde. The electrodes were assayed in the flow injection mode. The carrier was 0.1 M phosphate buffer (pH 6) and the flow-rate was 0.7 ml min^{-1}. The applied potential was −50 mV *vs.* SCE. n.d. = Not determined; t_w = peak shape (see text); i = current intensity.
[a] 100 μM.

in s, at half and tenth of the peak height ($t_{w1/2}$ and $t_{w1/10}$, respectively). The covalent coupling also gives the most favourable peak shapes *versus* adsorption where broad peaks are obtained. When adsorption of the TYRase on the surface of the electrode was made by dipping the electrode into an enzyme solution (instead of adding 12 μl of enzyme solution on the electrode surface) the peak width became much larger (data not shown). Narrower peaks was observed for catechol compared with those of phenol which proves the influence of the catalytic reaction on the peak dispersion [37]. This fact supports the assumption of the existence of two different active centres in the same enzyme working independently but consecutively for monophenols. In fact, the hydroxylase reaction rate will determine the concentration profile for phenol and consequently any approach which favours this slow kinetic step will also favour the peak shape.

All the following experiments with solid graphite electrodes were therefore made with the

TYRase electrode prepared by covalent coupling and cross-linking with glutaraldehyde.

Effect of Tween 80

A concentration of 0.025% Tween 80 was added into the carrier consisting of 0.1 *M* phosphate buffer pH 6. The flow-rate was kept at 0.7 ml min^{-1}. After 20 consecutive injections of 20 μM catechol, a 40% decrease in response was observed, while in the absence of surfactant this dramatic decrease in response was not seen. It has to be stressed that the addition of a surfactant to the solid electrode was motivated mainly for comparative reasons. The absence of an organic phase in the solid electrode simplifies the reaction mechanism and it makes the addition of a surfactant presumably unnecessary. The decrease in signal response, understood as a catalytic inactivation or a deteriorated electrochemical performance, requires further investigation. It has been reported that amphiphilic molecules aggregate on solid surfaces and reduce the rate of charge transfer from redox couples in solution to gold, tin oxide [38,39], and carboneous [40] electrodes [41].

pH dependence

The pH profile obtained for phenol and catechol, ranging from 4.0 to 9.0 in the carrier, is presented in Fig. 5. For both, phenol and catechol, an optimum is found at pH 6 as for the CP electrode. A sharper decrease for phenol than for catechol is noticeable at higher pH values, while at lower pH values the response for catechol decreases more dramatically than for phenol.

Flow dependence

Fig. 6 shows the variation of the response for a TYRase–solid electrode with the flow-rate. An increase in the current intensity can be expected when assuming that the enzyme is working under first-order conditions. This general behaviour is not observed for the response of this biosensor to injections of phenol. The plateau obtained indicates that at high flow-rates the kinetics of the overall enzymatic and electrochemical steps are the rate-limiting factors for the response.

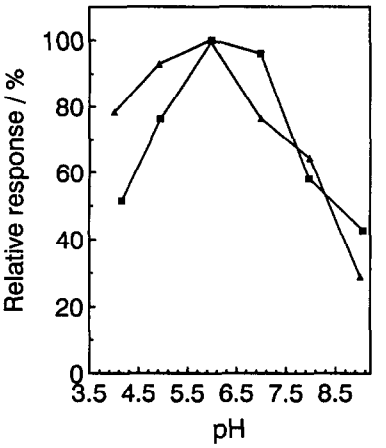

Fig. 5. Variation of the current intensity with the pH obtained with a tyrosinase graphite electrode in the flow injection system. A 25-μl volume of 10 μM phenol (▲) and 25 μl of 20 μM catechol (■) were injected into the carrier stream. The carrier was 0.1 *M* phosphate buffer at different pH values and the flow-rate was 0.7 ml min^{-1}. The applied potential was −50 mV *vs.* SCE.

The faster the phenol plug reaches the electrode surface, the lower enzymatic conversion and additionally, the produced quinone is more easily transported back into the bulk solution. The plateau response is already achieved at 1.1 ml min^{-1} in the flow injection system, and the decrease in response is 60% in relation to the response obtained at low flow-rate. Between 1.1 and 0.1 ml min^{-1} the response increases as a consequence of an increase in the time allowing the sample to be in contact with the enzyme. The flow-rate profile for catechol appears rather different. Increasing the flow-rate between 0.4

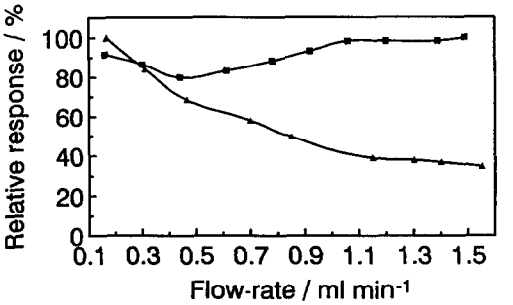

Fig. 6. Variation of the current intensity with the flow-rate obtained with the tyrosinase solid graphite electrode. Same conditions as in Fig. 5.

and 1.0 ml min^{-1} results in an increase of the response showing a mass transport limitation in this flow range, *i.e.* the overall enzymatic and electrochemical reactions are not limiting the current intensity. Similar as for CP electrodes, the behaviour observed for phenol concludes that it is the hydroxylase activity, which is limiting the overall conversion for monophenols. Between 0.4 and 0.1 ml min^{-1} the increase in response can be equally understood considering the longer residence time of the catechol on the surface of the electrode. At higher flow-rates, between 1 and 1.5 min ml^{-1}, the enzymatic oxidation becomes the limiting step, also for catechol.

O_2 dependence

The effect of molecular oxygen was studied in the flow injection system flowing at 0.7 ml min^{-1} for a 2 μM phenol solution (pH 6) in the carrier stream. Once a steady current was achieved with an aerated phenol solution, nitrogen was introduced by sparkling the gas into the carrier stream for 50 min. A decrease of the phenol response could be observed during the time that oxygen was removed from the carrier stream. After 50 min, nitrogen sparkling was stopped and phenol was removed by introducing phosphate buffer into the carrier stream to restore the baseline. Recovery of the response for phenol was evaluated which resulted in that 30% of the TYRase activity was irreversibly lost by anaerobic conditions.

Calibration of solid graphite TYRase electrode

Strictly linear calibration was obtained from 10 nM to 20 μM at pH 6 in the flow injection system with 25-μl injections of catechol at a flow-rate of 0.7 ml min^{-1}. Regression analysis gave an equation $y = 134.05(\pm 0.97)x + 12.85(\pm 7.07)$, where y is the response in nA and x the catechol concentration in μM, $r = 0.99979$. With a 25-μl injection loop a limit of detection (LOD) of 2.3 nM was calculated from $LOD = y_B + 3s_B$, where y_B and s_B are, respectively, the blank signal and its standard deviation ($n = 10$). The average sensitivity was 0.16 $\mu A/\mu M$.

TYRase-modified solid graphite or CP electrode?

The question whether to use a solid graphite electrode or a CP electrode as the post-column detector for CLC was at this stage not difficult. Since a stable response was not obtained for the CP electrode unless Tween was added to the carrier stream, this configuration was ruled out, otherwise a make-up flow would have been necessary. Additionally, the sensitivity of the solid graphite electrode was generally ten times higher than for the CP electrode. However, ongoing work with TYRase-modified CP electrodes has shown that this type of configuration has a promising future. The sensitivity can be increased considerably by bulk modifying the CP electrode with different types of activators and/or stabilisers (data not shown). Additionally, the noise and background currents are lower than for solid electrode materials. The above-mentioned possibilities and the fact that a whole new catalytic biosensor surface can be obtained by simply removing the outer layer, call for further investigation of the TYRase-modified CP electrode.

3.4. Analysis of industrial wastewater samples

Applications were made as described earlier [16] by comparing the use of UV detection and the use of the TYRase-modified solid graphite electrode as detection device for the CLC system. Wastewater samples were obtained from a pulp industry in the south of Sweden. SPE was introduced as a sample handling step using disposable Sep-Pak columns of the silica-based amino phase type (see Experimental). This SPE step was used to eliminate some of the brown components present in the almost black wastewater samples. Removal of disturbing brown-coloured components such as humic substances and lignin-derived oligomers and polymers, was not complete although the levels were lowered considerably. This was investigated by running UV spectra before and after the clean-up and recovery studies with phenol and *o*-cresol as phenolic standards. Even though a quaternary amine phase (strong anion exchanger) was found

to have a higher sorption capacity for the brown-coloured interferents, these Sep-Pak phases showed much lower recovery values for both phenol and *o*-cresol.

Fig. 7A shows the separation of a wastewater sample spiked with phenol, *p*-cresol and catechol after sample pretreatment, as described in the Experimental section. The phenol peak (peak 2) is somewhat tailing which is probably due to limitations in enzyme reaction kinetics. The blank injection is shown in Fig. 7B and it can be seen that no other peaks are found in the chromatogram. The same sample as above was separated and detected with a UV detector at 270 nm, see Fig. 8. The spiked sample (Fig. 8A) shows many early-eluting compounds in the chromatogram, in spite of that many of these first-eluting compounds were successfully eliminated in the clean-up step. The separation of the blank in Fig. 8B shows the same separation of early-eluting compounds but also the possible background levels of catechol and other polar possible phenolic compounds present in the sample. These could not be confirmed by using

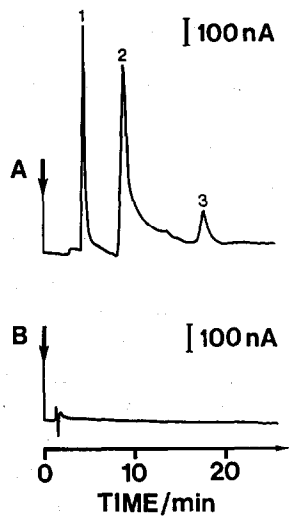

Fig. 7. Chromatographic separation of (A) spiked wastewater and (B) blank samples after solid-phase extraction. Chromatographic conditions: analytical column, silica C_{18} (LiChrospher); mobile phase, acetonitrile–phosphate buffer (100 m*M*, pH 6.2) (5:95); injection volume 20 μl, and an applied potential of −50 mV *vs.* Ag/AgCl. Peaks: 1 = 100 μM catechol; 2 = 100 μM phenol; 3 = 100 μM *p*-cresol.

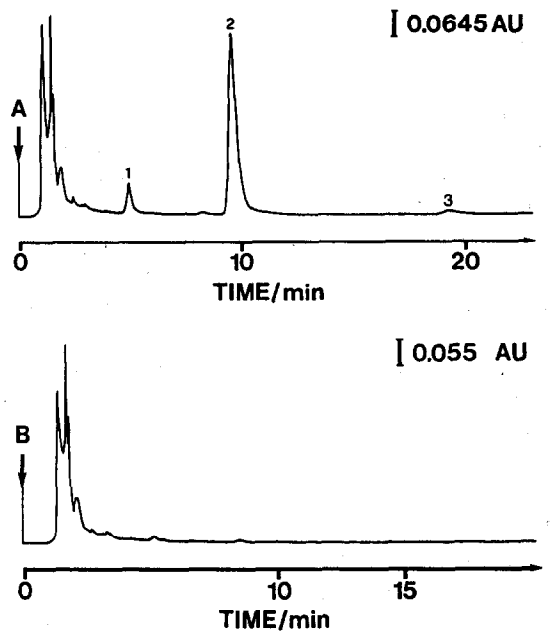

Fig. 8. As Fig. 7, using a diode array detector at 270 nm. Peaks: 1 = 100 μM catechol; 2 = 100 μM phenol; 3 = 100 μM *p*-cresol.

the retention data and the UV spectrum alone. These compounds are not found in the separation of the blank using the enzyme electrode, see Fig. 7B, which is due to the higher selectivity obtained with the TYRase biosensor compared with UV detection. The biosensor is operated in the optimal potential range for electrochemical measurements [in the vicinity of 0 V *versus* the saturated calomel electrode (SCE)]. Here, the enzymatic and electrochemical reactions occur at their optimal reaction rates, the capacitive current switches signs and thus background and noise currents take their lowest value, the electrochemical reduction of molecular oxygen and oxidation of interfering compounds also present in the sample are eliminated and thus not contributing to the analytical signal. Calculation of the concentration of these possible phenolic compounds obtained in Fig. 8A showed that if they were of phenol origin they would readily be detected by the biosensor detection mode. The conclusion can therefore be drawn that these peaks are not of phenol or catechol origin.

4. Conclusions

We have shown that it is possible to obtain a more selective detection system for phenolic compounds by using a TYRase-based biosensor as a post-column detector for CLC compared with UV detection. The TYRase-modified solid graphite electrode was used in the post-column mode, since at this initial stage, the sensitivity and stability of this electrode was greater than with the CP electrode. As mentioned earlier, the CP electrode configuration should not be ruled out since ongoing work has shown that various so-called promoters (activators and/or stabilisers) have shown to have great influence on the CP electrode biosensor performance.

Another matter that should be addressed is the observed instability of the biosensor in the presence of organic modifiers. However, this instability can be improved by casting a membrane on top of the biosensor, thereby protecting the enzyme from the organic solvent. Additionally, the chromatographic separation can be optimized so that lower levels or even pure aqueous mobile phases can be used.

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