

# MODIFIED SCREEN-PRINTED CARBON ELECTRODES WITH TYROSINASE FOR DETERMINATION OF PHENOLIC COMPOUNDS IN SMOKED FOOD

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**Abstract:** A screen-printed carbon electrode modified with tyrosinase (SPCE-Tyr/Paa/Glut) has been developed for the determination of phenol concentration in real samples. The resulting SPCE-Tyr/Paa/Glut was prepared in a one-step procedure, and was then optimized as an amperometric biosensor operating at 0 mV versus Ag/AgCl for phenol determination in flow injection mode. Phenol detection was realized by electrochemical reduction of quinone produced by tyrosinase activity. The possibility of using the developed biosensor to determine phenol concentrations in various smoked products (bacon, ham, chicken and salmon) was also evaluated.

Gas chromatography (GC) method was used for result validation obtained in flow injection mode using amperometric biosensor. The result showed good correlation with those obtained by flow injection analysis (FIA).

**Keywords:** screen-printed carbon electrode SPCE; biosensor; phenol; tyrosinase; gas chromatography.

## 1. Introduction

Quality evaluation of smoked products is needed because of the wide range of quality of these products on the market. Several previous studies of smoke or smoked foods included estimates of total phenolic compounds [1]. Phenolic derivatives are very important compounds in smoked products.

The determination of phenol and its derivative compounds are important for the environment because these substances are toxic and are a result of various industrial processes. They are present in any wastewater streams, resulting from the oil, paint, polymer and pharmaceutical processing industries [2]. Ingestion of phenol and cresol causes intense burning of mouth and throat, followed by marked abdominal pain and distress. Phenol is considered to be very toxic to humans through oral exposure; ingestion of 1g are reported to be lethal with symptoms including muscle weakness and tremors, loss of coordination, paralysis, convulsion, coma, and respiratory arrest [3].

The detection of mono- and polyphenols is usually carried out by HPLC and/or spectrometry methods [4]. However, these methods are used with sample pretreatment and unsuitable for "in situ" monitoring. Many efforts have been made for simple and effective determination of phenol to solve these problems. Electrochemical methods have been widely used for measuring these compounds due to their advantages such as good selectivity in presence of phenol oxidizes, relatively low-cost of realization and storage and the potential for miniaturization and automation [5-7].

Enzymatic biosensors arrays represent promising prescreening method for rapid and simple measurements and express analysis of many pollutant phenols derivatives. Biosensors based on tyrosinase have proved to be sensitive and convenient tools for this purpose.

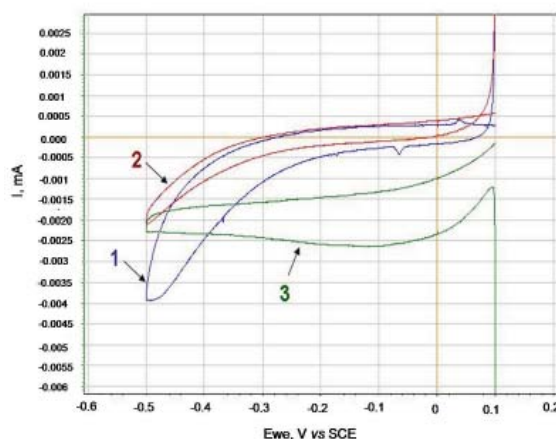
Various methods have been reported for the immobilization of tyrosinase on different suitable substrates. These reports have employed conventional electrode materials as substrates, such as glassy carbon [8-9], graphite-epoxy resin [10], gold [11] and other materials [12-14], etc. However, tyrosinase-based electrochemical biosensors on some substrates suffer from low stability and significant inhibition of enzyme by reaction products; both these factors deteriorate electrode characteristics in phenolic compounds determination [15]. One of the major causes of poor stability is desorption of enzyme from electrode materials. Therefore, the search for reliable methods or electrode substrates that would be a strong and efficient bonding of tyrosinase is still interesting. The aim of this study is to apply an analytical technique for determination of volatile phenolic derivatives in traditional smoked meat.

## 2. Results and discussion

### 2.1. Optimization of Membrane

The starting point for the tyrosinase biosensor development was the study of its electrochemical behaviour in the cyclic voltammetry. The electroenzymatic effect of the modified electrode was examined in the presence and absence of the phenol in the buffer phosphate solution. In figure 1 there are represented three voltammograms: the voltammogram 1

represents the behaviour of a bare screen-printed electrode in the presence of phenol solution and voltammograms 2 and 3 show the behaviour of a screen-printed electrode modified by tyrosinase in the absence (2) or presence (3) of phenol. Adding the phenol in the solution leads to a change in the shape of the voltammogram.



**Fig. 1.** The Voltammograms were performed on a bare screen-printed electrode (1) and modified by tyrosinase (2 and 3) in the absence (2) and presence (3) of phenol. The scan rate of 5 mV/s, Phosphate Buffer (0.1 mol / L, pH = 6.0 + KCl 0.1 mol / L). C (Phenol) = 1 mmol / L

In the initial potential (0.1 V) there is a reduction current which shows the formation of quinone as a result of phenol oxidation by tyrosinase.

In order to find an optimal composition of the used membrane and to increase the analytical performance of the biosensor in terms of sensitivity and operational stability, a range of experiments were used. For the choice of parameters (factors) to study, it was decided to vary the tyrosinase, the PAA and the Glut that seemed to be able to affect the response of the biosensor. The effect of these three factors and their interactions were assessed at two levels denoted minimum (low) ‘- 1’ and maximum (high) ‘+1’. These levels were selected to build this plan of experiments that are based on various preliminary work and the results obtained previously for electrodes already tested in the laboratory. The code and levels fixed for each factor are displayed in Table 1.

**Table 1**

**Factor and Levels Used in the Factorial Design for Biosensor Optimization**

	Variable	Low level (- 1)	High level (+1)
1	Tyrosinase (Tyr), mg/ml	2,5	5,0
2	Polyallylamine (Paa),%	0,025	0,05
3	Glutaraldehyde (Glut), %	0,0125	0,025

The organization of the needed achieved manipulations for a comprehensive plan 2<sup>3</sup> is summarized in Table 2.

**Table 2**

**Design Matrix and Responses for Biosensor Optimization**

Experiment	Factor 1	Factor 2	Factor 3	Response
1	-	-	-	y <sub>1</sub>
2	+	-	-	y <sub>2</sub>
3	-	+	-	y <sub>3</sub>
4	+	+	-	y <sub>4</sub>
5	-	-	+	y <sub>5</sub>
6	+	-	+	y <sub>6</sub>
7	-	+	+	y <sub>7</sub>
8	+	+	+	y <sub>8</sub>
<b>Effect</b>	<b>E<sub>1</sub></b>	<b>E<sub>2</sub></b>	<b>E<sub>3</sub></b>	

To solve the plan of experiments we proceed and calculate the effects of each factor and the interactions on each response (sensitivity, operational stability). The value of effects is obtained by calculation in three steps:

Step 1: Multiplying each response by the sign of the corresponding column of the matrix effects;

Step 2: multiplying the obtained results;

Step 3: dividing by the number of experiments (8).

If the calculated effect (or interaction) is the same order of magnitude as the error, it may or may not influence the response. The significance of effects has been evaluated using Student-test in which  $t$  values are calculated by dividing the effect values  $E_i$  by the standard error  $\sigma_E$  which has been evaluated using the standard error  $\sigma_k$  on the response obtained for each run ( $k \in [1,8]$ ) according to the following equation :

$$\sigma_E = \frac{\sigma_y}{\sqrt{16}} \text{ avec } \sigma_y = \sqrt{\frac{1}{8} \sum_k \sigma_k^2}$$

In this way, eight types of electrodes are provided.

In conclusion, the effects of the three parameters studied on the electrode performance can be presented as follows:

- Increasing the amount of used tyrosinase influences significantly the sensitivity of the electrode;
- Increasing the rate of Paa in the membrane induces an increase in the operational stability of the biosensor;
- The simultaneous increase of Paa and that of tyrosinase improves measurement repeatability;
- The decrease in Glut improves the linearity of the modified electrode and operational stability.

Table 3

### Principal and Interactions effect values for 2<sup>3</sup> factorial

Effects and Interactions on the Sensitivity			Effects and Interaction on the operational stability of Electrodes		
	Average			Average	
E <sub>1</sub> =1	1 (Tyr.)	10,63	E <sub>1</sub> =1	1 (Tyr.)	<b>0,6425</b>
E <sub>2</sub> =2	2 (Paa)	-1,23	E <sub>2</sub> =2	2 (Paa)	<b>-0,5625</b>
E <sub>3</sub> =3	3 (Glut.)	2,94	E <sub>3</sub> =3	3 (Glut.)	-0,2138
E <sub>12</sub> =12	12	-2,62	E <sub>12</sub> =12	12	<b>-0,4425</b>
E <sub>13</sub> =13	13	4,67	E <sub>13</sub> =13	13	-0,0013
E <sub>23</sub> =23	23	2,57	E <sub>23</sub> =23	23	-0,2163
E <sub>123</sub> =123	123	0,58	E <sub>123</sub> =123	123	-0,2113
<b>Erreur-type on the effect : 0,5905</b>			<b>Erreur-type on the effect : 0,2675</b>		

Given the results of experimental sessions, it was chosen to modify the electrodes from the mixture with test 4 in the matrix of experimental design.

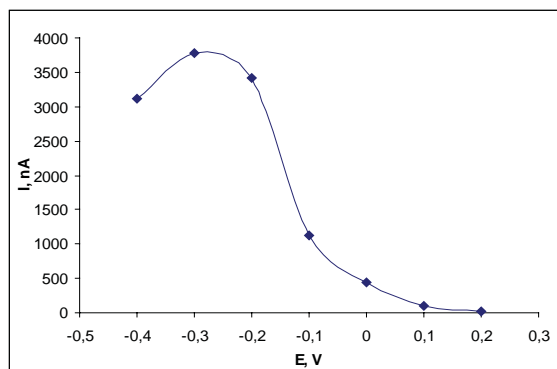
- Tyrosinase 5mg/ml (equivalent of 16 unities)
- Paa - poly (allylamine), 0,05%;
- Glutaraldehyde (Glut), 0,0125%.

This composition gives a better compromise between sensitivity and operational stability. The modified electrodes are prepared by putting manually on the working electrode a solution composed of these three compounds mixed in equal volumes (50μL)

#### 2.2. Influence of Applied Potential

The use of the modified electrode with tyrosinase for the detection of phenol in flow injection analysis necessitated the search for optimum conditions of analysis. We studied the potential, the pH and the organic solvent influences on the biosensor response.

In order to determine the choice of potential, the current response of the biosensor for a phenol concentration of 0,050 mmol/L was measured in the comprised potential range between -0.3 and + 0.3V/Ag/AgCl, a throughput and a constant injection volume.



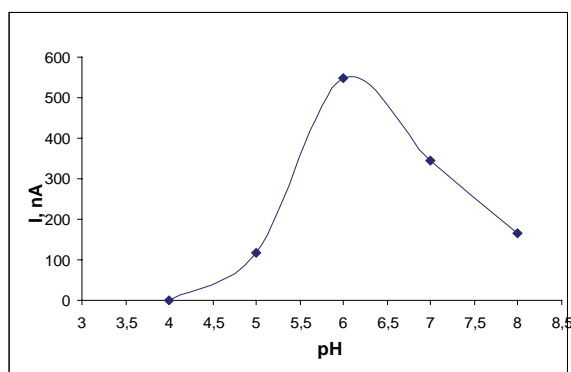
**Fig. 2.** Influence of applied potential on amperometric response of the SPCE-Tyr/Paa/Glut in the Flow injection analysis. Phosphate buffer (0.1 mol/ L, pH=6)

It was found that the maximum reduction current is at a potential of  $E = -0.3\text{V}$  (Figure 2). Furthermore, it was found out that although the biosensor sensitivity has a maximum potential of  $-0.3\text{ V}$  versus Ag/AgCl, however this has some potential drawbacks such as the time required to stabilize the baseline. Approximately two hours are needed for the baseline to stabilize when the working potential is set at  $-0.3\text{ V}$  and about 1 hour at a potential of  $-0.1\text{ V}$ . This relatively long, waiting time to obtain a stable baseline is incompatible with a rapid method. In order to obtain a potential value of  $0\text{ V}$ , it has been determined that only 5 to 7 minutes are required to obtain a stable baseline with very low background noise. Also, it must be noted that at  $0\text{ V}$ , the reactions of electrochemical reduction of oxygen are minimized. For further experiments and to minimize the base current, it was chosen to conduct a study to a potential value of  $0\text{V} / \text{Ag} / \text{AgCl}$ .

### 2.3. pH-dependence

The kinetics of an enzymatic reaction is always dependent on the pH value in the enzyme environment. For this reason, we examined the influence of this parameter on the response of amperometric biosensor.

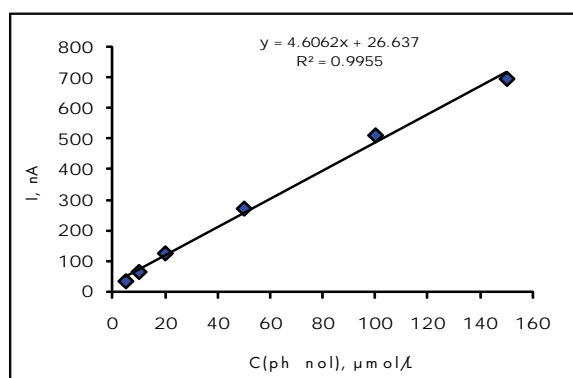
The isoelectric point of tyrosinase is located around  $\text{pH}_i = 4.7 - 5$ , it was chosen to conduct the study between pH values of 4 and 8, for a 0,050 mmol/L phenol concentration. The figure 3 shows the variation of the current function in comparison with the environment reaction's pH. The dependence presents a bell-characteristic shape with a maximum response for pH values between 5.5 and 6.5. An optimal amperometric response at pH 6.0 was obtained. Then for the tyrosinase electrode, a pH value of 6 for the analysis was chosen.



**Fig. 3.** pH effect on response of the SPCE-Tyr/Paa/Glut in the Flow injection analysis. Phosphate buffer (0.1 mol/ L, pH=6). For 0,050 mmol/L phenol. Operating potential 0 mV vs. SCE

#### 2.4. Linearity and limit of detection

The optimal conditions for using the modified electrodes with tyrosinase in order to detect the phenol were determined and furthermore the electrode calibration was performed (Figure 4).



**Fig. 4.** FIA calibration curves for phenol using SPCE– Tyr/Paa/Glut. Phosphate buffer (0.1 mol/ L, pH = 6,0 + KCl 0,1 mol/L) Operating potential 0mV vs. Ag/AgClspce. Flow rate: 0.7mL/min.

The curve I-intensity resulting from the phenol concentration  $I=f([\text{phenol}])$  is linear between 5 and 150  $\mu\text{mol/L}$ , the sensibility is of 4,7 nA.cm-1/ $\mu\text{M}$ .

#### 2.5. Applications to Standard and Real Samples

In the study the focus was set on ten major phenolic compounds contained in smoked products. According to T. Serot and his collaborators these are: Phenol, o-Cresol, p-Cresol, Guaiacol, 4-Methylguaiacol, 4-Ethylguaiacol, Syringol, Eugenol, 4-Propylguaiacol and Isoeugenol. One eleventh compound was also studied, m-cresol, which is present in smoked bacon, according to Chi-Kuen and his collaborators.

Only phenol, m- and p-cresol gave a significant response. Therefore it was decided to carry out the calibration of the electrode with different standard solutions of phenol, p-cresol and m-cresol to determine the linear range. The obtained results show that the best sensitivity of the biosensor is obtained with p-cresol, while the linearity range is broader for phenol. The phenol was chosen as a standard for calibration of tyrosinase electrodes during the dosage of real samples. At the same time the dosage of standard solutions of phenolic compounds was carried out in gas chromatography. The dosage of real samples was effectuated using the method “additions and assayed” using an internal standard 2.4-dichlorophenol.

In order to validate the measurement results obtained for the determination of phenolic compounds in smoked products with the biosensor, a comparison with gas chromatography was made (Table 4). The following results are averages of the three successive determinations.

Table 4

#### Concentrations ( $\mu\text{mol/L}$ ) of phenol, p-cresol in various smoked products determined using Flow Injection Analysis (FIA) and gas chromatography (CG) methods

ANALYZED PRODUCTS	FIA, C, Mmol/ L	CG C, Mmol/ L
Smoked ham	Phenol + p-cresol : <b>44.44 ± 1.09</b>	Phenol : 40.23 ± 1.38 p-cresol : 2.92 ± 0.42 Total : <b>45.10 ± 1.54</b>
Smoked bacon	Phenol + p-cresol : <b>39.10 ± 0.99</b>	Phenol : 36.68 ± 7.31 p-cresol : 2.32 ± 0.39 total : <b>39.00 ± 7.70</b>
Smoked chicken	Phenol + p-cresol : <b>41.40 ± 2.35</b>	Phenol : 35.13 ± 1.12 p-cresol : 17.21 ± 1.73 total : <b>52.34 ± 2.85</b>
Smoked salmon	Phenol + p-cresol : <b>37.07 ± 1.86</b>	Phenol : 30.76 ± 0.86 p-cresol : 7.5 ± 1.34 total : <b>38.26 ± 2.20</b>

In the case of phenolic derivatives content determination in smoked products, a good degree of correlation was found between the results obtained in CPG and the ones obtained by biosensor.

### 3. Conclusions

This research has demonstrated the feasibility of preparing a phenol SPCE based on one-step screen-printing procedure.

A simple to be implemented biosensor for the phenol detection was developed. The biosensor was used in scanning mode by flow injection. Thus, the electrode composition was optimized in order to improve the analytical performance parameters such as the sensitivity and operational stability.

The modified electrode was used in order to determine the low concentration of phenol in real samples of smoked food such as smoked ham, bacon, chicken and salmon. The results obtained when using the biosensor in the FIA have a good correlation with results obtained by CPG.

### 4. Experimental

#### 4.1. Reagents

Phenol and glutaraldehyde were bought from SIGMA. P-cresol, m-cresol, guaiacol, creosol, eugenol, 4-ethylguaicol were bought from ACROS, syringol, o-cresol were purchased from ALDRICH, isoeugenol from LANCASTER, 4-propylguaiacol for SAFC, dichlorophenol, tyrosinase (E.C. 1.14.18.1, approximately 3216 U/mg) were obtained from FLUKA, poly(allylamine hydrochloride) for ALFA ACSAR. Stock standard solutions of phenolic compounds were prepared by dissolution in HPLC grade methanol from PROLABO. All solutions were stored in brown glass bottles at -20 °C.

The used commercial products such as smoked bacon, ham, chicken and salmon were purchased from the local market (Nantes, France).

#### 4.2. Screen-Printed Electrodes Preparation

DEK Albany model 245 screen printer machine and stainless screens with a 200 mesh and variable thickness (13, 23 or 36 μm) have been used to prepare the three electrodes system in four printing steps: (a) printing of Ag/AgCl electrode (13 μm) using the commercially available ink Ag/AgCl (GEM-Gwent), the resulted printed Ag/AgCl electrode presents a stable half-cell potential (0.276V versus NHE), (b) printing of both the counter electrode and the conducting tracks of working electrode using graphite – CA ink, (c) printing of the activated surface using the graphite-binder (CA) ink (the diameter of the work surface is 2 mm) (d) printing of nonconductive dielectric layer to define the working surface area. For each printing step a group of four electrodes was simultaneously printed on alumina ceramic substrate (1.5 cm×1.5 cm). All printed layers were cured at room temperature overnight.

#### 4.3. Tyrosinase Biosensor: Preparation and Analytical Performances

The modified electrodes were prepared by the deposition of the worked electrodes in a three compounds mixture: tyrosinase (Tyr), the Paa – poly(allylamine) 0.05% and Glutaraldehyde (Glut) 0.0125%.

The modification of electrodes was made manually by depositing on the working electrode 3 μL of mixture. The electrodes were then left to dry for 1 hour.

#### 4.4. Sample Preparation

The extraction of phenolic compounds from smoked products was realized using methanol because it permitted the dilution of samples using aqueous solution of electrolytes. The extraction of phenolic real samples was carried out in several stages. First of all, phenolic compounds were extracted from smoked products then they were filtered and finally concentrated.

#### 4.5. Appliances and Procedures

Flow Injection Analysis (FIA) measurements were performed using a three-electrode flow-through amperometric homemade cell of wall-jet type adapted to the screen-printed configuration. The cell was connected to a potentiostat (BAS model Small Ampere CV-1B). A strip chart recorder (Linseis model L200E) was used to follow the electrode response. The flow-injection system consisted of a flow carrier and sample pump (Ismatec) and an electrical six way-valve (Rheodyne) for sample injections by means of 100 μL injection loop. The flow carrier, 0.1 mol/L phosphate buffer (pH 6.0) with 0.1 mol/L KCl added, was pumped at a flow rate of 0.7 ml·min<sup>-1</sup>. All presented results were the mean of at least three similar electrodes.

Flow Injection Analysis (FIA) technique was used to characterize the amperometric response of the biosensor in terms of sensitivity, repeatability and linear range. In the second part of this work, it was investigated the possibility to

use the proposed biosensor in flow injection analysis mode in order to determine the phenol concentration in various real samples such as smoked products. The obtained results were compared with those obtained using the gas chromatography method.

#### • GC-analysis

Gas chromatography analysis was carried out using a HP-6890 Gas chromatograph equipped with a split / splitless injector and FID detector. A capillary column (Agilent 19091J-413 HP5; 30 m × 0.25 mm I.D. fused-silica column coated with a 0.25- $\mu$ m layer of poly (5% phenyl: 95% methylsiloxane) was used to separate phenolic compounds.

Injector temperatures were of 270 °C, the flow rate of carrier gas (helium) was 1.4 ml/min, and oven temperature was programmed from 80 °C (1.5 min) to 290 °C at 50 °C/min, and the final temperature (290°C) was maintained for 10 min finally). The split mode (split ratio: 10:1 was used in all cases. The flame ionisation detector (FID) used in the chromatographic analysis was set at 260 °C throughout the experiment. Chromatographic data acquisition and processing were carried out using HP ChemStations system.

Compounds were identified by matching their GC retention times with those obtained from authentic standards analysed under the same experimental conditions.

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