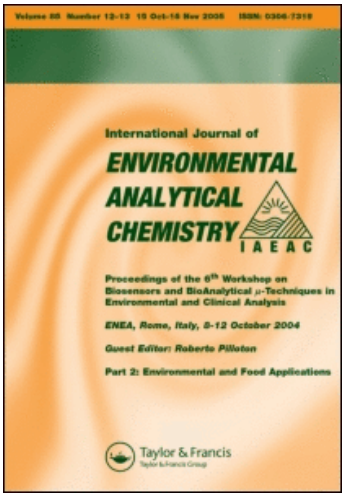

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Bioelectrochemical evaluation of the total phenols content in olive oil mill wastewaters using a tyrosinase-colloidal gold-graphite-Teflon biosensor

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Bioelectrochemical evaluation of the total phenols content in olive oil mill wastewaters using a tyrosinase–colloidal gold–graphite–Teflon biosensor

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The use of a robust tyrosinase biosensor, fabricated from graphite–Teflon rigid electrode matrices modified with gold nanoparticles, for the estimation of the total phenols content in olive oil mill wastewaters (OMW), is proposed. The performance of this bioelectrode using both continuous stirring and flow-injection amperometry was studied. A potential value of −0.10 V was selected for the sensitive and stable detection of various phenolic compounds present in OMW samples: catechol, 3,4-dihydroxycinnamic acid (caffeic acid), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxyphenylacetic acid, 4-hydroxyphenylethanol (tyrosol), and 4-hydroxyphenylpropionic acid. Using catechol as the target phenol, linear calibration graphs were obtained in the $1.0 \times 10^{-8} - 8.0 \times 10^{-6}$ mol L^{-1} (batch) and $1.0 \times 10^{-7} - 1.0 \times 10^{-5}$ mol L^{-1} (FI) concentration ranges, with slope values of 750 mA L mol^{-1} and 103 mA L mol^{-1}, respectively. Batch amperometry was chosen for the analysis of real samples because of its higher sensitivity. For example, the limit of detection for caffeic acid was 80 nM. The 'pool' of phenolic compounds was estimated in OMW obtained from different extraction systems and containing phenols at diverse levels of concentration. A comparison of these results with those obtained by applying the Folin–Ciocalteau spectrophotometric reference method was carried out.

Keywords: Phenols; Olive oil mill wastewaters; Tyrosinase biosensor; Gold nanoparticles

1. Introduction

Biosensors nowadays constitute versatile analytical tools with more and more applications in environmental analysis [1]. This is probably due to the great impact of pollution on human health, and the increasing demand for sensitive and selective methods for the quantitative determination of target analytes [2]. Amperometric biosensors satisfy most of the requirements of modern environmental analytical chemistry, such as low cost, fast response, reliability, simplicity, and stability. An additional advantage resides in their use for *in situ* monitoring. So, for example,

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many applications of amperometric enzyme biosensors for water analysis have been focused on real-time detection of compounds with environmental relevance [3, 4].

Olive oil mill wastewater (OMW) is produced by the olive oil extraction industry and is a strong pollutant mainly because of the high concentration of phenolic compounds, which are phytotoxic and also resistant to biological degradation treatments [5]. Environmental contamination from OMW can be produced when the liquid from the extraction process is discharged to the soil or into a water stream. This becomes a major environmental problem in the main olive-producing countries of the Mediterranean region [6]. Several analytical methods involving techniques such as HPLC [7, 8], LC-MS [9], GC-MS [10, 11], and capillary electrophoresis [12] have been reported for the determination of phenolic compounds in olive oil and related products, including residues. However, there is still a demand for relatively simple analytical devices suitable for screening and rapid assays of the total content of this type of compound in complex real samples. In this work, we propose the use of a robust tyrosinase amperometric composite biosensor for the evaluation of the polyphenols content in OMW.

Tyrosinase carbon paste electrodes have been prepared since the early 1990s [13–16]. The analytical performance of these biosensor designs can be improved by using rigid composite electrode matrices, such as matrices composed of graphite and Teflon, into which the enzyme is incorporated by simple physical inclusion [17]. The resulting bioelectrodes are easily renewable by polishing and permit the incorporation of modifiers to the electrode matrix to enhance the analytical properties of the biosensors. In this context, we recently described a new tyrosinase biosensor design based on the construction of a graphite–Teflon composite electrode matrix in which the enzyme and colloidal gold nanoparticles were incorporated by physical inclusion [18]. The analytical performance of this new biosensor design was checked for catechol, phenol, 3,4-dimethylphenol; 4-chloro-3-methylphenol; 4-chlorophenol, 4-chloro-2-methylphenol; 3-methylphenol and 4-methylphenol. Gold nanoparticles allow proteins to retain their biological activity upon adsorption, and improve the kinetics of the reactions involved in the biochemical recognition process and in the electrochemical transduction, thus yielding a high sensitivity for the detection of phenolic compounds.

In this work, we describe an application of the tyrosinase–colloidal gold–graphite–Teflon biosensor for a bioelectrochemical evaluation of the total phenolic compounds content in a complex real environmental sample such as OMW. In order to do this, the operational performance of the biosensor for different phenolic compounds, which are currently present in OMW (catechol; 3,4-dihydroxycinnamic acid (caffeic acid), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxyphenylacetic acid, 4-hydroxyphenylethanol (tyrosol), and 4-hydroxyphenyl-propionic acid) is evaluated both in batch amperometry and flow injection with amperometric detection. Furthermore, the results obtained were compared with those achieved by applying the reference Folin–Ciocalteau method.

2. Experimental

2.1 *Apparatus and electrodes*

Amperometric measurements under batch conditions (i.e. in stirred solutions) were carried out with an ECO Chemie Autolab PGSTAT 10 potentiostat using the software

package GPES 4.7 (General Purpose Electrochemical System). A P-Selecta Agimatic magnetic stirrer was also used. A three-electrode cell (a BAS VC-2 10-mL glass electrochemical cell) equipped with a platinum wire counter electrode, a BAS MF-2063 Ag/AgCl/3M KCl reference electrode and a tyrosinase–colloidal gold–graphite–Teflon (Tyr–Au_{coll}–graphite–Teflon) biosensor as the working electrode, was used. The flow-injection arrangement consisted of a Gilson Minipuls-2 peristaltic pump and a Rheodyne 7725i valve. Electrode potentials were controlled by means of a μ-Autolab (ECO Chemie) potentiostat, using the GPES 4.7 software. A Metrohm EA 1096 wall-jet cell equipped with an Ag/AgCl/3M KCl reference electrode and a gold counter electrode was also used.

2.2 *Reagents and solutions*

Tyrosinase (from mushrooms, EC.1.14.18.1, activity of 2590 units per mg of solid) was purchased from Sigma (St. Louis, MO) and used as received. Graphite powder (Ultra Carbon, Bay City, MI) and Teflon powder (Aldrich) were used for the fabrication of the composite electrode. An aqueous 1% $HAuCl_4.3H_2O$ solution (Sigma, >49% as Au) and 1% sodium citrate solution were used for the preparation of colloidal gold.

Stock 0.1 mol L^{-1} solutions of phenolic compounds from Aldrich: catechol, 3,4-dihydroxycinnamic acid (caffeic acid), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxyphenylacetic acid, 4-hydroxyphenylethanol (tyrosol), and 4-hydroxyphenylpropionic acid were prepared daily by dissolving the appropriate amount in a 0.1 mol L^{-1} phosphate buffer of pH 7.4, or in methanol (Scharlab), depending on the solubility of these compounds in water. More diluted standards were prepared by suitable dilution with the 0.1 mol L^{-1} phosphate buffer, which was also used as the supporting electrolyte in batch amperometric measurements, and as the carrier solution under flow-injection conditions.

2.2.1 Samples. OMW samples containing phenolic compounds at diverse concentration levels came from three olive oil cooperatives in Spain (Almendralejo, Badajoz; Martos, Jaén and Villarejo de Salvanés, Madrid). Olive oil was extracted using a centrifugation process in the case of Martos (sample 1), Villarejo, and Almendralejo samples, and a pressing discontinuous process in the case of Martos 2 and 2a–c samples. All samples corresponded to the 2004–2005 harvest season.

2.3 *Procedures*

2.3.1 Preparation of tyrosinase–colloidal gold–graphite–Teflon biosensors. Prior to the fabrication of the biosensor, colloidal gold nanoparticles of 16 ± 2 nm in diameter were prepared by adding 2.5 mL of sodium citrate solution to 100 mL of a boiling aqueous solution containing 1 mL 1% (w/w) $HAuCl_4$. Tyr–Au_{coll}–graphite–Teflon biosensors were fabricated in the form of cylindrical pellets as described earlier [18]. Briefly, graphite, 150 mg, and 900 µL of colloidal gold were thoroughly mixed by mechanic stirring for 2 h, after which water was evaporated under air current. Then, 34.75 mg of tyrosinase and 400 µL of 0.1 mol L^{-1} phosphate buffer solution, pH 7.4, were

incorporated to the mixture by stirring for 2 h in an ice bath. The resulting mixture was dried, and 415.25 mg of Teflon was added and thoroughly hand-mixed. Then, the mixture, which contained 70% Teflon, was pressed into pellets, by means of a Carver pellet press (Perkin-Elmer, Norwalk, CT) at 10 000 kg cm^{-2} for 10 min. Five or six 3.0 mm cylindrical portions of this main pellet were bored, and each portion was press-fitted into a Teflon holder. Electrical contact was made through a stainless steel screw.

2.3.2 Evaluation of the phenols content in OMW. An appropriate aliquot of homogenized sample was diluted to 10 mL with phosphate buffer solution of pH 7.4 and transferred to the electrochemical cell. Amperometric measurements in stirred solutions at −0.1 V using the Tyr–Au_{coll}–graphite–Teflon were carried out by recording the current and allowing the steady state to be reached. The content of phenolic compounds was estimated by applying the standard additions method, which implied the addition of five successive 20 μL aliquots from a caffeic acid stock solution.

As a reference method, the samples were also analysed by means of the spectrophotometric method involving the use of Folin–Ciocalteau reagent [19]. In this method, 4.2 mL of deionized water and 0.5 mL of Folin–Ciocalteau reagent (phosphotungstic-phosphomolybdic acid) were added to a 0.5 mL aliquot of sample previously diluted with deionized water. The mixture was stirred for 1 min, and 1.0 mL of an 80% sodium carbonate solution and 4.2 mL of deionized water were added. The resulting solution was allowed to stand for 2 h at room temperature in darkness, and then the absorbance was read at 730 nm. The absorbance value was interpolated into a calibration plot for caffeic acid constructed with standard solutions of this compound in the $2.0 \times 10^{-6} - 1.0 \times 10^{-4}$ mol L^{-1} concentration range, which were subjected to the same procedure.

3. Results and discussion

As has been widely reported, the tyrosinase enzyme reaction with phenolic compounds involved the catalytic oxidation of these compounds to their corresponding *o*-quinones [20]. The electrochemical reduction of these quinones at the modified electrode by transferring two electrons and two protons was used to monitor the enzyme reaction.

3.1 *Operational performance of the Tyr–Au_{coll}–graphite–Teflon biosensor for phenolic compounds currently present in OMW*

The composition of the Tyr–Au_{coll}–graphite–Teflon bioelectrodes, as well as the potential value to be applied for the amperometric detection of the different phenolic compounds which are currently present in OMW, were the same than those used for other phenolic compounds in reference [18]. The analytical characteristics of the calibration plots constructed by batch amperometry for catechol, 3,4-dihydroxycinnamic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-hydroxyphenylethanol, and 4-hydroxyphenylpropionic acid, are summarized in table 1. The limits of detection were calculated according to the $3s_b/m$ criterion, where m is the slope value of the corresponding calibration graph, and s_b was estimated as the standard

Table 1. Analytical characteristics of the calibration graphs and kinetic parameters for different phenolic compounds currently present in OMW, obtained by amperometry in stirred solutions at −0.1 V with Tyr–Au_{coll}–graphite–Teflon biosensors.

Phenolic compound	Slope (mA M^{-1})	Linear range (μM)	LOD[a] (μM)	K_M^{app} (μM)	V_{max}
Catechol	746 ± 42	0.01–8.0	0.003	6.6	11.3
3,4-Dihydroxycinnamic acid	43 ± 6	0.2–70	0.08	64	5.3
3,4-Dihydroxyphenylacetic acid	39 ± 6	0.2–100	0.09	250	14
4-Hydroxyphenylacetic acid	56 ± 6	0.1–100	0.05	79	8
4-Hydroxyphenylethanol	43 ± 2	0.2–100	0.08	45	9
4-Hydroxyphenylpropionic acid	31 ± 5	0.5–7	0.02	215	16

[a] $3s_b/m$.

deviation ($n = 10$) of the amperometric signals from different solutions of the compounds at the lowest concentration level of the respective calibration plot. As expected for a tyrosinase biosensor, the highest sensitivity observed was for catechol, whereas the presence of carboxylic or hidroxy- substituents in the fourth position of the aromatic ring resulted in a considerable decrease in sensitivity for the other phenolic compounds tested. Nevertheless, it should be remarked that the detection limits obtained for these compounds are considerably better than those achieved for the same compounds with other biosensor configurations using phenol oxidases [21, 22]. For example, the detection limit for caffeic acid, 80 nM, is sevenfold lower than that obtained using a laccase biosensor constructed by adsorption of the enzyme on a graphite electrode, even though this compound is one of the most sensitive substrates of laccase [23].

All the enzyme reactions for the different phenolic compounds obeyed a Michaelis–Menten type kinetics, as demonstrated by calculating the 'x' parameter from the corresponding Hill plots [$\log(i_{max}/i) - 1$] vs. the log of the substrate concentration, which ranged between 0.995 and 1.12. Consequently, the apparent Michaelis–Menten constants (K_M^{app}) and the maximum rate values of the reaction (V_m) were calculated from the corresponding Lineweaver–Burk plots (see table 1). As expected, the lowest K_M^{app} value was obtained for catechol, which exhibited the highest sensitivity at the Tyr–Au_{coll}–graphite–Teflon bioelectrode. This value was remarkably lower than those reported previously in the literature [18]. Furthermore, the K_M^{app} value obtained for caffeic acid was also one order of magnitude lower than that reported in the literature [22].

The performance of the enzyme electrode was also evaluated under flow-injection conditions. In this case, the influence of the applied potential on the amperometric response was checked by injection of 160 μL of a 1.0×10^{-5} mol L^{-1} catechol solution into the carrier solution consisting of 0.1 mol L^{-1} phosphate buffer of pH 7.4. As can be observed in figure 1, the peak current increased from +0.20 V up to −0.10 V, following which a decrease was observed for more negative potentials, which can be attributed to polymerization of the corresponding *o*-quinones at these negative potentials [24]. Therefore, according to these results, a potential of −0.10 V was selected again for the amperometric detection.

Characteristic flow-injection parameters, such as flow rate and sample volume injected, were also optimized. Concerning flow rate, its influence on peak current signals was investigated in the 0.30–1.35 mL min^{-1} range. The results showed a peak current maximum at 0.4 mL min^{-1} and then a slight decrease in the response with flow

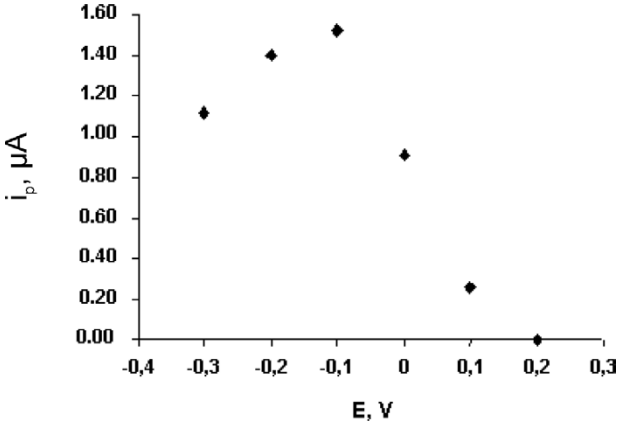

Figure 1. Influence of applied potential on the steady-estate current for 1.0×10^{-5} mol L^{-1} catechol at a Tyr–Au_{coll}–graphite–Teflon biosensor.

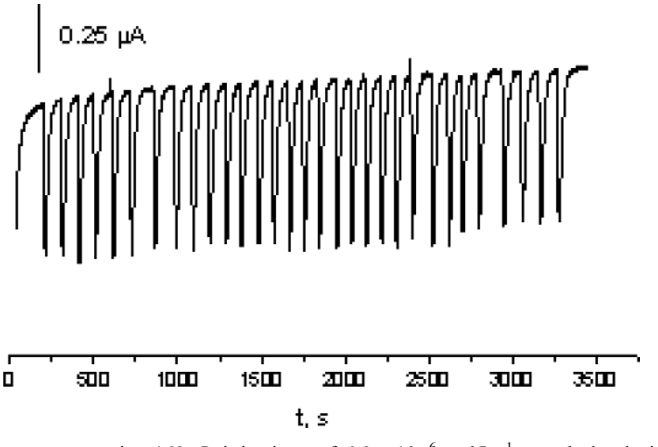

Figure 2. Response to successive 160 μL injections of 6.0×10^{-6} mol L^{-1} catechol solution in 0.1 mol L^{-1} phosphate buffer solutions of pH 7.4 at a Tyr–Au_{coll}–graphite–Teflon biosensor. $E_{app} = -0.10$ V.

rate. A similar behaviour was observed for a graphite laccase biosensor using the same wall-jet flow cell configuration, which agrees with a highly kinetic control of the response [23]. Further studies were carried out at a flow rate of 0.4 mL min^{-1}. On the other hand, a sample volume of 160 μL was chosen as the injection volume taking into account the $i_p/W_{1/2}$ ratio, where $W_{1/2}$ is the peak width at half height. Using these experimental conditions, a good repeatability of the FI responses was observed. As an example, figure 2 shows amperometric signals from 30 successive injections of 6.0×10^{-6} mol L^{-1} catechol solutions, yielding a RSD value for i_p of 3.7%.

Linear calibration graphs were obtained for both catechol and caffeic acid over the concentration ranges $1.0 \times 10^{-7} - 1.0 \times 10^{-5}$ mol L^{-1} ($r = 0.996$) and $2.0 \times 10^{-6} - 6.0 \times 10^{-4}$ mol L^{-1} ($r = 0.998$), respectively, with slope values of 103 mA M^{-1} and 7.5 mA M^{-1}, respectively. As expected, the sensitivity obtained for these compounds was notably lower than that achieved using batch amperometry at the same detection potential (see table 1).

3.2 *Evaluation of the total phenols content in OMW*

The developed Tyr–Au_{coll}–graphite–Teflon biosensor was used for the rapid bioelectrochemical evaluation of the phenols content in OMW. This complex type of sample contains a variable number of organic compounds which are present at different concentration levels, and exhibit different properties such as volatility, solubility, etc. Moreover, OMW contain high concentrations of salts, lipids, pectins and polysaccharides, and show high BOD and COD levels. All these characteristics make evident the difficulties associated with the analysis of these samples.

Batch amperometry in stirred solutions was used for the analysis of OMW due to the extremely simple experimental procedure needed, which involved the direct addition of a diluted sample aliquot to the electrochemical cell and the application of the standard additions method using caffeic acid (3,4-dihydroxycinnamic acid) as the standard (see section 2). This compound was selected to express the total phenols content, as it is made in the Folin–Ciocalteau reference method [20], thus allowing a direct comparison of the results obtained by both methods. Table 2 summarizes the results obtained from five replicates of each sample, the confidence intervals being calculated for a significance level of 0.05. As can be observed, samples Martos 2 and Martos 2a, b and c, which corresponded to an olive oil extraction process by pressing, exhibited a higher phenols content. This type of extraction process provides a dry solid residue, and a mixture of oil and water which is introduced into decantation wells until phase separation occurs. The samples analysed were waters from this type of well. In contrast, samples obtained from a three-phases centrifugation system (Villarejo and Badajoz), involving the addition of water during the extraction process, thus generating high volumes of OMW, yielded lower phenolic compound contents. Finally, the sample called Martos 1, which was obtained from a two-phase centrifugation system, producing olive oil and wet solid residue without water addition, gave an intermediate concentration of phenols. The latter is an ecologically attractive process [7] because of the lower volume of waste. It is important to point out that, in spite of the differences in the origin and concentration levels of phenolic compounds between the samples analysed, similar slope values, ranging between 13 and 17 $mA\,M^{-1}$, were obtained in all cases from the calibration plots constructed by applying the standard additions method. The existence of a matrix effect was demonstrated by comparison of these slope values with that given in table 1 for caffeic acid (43 $mA\,M^{-1}$), using standard solutions.

Table 2. Estimation of the content of phenolic compounds[a] in OMW samples by batch amperometry using a Tyr–Au_{coll}–graphite–Teflon biosensor.

Sample	Biosensor	Folin–Ciocalteau
Badajoz	0.255 ± 0.002	0.270 ± 0.009
Martos 1	0.416 ± 0.002	0.352 ± 0.005
Martos 2	3.5 ± 0.7	7.2 ± 0.5
Villarejo	0.22 ± 0.03	0.58 ± 0.02
Martos 2a	1.5 ± 0.1	3.6 ± 0.2
Martos 2b	1.7 ± 0.3	2.4 ± 0.2
Martos 2c	0.7 ± 0.1	1.8 ± 0.1

[a] As caffeic acid in $g\,L^{-1}$: $x \pm (ts/\sqrt{n})$ $(n = 5)$

As commented above, the results obtained using the biosensor were compared with those achieved by applying the reference spectrophotometric method involving the use of Folin–Ciocalteau reagent (table 2). Both sets of results must be considered as phenols indexes since both methods yield responses for these compounds whose sensitivity depends markedly on their structure. Therefore, it was expected that the values given in table 2 as 'estimations of the content of phenolic compounds' will be different to some extent, as a consequence of the completely different analytical methodologies employed. Nevertheless, from a rough comparison of the indexes for both methods, it can be deduced that the values are rather similar for a given sample. When the results obtained with the biosensor were plotted vs. the results obtained with the Folin–Ciocalteau method, a linear plot with a remarkably good correlation coefficient ($r = 0.996$) and an intercept including zero ($0.1 \pm 0.3\,\mathrm{g\,L^{-1}}$ of caffeic acid) were found. These results indicated that no systematic errors exist in any of the two methods. Although, as expected considering the different values for the phenolic compounds indexes obtained by the two methods, the slope of such a linear plot was far from the unity ($m = 0.54 \pm 0.09$), the correlation results reveal that the Tyr–Au_{coll}–graphite–Teflon biosensor can be used for the rapid and easy evaluation of the total phenolic compounds content in this type of complex sample.

4. Conclusions

This study has demonstrated the possibility of using a robust tyrosinase composite biosensor, into which colloidal-gold nanoparticles have been incorporated, for rapid and *in situ* bioelectrochemical evaluation of the phenolic compounds content in a complex real environmental sample such as olive oil mill wastewaters. The proposed analytical methodology, which can be implemented using both batch amperometry in stirred solutions and flow injection with amperometric detection, exhibits several important practical advantages with respect to the Folin–Ciocalteau reference method. The most important is the simplicity of the sample treatment, involving only the direct addition of a diluted sample aliquot to the electrochemical cell, which dramatically reduces the time required for the analysis. Thus, by applying the batch amperometric method, the time of analysis for an OMW sample is of approximately 5 min, in contrast to the 2.5 h needed when the Folin–Ciocalteau method is used. Dark and cloudy samples containing oil residues can also be directly analysed. Moreover, the use of an enzyme biosensor ensures a high selectivity, in contrast to the Folin–Ciocalteau method where other compounds can produce positive interferences.

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