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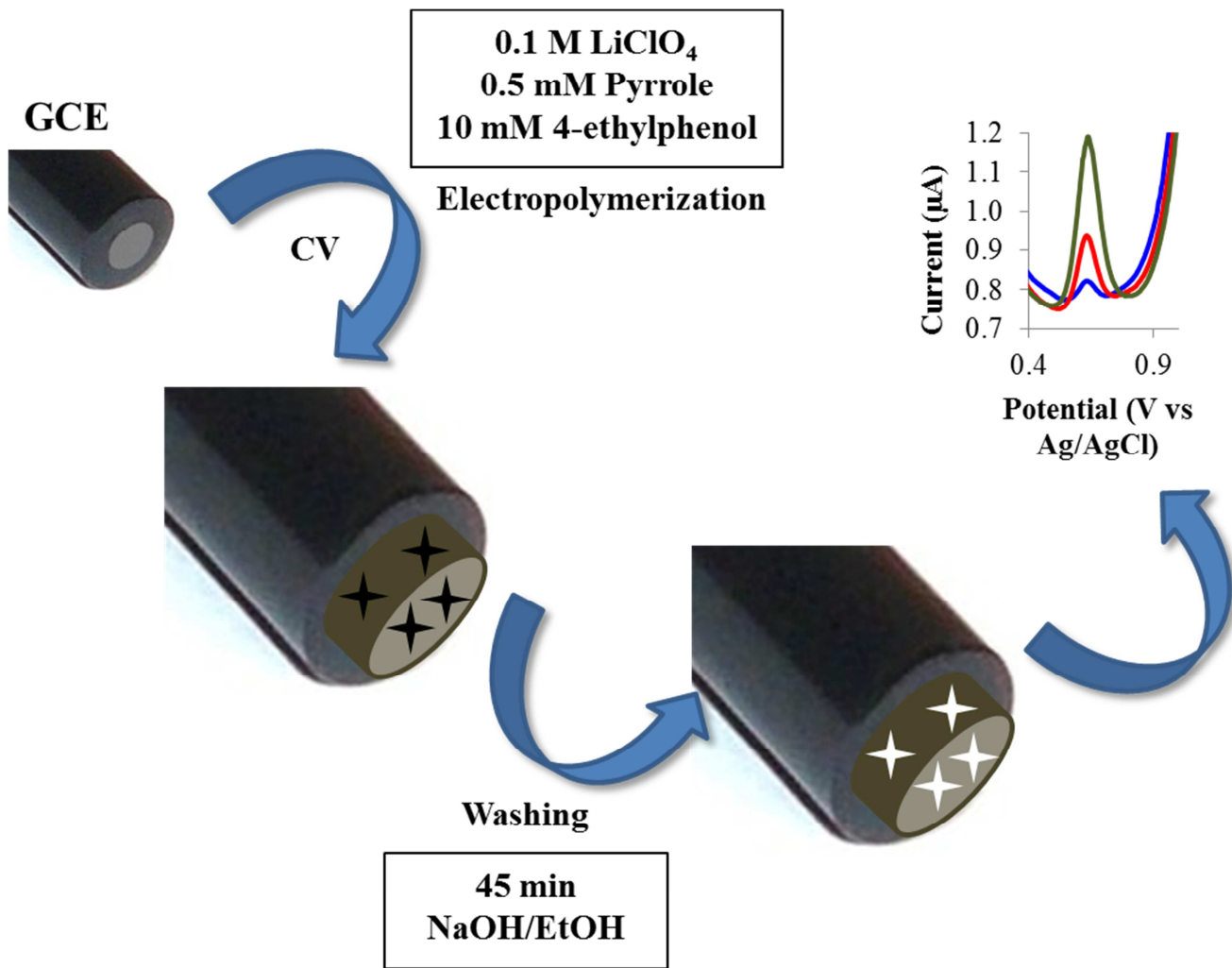
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**MOLECULARLY IMPRINTED POLYPYRROLE BASED
ELECTROCHEMICAL SENSOR FOR SELECTIVE DETERMINATION OF 4-
ETHYLPHENOL**

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Abstract

This work describes the development of an electrochemical sensor based on a molecularly imprinted polymer (MIP) for sensitive and selective determination of 4-ethylphenol in wine. The sensor has been built by means of the electrosynthesis of the MIP on a glassy carbon electrode surface using cyclic voltammetry. The electropolymerization has been performed in the presence of 4-ethylphenol and pyrrole as template molecule and functional monomer, respectively. The influence of the molar ratios of template molecules to functional pyrrole monomers and the time needed to remove the template have been optimized taking into account the differential pulse voltammetric response of 4-ethylphenol. Under the optimal experimental conditions the developed MIP/GCE sensor shows good capability of detection ($0.2 \mu\text{M}$, $\alpha = \beta = 0.05$)

and reproducibility (3.0%) in the concentration range from 0.2 to 34.8 μM . The influence of possible interfering species in the analytical response has been studied and the sensor has successfully been applied to the determination of 4-ethylphenol in different wine samples.

Keywords: 4-ethylphenol; Molecularly imprinted polymer; Polypyrrole; Electrochemical sensor; Wine

1. Introduction

In wine industry, the quality of the final product that reaches the consumer is definitely related to its organoleptic properties. These properties are determined by the presence of a wide variety of volatile compounds, such as 4-ethylphenol, in a wide range of concentrations [1]. 4-ethylphenol can be found in wine due to the action of yeast of the species *Brettanomyces/Dekkera bruxellensis* through enzymatic decarboxylation and subsequent reduction reactions of the *p*-coumaric acid. This phenolic molecule confers a very negative impact on the perception of wine quality due to its undesirable off-flavours that have often been described as wet animal, horse sweat, barnyard or leather [2, 3]. Thus, the determination of this compound is extremely important for wine producers in order to guarantee wine quality and avoid serious economic problems.

Different chromatographic techniques have been employed in the detection of 4-ethylphenol including gas chromatography [1-16] and high performance liquid chromatography (HPLC) [9, 17-21]. These chromatographic methods are characterized by a high degree of selectivity and sensitivity (Table 1). However, all of them are time consuming; require expert analysts, tedious sampling methods and high cost equipment, being often not useful for real-time and field analysis.

Electrochemical techniques are also characterized by high sensitivity and selectivity but, in addition, these techniques have wide linear range and low-cost instrumentation. Moreover, electrochemical devices can be easily miniaturized for real in situ applications. This kind of techniques has also been described for the analysis of

4-ethylphenol using different modified electrodes (Table 2). However, only a few of these works have been applied in the determination of this molecule in wine [22, 23].

Molecularly imprinted polymers (MIPs) have been successfully applied to the fabrication of electrochemical sensors based on their selective biomimetic recognition of target molecules, providing interesting substitutes to natural receptors [24, 25]. In addition, MIPs are characterized by important properties including high mechanical, physical and chemical stability, reusability, along with easy and low cost fabrication processes [26, 27]. MIP based electrochemical sensors may then combine these properties with the above described advantages of electrochemical detection.

Different conducting polymers have been used in the construction of MIP based electrochemical sensors including polyaniline [27], poly(o-aminophenol) [28], poly(o-phenylenediamine) [29, 30] and polypyrrole [24-26, 31-34], being the last one of the most widely used due to its high electrical conductivity, suitable redox properties, good biocompatibility and easier polymerization procedure compared to other conducting polymers.

Among the different methods for MIP preparation, electropolymerization of the MIP film on the electrode surface results highly advantageous. This method involves an easy way of preparation and control of film thickness and morphology. Furthermore, the electropolymerized films have a strong adherence to the transducer and a rapid response to template molecules [29-32].

The present work describes the preparation of a new electrochemical sensor for 4-ethylphenol using a molecularly imprinted polypyrrole electrosynthesized on a glassy carbon electrode (GCE). MIPs have already been used for the analysis of 4-ethylphenol in pretreatment procedures of extraction/preconcentration in HPLC [18] and in the development of a bioelectronic tongue analytical system [22]. However, to the best of authors' knowledge, this kind of handy electropolymerized MIP sensors have not been used in the analytical determination of 4-ethylphenol, much less in wine.

The performance of the developed method has also been studied in terms of reproducibility, capability of detection, as well as by its application to the quantification of 4-ethylphenol in different commercial wine samples.

2. Experimental

2.1. Reagents

All reagents used were of analytical-reagent grade. Ultrapure water obtained from a Milli-Q water purifier (Millipore, Bedford, MA, USA) was used for the preparation of all solutions. Britton Robinson (BR) buffer solutions, containing 0.04 M phosphoric acid (Panreac, Barcelona, Spain), 0.04 M acetic acid (VWR Chemical, Fontenay, France) and 0.04 M boric acid (Panreac, Barcelona, Spain), were used as supporting electrolyte for the electrochemical measurements. 1 M NaOH (Ercros, Barcelona, Spain) solutions were used to adjust the pH value of the buffer solutions.

Stock standard solutions of 4-ethylphenol (Alfa Aesar, Haverhill, Massachusetts, USA) were prepared by dissolving the adequate amount in Milli-Q water. Pyrrole and LiClO_4 , used to modify the working electrode surface, were purchased from Sigma-Aldrich (Steinheim, Germany) and Panreac (Barcelona, Spain), respectively. Ethanol (EtOH), used to remove the template molecules, was purchased from Scharlau (Barcelona, Spain).

2.2. Apparatus

Electrochemical measurements were carried out in a bulk solution using a CHI1030 potentiostat (CH Instruments, Texas, USA), using three electrodes: an Ag/AgCl electrode as reference electrode, a platinum electrode as auxiliary electrode and a GCE or a MIP modified GCE (MIP/GCE) as working electrode.

pH measurements were performed by means of a Crison Model 2002 (Barcelona, Spain)

2.3. Software

STATGRAPHICS Centurion XVI was used for experimental design, data analysis and robust regressions [35].

DETARCHI was used for the estimation of the capability of detection with a given probability of false positive and negative [36].

2.4. Preparation of molecularly imprinted (MIP) and non-imprinted (NIP) modified GCE

The MIP/GCE was obtained by electropolymerization using a solution containing the target analyte and functional pyrrole monomers. Prior to this electropolymerization process, the GCE was polished using alumina slurry, followed by rinsing with deionized water. After that, the GCE was immersed in a 10 mL solution containing 0.1 M LiClO₄, 0.5 mM pyrrole and 10 mM 4-ethylphenol as a template (except for the optimization process). The electropolymerization was performed by CV scanning from - 0.6 to + 1.8 V with a scan rate of 50 mV s⁻¹ for 8 cycles. The MIP/GCE was finally immersed in a NaOH (0.2 M):EtOH (8:2, v/v) stirred solution during 45 min to remove the template [26].

The non-imprinted polymer (NIP) modified GCE (NIP/GCE) was also prepared by using the above-described electropolymerization procedure, without addition of 4-ethylphenol template molecules.

2.5. Electrochemical measurements

Differential pulse voltammetric (DPV) measurements were performed at room temperature (approx. 20 °C) in a cell containing 5 mL of BR buffer solution (pH 6, except for the optimization process). The potential was scanned from + 0.0 V to + 1.0 V with amplitude, pulse width, sampling width and pulse period of 50 mV, 50 mV, 0.0167 s and 0.2 s, respectively.

3. Results

3.1. DPV determination of 4-ethylphenol using a GCE

The oxidation response of 4-ethylphenol was studied by DPV at a GCE in BR solutions with different pH values. The DP voltammograms obtained under the different experimental conditions showed no significant effect of the pH in the current intensity of the oxidation peak of the analyte. Moreover, the sensitivity of the method was studied using the calibration curves constructed in the concentration range from 37.8 to 175.5 μM for pH values ranging from 4 to 10. Sensitivity values of the same order for the different pH values were obtained. In view of these results, a BR buffer solution pH 6 was selected for next experiments since it has led to the best results in the analysis of other different analytes in wine samples [37-40], matrix that is the main objective of this work.

Fig. 1 shows DPV curves of different concentrations of 4-ethylphenol using a bare-GCE. The peak current at approx. + 0.63 V increased with the growth of the analyte concentration and a linear range between 3.2 and 168.5 μM was established. Different calibration curves were built using ordinary linear regressions. In order to provide a correct evaluation of the calibration parameters, outlier points with a Studentized residual above 2.5 in absolute value were removed [35]. These properly evaluated linear regressions were used to estimate the precision and the capability of detection of the developed procedure.

The precision of the method was then determined in terms of the reproducibility of the slopes obtained for three different calibration sets. The relative standard deviation (RSD) value obtained was 1.9 %.

The capability of detection (CC_{β}) of the developed procedure was estimated as the lowest concentration level of analyte that the method is able to detect with a probability $1-\beta$ (β , false negative). The decision limit (CC_{α}) was also estimated as the lowest concentration level at which the method, with a statistical probability of $1-\alpha$ (α , false positive) can discriminate if the target analyte is in the analyzed sample. The values obtained for these parameters were 2.6 and 7.8 μM for CC_{α} and CC_{β} , respectively ($\alpha = \beta = 0.05$) [36, 41] (Table 3).

In order to study the selective recognition of the developed method, a volatile phenol usually found in wine samples (4-ethylguaiacol) was analyzed as a possible interference. This molecule showed a significant influence in the analytical response of 4-ethylphenol since its presence in a concentration level of 10 μM produces a decrease in the oxidation signal obtained for the same concentration of 4-ethylphenol of 72 %. Thus, the electrochemical determination of 4-ethylphenol using a bare-GCE shows a lack of selectivity.

3.2. DPV determination of 4-ethylphenol using a MIP/GCE

In order to improve the selectivity of the electrochemical method described above, a modification of the electrode surface with a MIP was performed following a similar procedure to the one described by Yang *et al.* [26]. This method consists of the

formation of the MIP by electropolymerization using a solution containing the target analyte and functional pyrrole monomers as it has been described in section 2.4.

The analytical performance of the MIP/GCE was characterized by DPV using $\text{Fe}(\text{CN})_6^{3-/4-}$ as the redox probe. Fig. 2 shows the oxidation responses obtained for this compound using different types of electrodes. As it can be seen in this figure, there is no analytical response for the NIP/GCE. However, the presence of the template molecule in the generation of the MIP leads to the formation of channels that allow the oxidation of the redox probe on the electrode surface.

Optimization of the electro-synthesis of MIP film

With the aim of achieving a highly sensitive sensor for the analysis of 4-ethylphenol, a series of experiments was performed in order to optimize the molar ratios of template molecules to functional pyrrole monomers and the time needed to remove the template. In this way, the electrochemical response of different modified electrodes was characterized by DPV with $\text{Fe}(\text{CN})_6^{3-/4-}$ as redox probe.

Three different MIPs, using different [4-ethylphenol]/[pyrrole] ratios, were fabricated following the procedure described in section 2.4. Two different washing times were also analysed using $\text{NaOH}(0.2 \text{ M})/\text{EtOH}(8:2, \text{ v/v})$ as washing solution:

	[4-ethylphenol]/[pyrrole]	Washing time (min)
MIP ₁ /GCE	2	30
		45
MIP ₂ /GCE	20	30
		45
MIP ₃ /GCE	200	30
		45

Fig. 3 shows the DPVs recorded in the blank solution using the different built MIP/GCE. In the case of MIP₃/GCE, the peak corresponding to 4-ethylphenol at +0.63 V was observed at any washing time (Figure 3c). A washing time longer than 45 min would be necessary for this modified electrode to completely eliminate the template molecule during the modification process, leading to an unnecessary increase of the time of analysis. Thus, MIP₃/GCE was not considered adequate for the analysis of 4-ethylphenol. Regarding the other [4-ethylphenol]/[pyrrole] ratios tested, the best results were achieved for MIP₂/GCE since this modification process led to a higher response for Fe(CN)₆³⁻ (Figure 3a-b). A washing time of 45 min was also selected taking into account the better response obtained for the redox probe. Furthermore, the oxidation of 4-ethylphenol was also evaluated using MIP₁/GCE and MIP₂/GCE (Figure 3d). A higher oxidation peak for 4-ethylphenol was also recorded using a MIP₂/GCE. Thus, a [4-ethylphenol]/[pyrrole] of 20 and a washing time of 45 min were selected as the optimum conditions for the fabrication of the MIP/GCE following the procedure described in section 2.4. Under these optimum conditions, an oxidation peak at approx. + 0.63 V increased with the growth of the analyte concentration, while no analytical response for the analyte was observed when a NIP/GCE was used (Figure 4a). A linear range between 0.2 and 34.8 μM was established (Figure 4b).

Precision and capability of detection of the MIP/GCE

Reproducibility was calculated in terms of the RSD value obtained for the slopes of different calibration sets performed for concentrations of 4-ethylphenol ranging from 0.2 to 1.8 μM using different MIP/GCEs. The RSD value obtained for the slopes was 3.0 %.

In order to study analytical abilities of the developed MIP/GCE, CC_β and CC_α values were also calculated for $\alpha = \beta = 0.05$ [36, 41]. The values obtained for these parameters were 0.2 and 0.1 μM , respectively (Table 3).

Interference studies

In order to study the selective recognition of the MIP/GCE developed, two phenolic compounds, 4-ethylguaiacol and dopamine, were studied as possible interferences due to their structural similarity to 4-ethylphenol. The influence of different concentrations of the interfering compounds in the analytical signal of a 10 μM 4-ethylphenol solution was analyzed (Table 4).

The interference analysis of 4-ethylguaiacol showed no influence on the oxidation signal of 4-ethylphenol even when the concentration of 4-ethylguaiacol was much higher than that of 4-ethylphenol. Therefore, 4-ethylguaiacol cannot be considered an interfering species in the analysis of 4-ethylphenol with the developed sensor. Moreover, the concentration of 4-ethylguaiacol in wine is always much lower than that of 4-ethylphenol, being generally the amount of 4-ethylphenol 10 times greater

[4, 8, 12]. Therefore, the determination of 4-ethylphenol in wine can be carried out successfully with the developed sensor in presence of 4-ethylguaiacol.

In the case of dopamine, some degree of interference in the determination of 4-ethylphenol was found at concentrations higher than 50 μM . Taking into account that the amount of dopamine present in wine has been reported to be below the detection limit of some techniques (0.16 μM) [42], its interfering effect was considered not significant.

Wine sample analysis

In order to deeply explore the performance of the developed sensor for the determination of 4-ethylphenol, different wine samples were directly analysed by using a calibration curve built between 0.2 and 1.8 μM . Six different commercial samples of white table wines from different origins and different grape variety were studied. Each determination was performed in triplicate (Table 5). 4-ethylphenol was quantified in 4 of the 6 analysed samples, being the concentration values obtained within the levels found in other works for this molecule [4, 5, 8, 12]. Recovery experiments were also performed by adding known concentrations of 4-ethylphenol. The recovery values obtained oscillate from 91 % to 115 %, which indicates a good applicability and reliability of the developed analytical method.

4. Conclusions

In this work, a sensitive and selective MIP based electrochemical sensor has been developed by electropolymerization of pyrrole on a GCE, using 4-ethylphenol as template. The effect of different preparation conditions including the ratio of template/monomer and the washing time needed to remove the template has been investigated. Under the optimum preparation conditions ([4-ethylphenol]/[pyrrole], 20; washing time, 45 min), the sensor showed high selectivity and sensitivity for the analysis of 4-ethylphenol, wide linear range (from 0.2 to 34.8 μM), as well as good precision (3.0 %), capability of detection (0.2 μM) and limit of decision (0.1 μM) for $\alpha = \beta = 0.05$. These analytical characteristics are similar to those obtained by other authors in wine and other types of samples using different electrochemical sensors [22, 23, 44-46]. Moreover, this work implies a simpler analytical procedure with a simpler modification of the electrode surface than other previous electrochemical sensors used in the determination of 4-ethylphenol in wine [22, 23]. Additionally, the developed sensor has been successfully applied to the determination of 4-ethylphenol in different wine samples with recoveries ranged from 91 % to 115 %.

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Table 1. Chromatographic determinations of 4-ethylphenol in wine

Technique	Sample pretreatment	Limit of decision	Reproducibility (RSD)	Ref.
GC-MS	MHS-SPME	0.5 nM	2.6 %	[1]
		0.4 nM	5.5 %	[13]
	SPME	0.1 µM	12.1 %	[2]
		----	----	[7]
		----	----	[10]
		0.2 µM	0.5 %	[3]
	LLE	----	----	[9]
		----	----	[4]
	DLLME	0.4 µM	3.6 %	[5]
		0.4 nM	----	[6]
	Isotope dilution	4.1 nM	4.0 %	[15]
	SPE and DLLME	3.3 nM	2.9 %	[8]
	UEME	0.5 nM	----	[14]
	GC-FID	HS-SPME	16.4 nM	10 %
8.2 nM			----	[12]
24.6 nM			4.8 %	[16]
HPLC-MS	----	81.9 nM	9.2 %	[17]
HPLC-DAD	----	----	----	[9]
		81.9 nM	0.3 %	[17]
	MIP	21.5 nM	----	[18]
HPLC-FLD	----	8.2 nM	2.0 %	[17]
		32.7 nM	----	[20]
		0.1 µM	3.0 %	[21]
HPLC-CAD	----	21.2 nM	----	[19]

DLLME, Dispersive liquid–liquid microextraction; **GC-FID**, Gas chromatography with a flame ionisation detector; **GC-MS**, Gas chromatography–mass spectrometry; **HPLC-CAD**, High performance liquid chromatography with a coulometric array detector; **HPLC-DAD**, High performance liquid chromatography with a diode-array detector; **HPLC-FLD**, High performance liquid chromatography with a fluorescence detector; **HPLC-MS**, High performance liquid chromatography–mass spectrometry; **HS-SPME**, Headspace solid-phase microextraction; **LLE**, Liquid-liquid extraction; **MHS-SPME**, Multiple headspace solid-phase microextraction; **MIP**, Molecularly imprinted polymer; **SPE**, Solid phase extraction; **SPME**, Solid phase microextraction; **UEME**, Ultrasound-assisted emulsification-microextraction;

Table 2. Electrochemical determinations of 4-ethylphenol

Technique	Electrode	Limit of decision	Reproducibility (RSD)	Sample	Recovery	Ref.
	GCE	----	----	----	----	[43]
	GCE	2.6 μ M	1.9 %	----	----	This work
DPV	Molecularly imprinted nanoparticles modified Au electrode	0.6 μ M	----	----	----	[44]
	MIP modified GECE	10.6 nM	----	Wine	----	[22]
	MIP modified GCE	0.1 μ M	3.0 %	Wine	101 %	This work
CV	Tyrosinase-modified carbon nanotube GCE	0.2 μ M	----	Synthetic cocktail of VOs	----	[45]
	Array of 6 epoxy graphite modified electrodes	14.7 μ M	4.6 %	Wine	----	[23]
Amp.	Tyrosinase-modified SPE	11.5 nM	7.0 %	Water	----	[46]
	Tyrosinase-modified carbon nanotube GCE	0.1 μ M	----	Synthetic cocktail of VOs	108 %	[45]

Amp., Amperometry; **CV**, Cyclic voltammetry; **DPV**, Differential pulse voltammetry; **GECE**, Graphite epoxy composite electrode; **MIP**, Molecularly imprinted polymer; **VOs**, Volatile organic compounds; **SPE**, Screen-printed electrode

Table 3. Results obtained in the determination of 4-ethylphenol by DPV using different electrodes

	GCE	MIP/GCE
Linear Range	3.2 μM -168.5 μM	0.2 μM -34.8 μM
Slope [$\text{nA } \mu\text{M}^{-1}$]	21.1	22.3
Intercept [nA]	-51.7	8.2
Coefficient of determination (R^2)	0.99	0.99
Decision limit ($\text{CC}\alpha$) [μM]	2.6	0.1
Capability of detection ($\text{CC}\beta$) [μM]	7.8	0.2
Reproducibility (RSD)	1.9 %	3.0 %

Table 4. Percentage (%) of interference in the current intensity of a 10 μM 4-ethylphenol solution of interference compounds.

Conc (μM)	Interfering compound	
	Dopamine	4-ethylguaiacol
50	0.4 %	0 %
100	38.3 %	1.9 %
250	85.7 %	3.7 %

Table 5. Determination of 4-ethylphenol in different wine samples by DPV using a MIP/GCE (n=3).

Sample	Added (μM)	Found (μM)	Recovery (%)
Wine 1	-----	No detected	-----
	5.0	4.9 ± 0.05	98 %
	10.0	11.5 ± 0.2	115 %
Wine 2	-----	No detected	-----
	5.0	5.1 ± 0.5	102 %
	10.0	9.9 ± 0.5	99 %
Wine 3	-----	4.2 ± 0.5	-----
	5.0	9.2 ± 0.5	100 %
	10.0	14.5 ± 0.5	103 %
Wine 4	-----	9.1 ± 0.5	-----
	5.0	14.4 ± 0.8	106 %
	10.0	20.1 ± 1.2	110 %
Wine 5	-----	9.4 ± 0.5	-----
	5.0	14.0 ± 0.8	92 %
	10.0	19.6 ± 1.1	102 %
Wine 6	-----	10.3 ± 0.6	-----
	5.0	14.9 ± 0.8	92 %
	10.0	19.4 ± 1.1	91 %

Figure captions

Figure 1. DPVs for 4-ethylphenol solutions of concentrations ranging from 3.2 to 168.5 μM at GCE in BR pH6. The inset is the calibration curve.

Figure 2. DPVs obtained for 1 mM $\text{Fe}(\text{CN})_6^{3-}$ solutions in BR pH6 using (—) a NIP/GCE; (·····) a MIP/GCE and (- - -) a GCE.

Figure 3. (a-c) DPVs obtained for 60 mM $\text{Fe}(\text{CN})_6^{3-}$ solutions in BR pH6 using (a) MIP₁/GCE (b) MIP₂/GCE and (c) MIP₃/GCE (— 30 min; ···· 45 min of washing time).
(d) DPVs obtained for 4 mM 4-ethylphenol solutions in BR pH6 using (—) a MIP₁/GCE and (·····) a MIP₂/GCE.

Figure 4. (a) DPVs obtained for 4-ethylphenol 5 μM in BR pH6 using (—) a NIP/GCE: and (—) a MIP/GCE **(b)** Experimental points and regression for the calibration curve carried out to evaluate the linear range in the determination of 4-ethylphenol using a MIP/GCE.

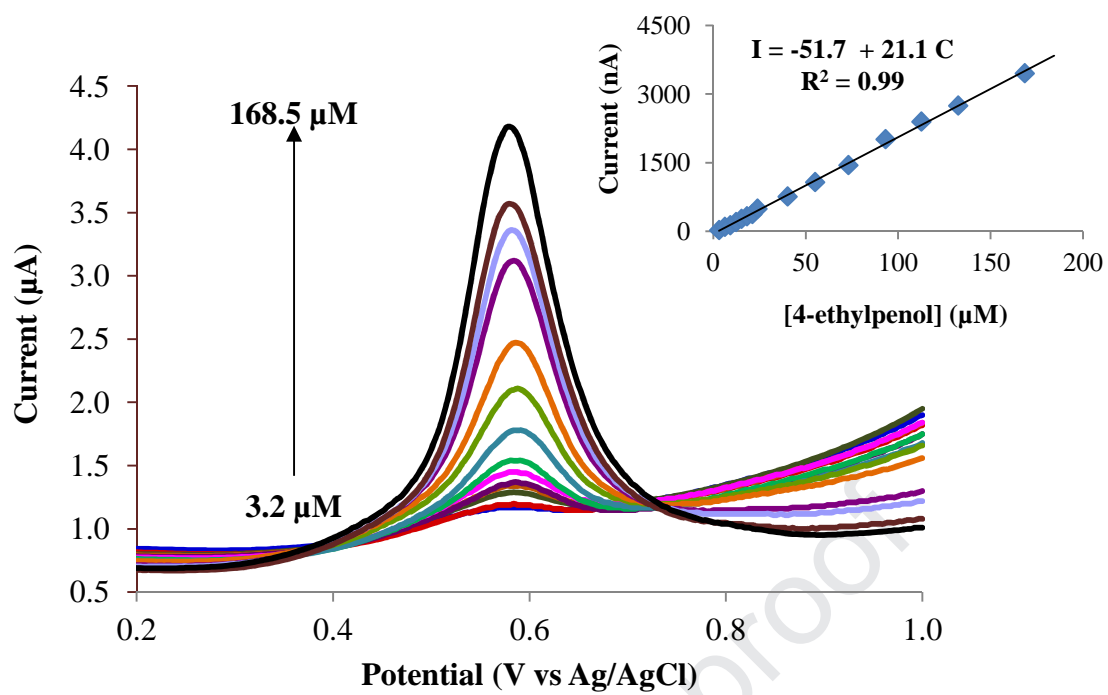
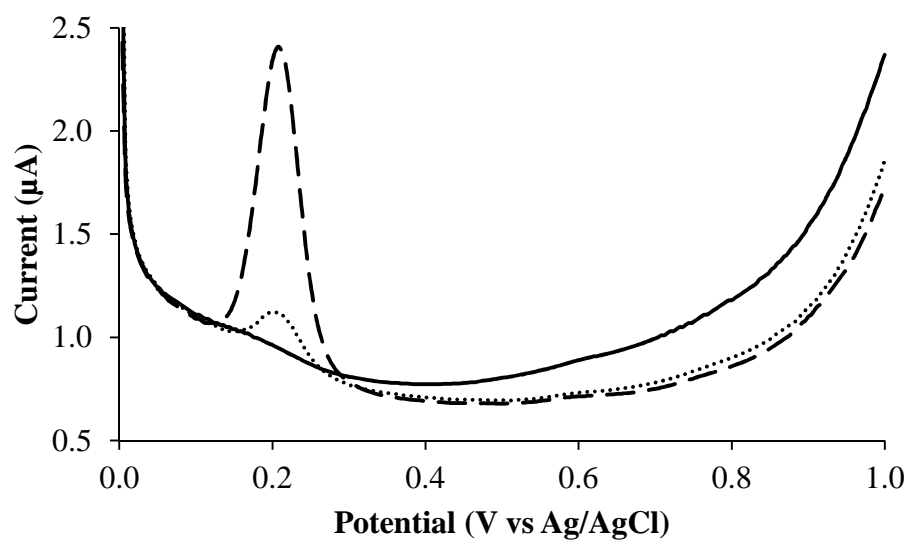


Figure 1

**Figure 2**

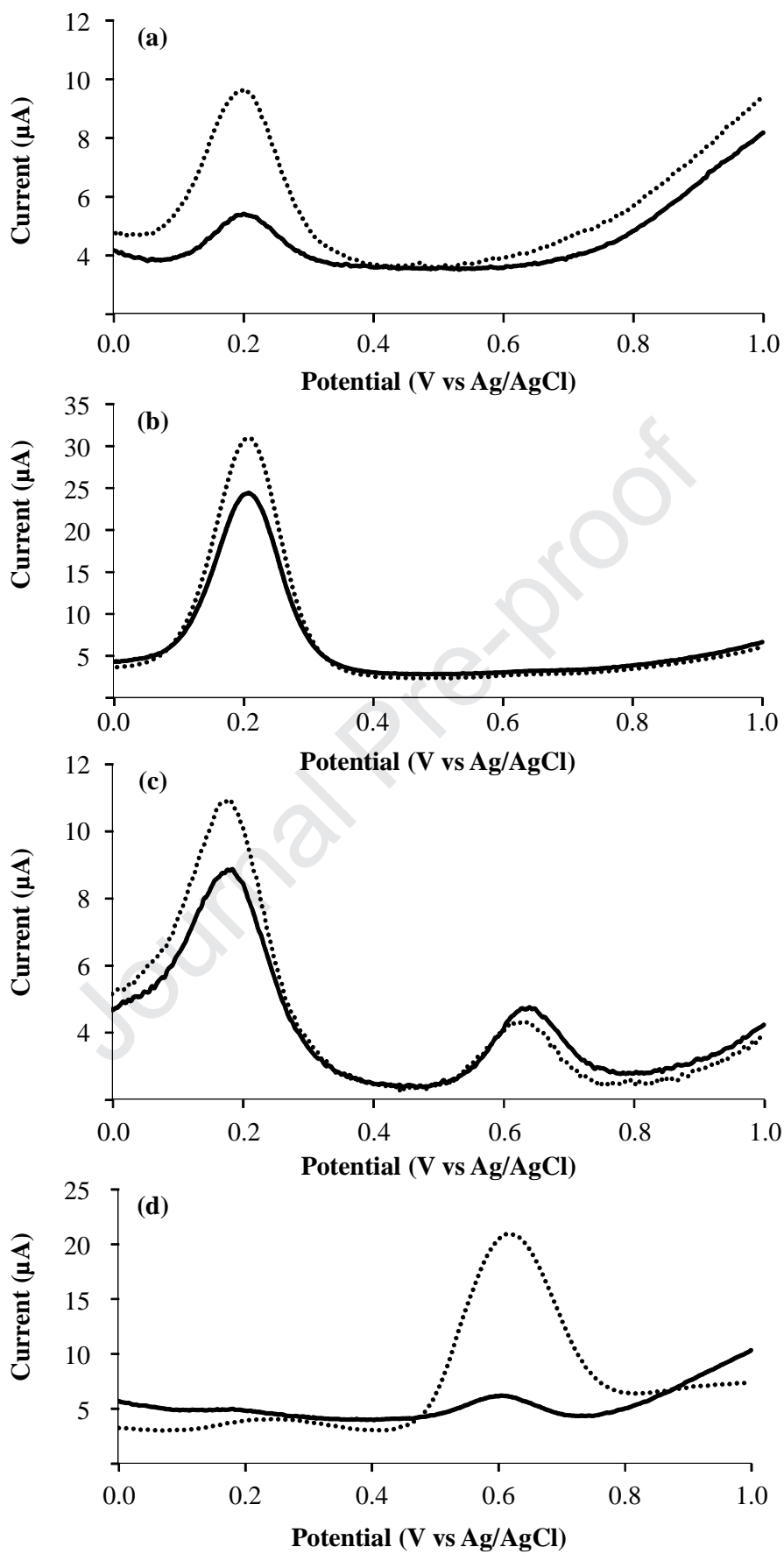


Figure 3

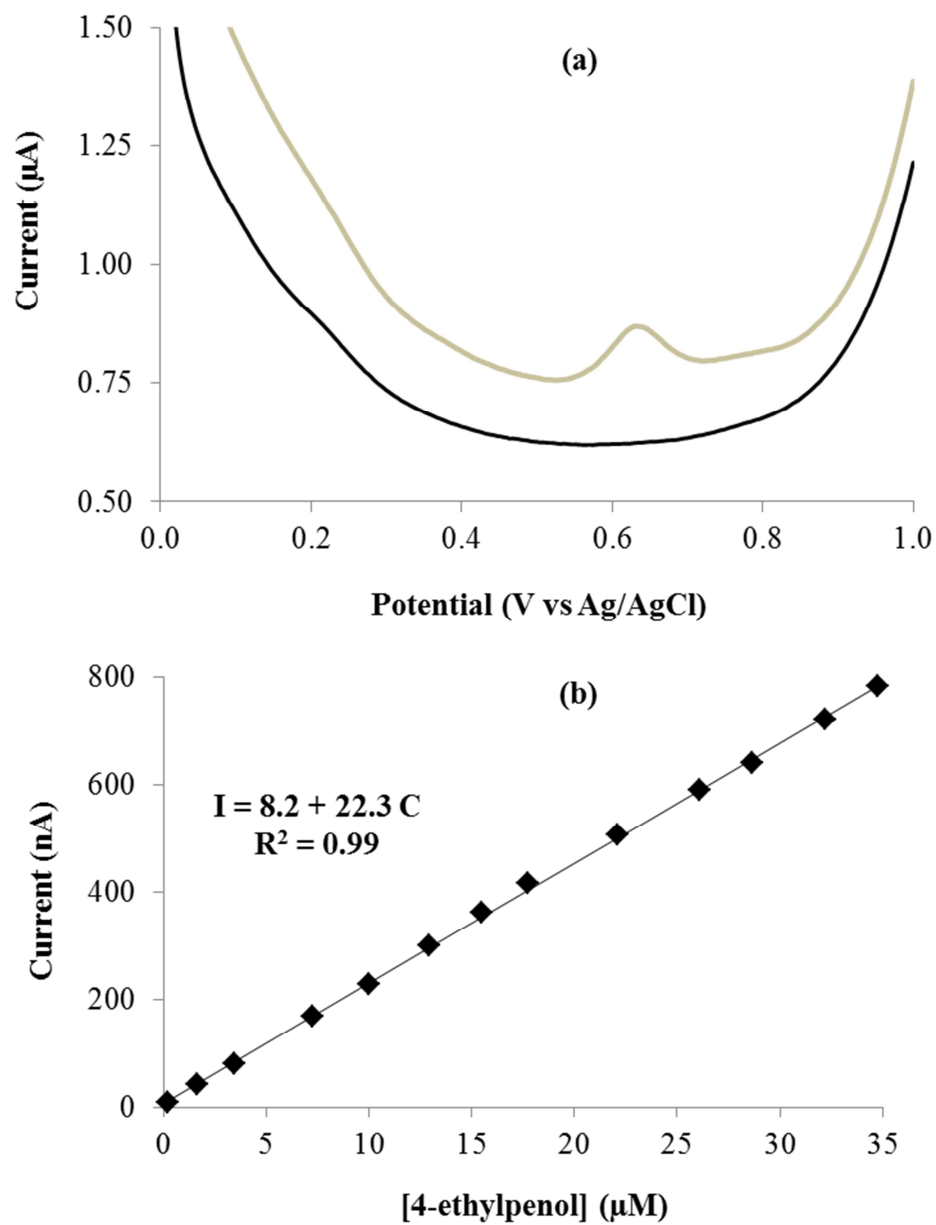


Figure 4

Highlights

- Voltammetric determination of 4-ethylphenol using a molecularly imprinted polypyrrole modified GCE.
- High sensitivity and selectivity with a wide linear range and good capability of detection.
- Successful determination of 4-ethylphenol in wine samples.