Electrochemical Studies of Ochratoxin A Mycotoxin at Gold Electrodes Modified with Cysteamine Self-Assembled Monolayers. Its Ultrasensitive Quantification in Red Wine Samples

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Abstract
The quantification of ochratoxin A is studied at cysteamine self-assembled monolayer modified gold electrodes in red wine samples by square wave voltammetry. Detection and quantification limits of 0.004 μg L^{-1} and 0.012 μg L^{-1}, respectively, were determined. The recovery percentages were in the range from 146% to 94.0% at spiking levels ranging from 0.02 to 5 μg L^{-1}. The variation coefficients for within-laboratory repeatability varied from 31.4 to 11.5% for spiked level from 0.02 to 2.0 μg L^{-1}. The developed electrochemical method is efficient, reproducible, and ultrasensitive for the quantification of OTA in red wine samples.

Keywords: Cyclic voltammetry, Ochratoxin A, Self-assembled monolayer, Square wave voltammetry, Sub-ppb electroanalysis

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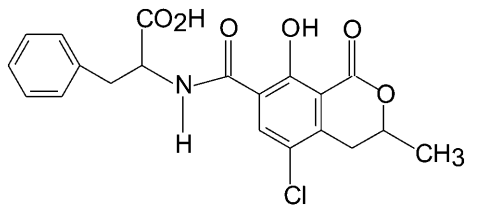
1 Introduction

Ochratoxin A, 7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin (OTA) is a mycotoxin mainly produced by several species of the *Aspergillus* and *Penicillium* fungi. Its molecular structure is shown in Scheme 1.

OTA has toxic properties for humans and animals via contaminated food, feedstuff and beverages [1–4]. The International Agency for Research on Cancer (IARC) has classified OTA in the Group 2B (carcinogenic to animals and possibly carcinogenic to humans) [1]. The Commission of European Union, in the Regulation N° 1881 [5], established that the OTA maximum permitted level in wines and fruits is 2.0 μg L^{-1}, and 0.5 μg L^{-1} for cereal-based processed food for infants and young children. Thus, the development of relatively fast analytical methods for OTA determination in contaminated samples is a mayor challenge, where sensitivity, selectivity, precision, and accuracy are required.



Scheme 1. Structure of Ochratoxin A, 7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin (OTA).

Several physicochemical-biological methods for OTA determination, which do not employ electrochemical devices, were published [6–17]. They were mainly chromatographic methods and enzyme-linked immunosorbent assays (ELISA). A recent review related to analytical methods for OTA determination has been reported [18]. Moreover, Wang et al. [19] published a review which includes electrochemical methods. Electrochemical biosensors for OTA determination with a high variety of platforms have also been described [20–27]. Recent reports dealing with immunosensors constructed on screen-printed electrodes based on ELISA format showed detection limits of about 0.3 μg L^{-1} [20,21]. However, to our knowledge, a few studies related to OTA electrochemical behavior have been described [28,29]. We have recently determined the thermodynamic and kinetics parameters of the surface redox couple of the OTA oxidation product adsorbed at glassy carbon electrodes in 10% acetonitrile + 90% 1 M $HClO_4$ aqueous [29].

On the other hand, monolayers from organosulfur compounds spontaneously self-assembled onto clean metallic surfaces (either obtained by contact with a compound solution or in vapor phase) have extensively been studied [30–32]. Molecules of monolayers exhibit a high order, orientation, and stability because they are covalently bonded to the corresponding metallic substrate. In addition, different interaction forces are present among adsorbed molecules, such as Van der Waals forces, hydrogen bond, and π-interactions [30]. Organosulfur compounds can be aliphatic or aromatic and have different chain length and variable tail functional groups [30]. Self assembled monolayer (SAM)-modified electrodes can enhance selectivity and sensitivity for substrate detection. However, the final response depends on the chain alkane-thiol length and/or the nature of tail functional groups [33–35]. The short alkane-thiol SAM probably forms thin monolayers with high defect sites or pinholes [36,37] where the electrocatalytic activity of different substrates has been observed, i.e.; epinephrine discharge at gold electrodes modified with homocysteine SAM [38].

In this work, we describe an ultrasensitive electrochemical method to determine OTA in red wine samples, which is based on cysteamine SAM modified polycrystalline gold electrodes in buffer solutions of pH 4.00. The electrochemical techniques used were cyclic (CV) and square wave (SWV) voltammetry.

2 Experimental

2.1 Chemicals

OTA was purchased from Sigma Chemical Company. 3-Mercaptopropionic acid and 2-amine-ethane-thiol (cysteamine) were from Fluka. Thiophenol, 1-undecanethiol, 4-aminothiophenol, 4-mercaptopyridine, and 2-(diethylamino) ethanethiol hydrochloride were from Aldrich. Diethyldithiocarbamic acid sodium salt trihydrate was from Sigma. Acetonitrile (ACN), H_2O, ethanol and $CHCl_3$ were from Sintorgan (HPLC grade). H_2SO_4, 30 % V/V H_2O_2 and citric acid were from Merck p.a. Polyvinylpirrolidone (PVP, MW: 360 000 g mol^{-1}) was purchased from Scientific Polymer Products, Inc. All reagents were used as received. Buffer solutions of pH 4.00, 7.00 and 8.00 were prepared by combining 0.1 M Na_2HPO_4 (Merck p.a.) with 0.1 M KH_2PO_4 (Merck p.a.). The final pH values were adjusted by adding different volumes of 1 M $HClO_4$ or 1 M NaOH. Buffer solution of pH 9.60 was prepared by mixing 0.05 M Na_2CO_3 (Merck p.a.) and 0.05 M $NaHCO_3$ (Merck p. a.).

Supporting electrolyte solutions were composed by 20 % ACN + 80 % buffer solutions of pH 4.00, 7.00, or 8.00. The glass filter paper was Wathman GF/A, MICROCLEAR, FFG070 WPH.

OTA standard solutions were prepared in ACN and kept at 4 °C in the dark. The final concentration was controlled by UV-vis spectroscopy, $\varepsilon = (5.97 \pm 0.02) \times 10^3$ M^{-1} cm^{-1} at $\lambda_{max} = 328$ nm [39]. Working solutions were prepared daily by adding aliquots of standard solution to the corresponding reaction media.

Cabernet Sauvignon red wine samples were purchased in a local supermarket. The wine samples were analyzed by AOAC official method 2001.01 and no OTA traces were detected. Thus, they were used as mycotoxin free wine samples (blank samples) and spiked with OTA known levels. OTA was extracted by a simple and inexpensive double liquid partition (L||L), i.e., an aliquot of 10.0 mL of wine sample was shaken twice with 100 mg of PVP to remove polyphenolic compounds, potential interferences to OTA electrochemical determination [40], and filtered with glass filter paper. The liquid (5.00 mL) was acidified with 2.50 mL of 1 M citric acid and extracted with 5.00 mL of $CHCl_3$. The phases were separated by centrifugation (5 min; 4600 rpm) and the aqueous phase was discarded. OTA in the organic phase was extracted with 5.00 mL of pH 9.60 $Na_2CO_3 + NaHCO_3$ buffer. These two new phases were centrifuged (5 min; 4600 rpm) and the organic phase was discarded. Then, 1.00 mL of the aqueous phase, which contained the OTA salt, was mixed with 1.00 mL of the corresponding buffer solutions (pH 4.00, 7.00, or 8.00). The exact pH values were achieved by addition of $HClO_4$ or NaOH solutions, with negligible volume modification. These extracts were used for performing the electrochemical or chromatographic analysis.

2.2 Apparatus and Experimental Measurements

CV and SWV measurements were performed with an AutoLab PGSTAT30 potenciostat controlled by GPES 4.9 electrochemical software from Eco-chemie, Utrecht, The Netherlands. The scan rate (v) was varied between 0.025 and 0.200 V s^{-1} for CV. A square wave amplitude of $\Delta E_{SW} = 0.050$ V and a staircase step height of $\Delta E_s = 0.010$ V were mainly employed in SWV. The frequency (f) was varied from 10 to 150 Hz. In some SW experiments, ΔE_{SW} was varied from 0.025 to 0.150 V.

Electrochemical measurements were carried out in a two-compartment Pyrex cell [29]. The working electrode was a polycrystalline gold disk (BAS, 1.6 mm diameter). Previous to perform the experiments, the electrode was successively polished on BAS™ cloth with diamond paste of 15, 3 and 1 μm, respectively, washed with water and cleaned in an H_2SO_4 + 30 % H_2O_2 (3 : 1 V/V) solution during 5 min. Then, it was placed in an ultrasonic bath for 5 min. Finally, it was cycled in 1 M H_2SO_4 between −0.2 and 1.6 V until a typical voltammogram of a clean surface polycrystalline Au was obtained. The different SAM modified electrodes were obtained by immersing the clean gold disk electrode in 5 mM thiol/ethanol solutions during 60 min, with the exception of the diethyldithiocarbamic acid sodium salt trihydrate, which was dissolved in water. The counter electrode was a large-area platinum foil ($A \cong 2$ cm^2). The reference electrode was an aqueous saturated calomel electrode (SCE) fitted with a fine glass Luggin capillary containing a bridge solution identical to

that containing the sample being measured. Solutions were deaerated by bubbling purified nitrogen for at least 10 min previously to perform the measurements. The temperature was $20.0 \pm 0.2\,^{\circ}C$.

3 Results and Discussion

3.1 OTA Electrochemical Oxidation at Bare Gold Electrodes

Cyclic voltammograms for both the blank solution (20% ACN + 80% buffer pH 7.00) and two consecutive scans recorded in the presence of OTA are shown in Fig. 1. OTA voltammograms showed a prepeak centered at about 0.6 V during the first scan, which could be assigned to the strong adsorption of OTA anodic oxidation product (OTQ) [29,41]. The diffusion controlled OTA main oxidation peak was overlapped with the corresponding gold oxidation wave [29].

This peak showed a diminution in the current response during the second scan, which could be attributed to a diminution in the effective electrode area as a consequence of the adsorption of OTQ [29]. On the other hand, cyclic voltammogram recorded from 0.0 to 0.7 V showed that the prepeak response was reproducible (Figure 2, solid and short dash lines), with a well-defined cathodic peak after reversing the potential sweep. The anodic peak current ($I_{p,a}$) varied linearly with v, as it is theoretically predicted for a surface redox couple [41]. In addition, the width at half-height ($\Delta E_{p/2}$) of the anodic peak was 93 ± 4 mV, which agrees closely to the theoretical value predicted for a monoelectronic surface redox couple, i.e., $\Delta E_{p/2} = 90.6/n$ mV at 25 °C [41]. From these results, it is possible to infer that the exchanged electron number would be one, for Nernstian conditions and Langmuir isotherm as it was checked by fitting the experimental isotherm for OTQ (result not shown). This behavior is different to that found for OTQ oxidation in a very acidic medium, i.e., 1 M $HClO_4$ at glassy carbon surfaces [29], were $\Delta E_{p/2}$ of the anodic peak was 53 ± 3 mV from CV experiments, a value which implies $n = 2$ [41].

On the other hand, the surface film coverage (Γ_{OTQ}^*) was determined from the Equation 1 [41]:

$$I_{p,a} = (n^2F^2/4RT)vA\Gamma_{OTQ}^* \quad (1)$$

Therefore, from the slope of a plot of $I_{p,a}$ vs. v and assuming $n = 1$ and $A = 0.020\ cm^2$, a value of $\Gamma_{OTQ}^* = 2.83 \times 10^{-10}$ mol cm^{-2} was estimated. The results obtained by SWV reinforced the idea that the charge transfer of the surface process is monoelectronic, i.e., an average value of 98 ± 7 mV was obtained for the width of the net anodic peak at half-height ($W_{1/2}$) for five frequency values, which is expected for a redox reaction with n close to 1 [42].

The pH effect was also investigated at the bare Au. The surface quasi-reversible redox process occurs at a lower potential at pH 4.00 (results not shown). The OTA pK_a values cited in literature are 4.2–4.4 and 7.0–7.3 for the phenylalanine carboxyl group and isocoumarine phenolic group respectively [24]. Thus, species of OTA with negative charge decreases at pH 4.00 in comparison with those at pH 7.00. Therefore, repulsive interactions also decrease, which favors the adsorption of OTQ and the signal appears at less positive potentials.

Although OTA determination is feasible on bare gold electrodes, the sensitivity achieved with cysteamine SAM modified gold electrodes is about four times higher, as it is described in the next Section.

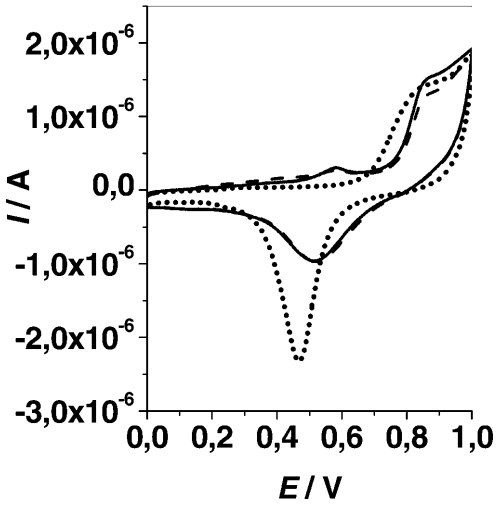


Fig. 1. Cyclic voltammograms recorded for the blank solution (20% ACN + 80% buffer pH 7, dotted line) and two repetitive scans (solid line: first scan; dashed line: second scan) in a solution containing OTA at a bare gold electrode. $c^*_{OTA} = 2.48 \times 10^{-4}$ M. $v = 0.025$ V s^{-1}.

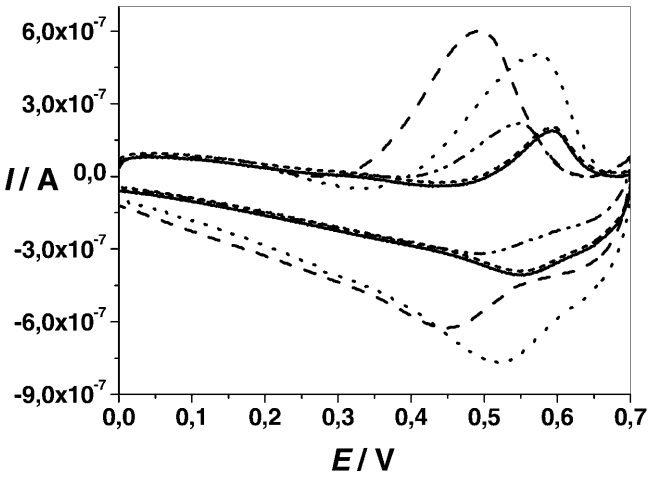


Fig. 2. Cyclic voltammograms recorded in 20% ACN + 80% buffer solutions at the bare gold electrode (pH 7.00) (—): first scan; (---) fifth scan) and at cysteamine SAM modified gold electrodes: (– – –) pH 4.00; (·····) pH 7.00 and (–··–) pH 8.00. $c^*_{OTA} = 2.48 \times 10^{-4}$ M. $v = 0.025$ V s^{-1}.

3.2 OTA Electrooxidation at Cysteamine SAM Modified Gold Electrodes

3.2.1 Cyclic Voltammetry

Several thiols (see Section 2.1) were employed to obtain modified gold electrodes to enhance the OTQ electroanalytical signal. The best response (the highest current and the lowest potential) was obtained with the cysteamine thiol (data for comparison between thiols are not shown). Therefore, the analytical data were obtained on the cysteamine SAM modified gold electrodes. Stability of cysteamine SAMs was evaluated after at least ten cyclic voltammetric scans. Typical CV profiles at pH values 4.00, 7.00 and 8.00 are depicted in Figure 2.

As it can be observed, the response at pH 8.00 is poorly defined. Signals at pH 4.00 and 7.00 show both a shift to a lesser positive potential (about 70 mV at pH 4.00 and 10 mV at pH 7.00) and an enhanced quasi-reversibility for OTQ signal with respect to the bare gold electrode.

Probably, the catalytic effect observed is a result of complex interactions (van der Waals, resonance, electrostatic forces and hydrogen bonding) between the adsorbent, adsorbate and the solvent [43]. The OTA pK_a value for the carboxyl group of the phenylalanine moiety is 4.25, so at pH 4.00 a fraction of molecules carries a negative delocalized charge, which can interacts with the opposite cysteamine tail group, i.e. $-NH_3^+$ [44]. Also, an electrostatic similar interaction was described for the OTA adsorption onto the silica gel surface positively charged at pH of wines (pH ~ 3.5) [43]. Although the main purpose of this paper was to demonstrate the high sensitivity of our developed technique for OTA determination, further studies are needed to clarify the nature of the process which leads to the catalytic effect. Electrochemical (EIS) or spectroscopic (ATR-FTIR, SEM) techniques would be appropriates, although not necessarily conclusive. For instance, in the catalytic effect for oxidation of ascorbic acid onto the supramolecular film modified gold electrode Fc-L-Cysteine/Au [44], the slight shift observed for different peaks in ATR-FTIR spectrum of the film was probably related to the coulombic interactions between $-COO^-$ and Fc^+.

Plots of $I_{p,a}$ vs. v were linear for both pH values and the sensitivity at pH 4.00 was about four times higher than that in the bare gold electrode. On the base of these results, the 20 % ACN + 80 % pH 4.00 buffer solution was chosen as the best reaction medium.

3.2.2 Square Wave Voltammetry

Typical responses were obtained for the surface quasi-reversible redox couple (adsorption pre-peak). Therefore, the forward (I_f), reverse (I_r) and net (I_n) currents were well defined (Figure 3). If it is considered that both redox species are confined onto a self-assembled structure by a strong adsorption, it is possible to perform a full kinetics and thermodynamic characterization by a combination of the "maximum quasi-reversible" and the "splitting of the net peak current" methods [45–47]. According to theoretical considerations, the relation between the net anodic peak current ($I_{p,n}$) and f is a parabolic function of the kinetics parameter $\kappa = k_s/f$, where k_s is the formal rate constant.

When $(I_{p,n}\, f^{-1})$ vs. f is maximum, i.e. $(I_{p,n}\, f^{-1})_{max}$, the frequency is $f_{max} = k_s/\kappa_{max}$ [48]. It is known that κ_{max} depends on both the anodic transfer coefficient $(1-\alpha)$ and $n\Delta E_{sw}$, but it is independent of $n\Delta E_s$ and the initial amount of the adsorbed reactive. Experimental plots of $I_{p,n}\, f^{-1}$ vs. f showed a maximum quasi-reversible centered at $f_{max} = (91 \pm 7)$ Hz.

On the other hand, the shape of the net SW response for adsorbed reversible redox couples is strongly influenced by the transfer coefficient, $(1-\alpha)$ [46,48,50].

For $(1-\alpha) > 0.2$, the ratio between the anodic (forward), $I_{p,a}$, and cathodic (reverse), $I_{p,c}$ peak currents can be approximated by a single exponential curve [49]:

$$I_{p,a}/I_{p,c} = 5.6414 \exp[-3.4606(1-\alpha)] \quad (2)$$

Therefore, the $(1-\alpha)$ estimation through Equation 2 is possible.

Moreover, the formal potential (E_f°) of the surface redox couple can be estimated as $E_f^\circ = 1/2\ (E_{p,a} + E_{p,c})$, where $E_{p,a}$ and $E_{p,c}$ are the corresponding forward (anodic) and reverse (cathodic) peak potentials, respectively. The forward and reverse peak potentials started to split as ΔE_{sw} is increased (Figure 3), at a given f, due to the relationship between the potential-dependent formal rate constant and the time scale of the experiment, being both the anodic and the cathodic peaks symmetrically located at about the E_f° of the surface redox couple [46,47].

Experimental values obtained for $E_{p,a}$, $E_{p,c}$, $I_{p,a}$, $I_{p,c}$, $I_{p,a}/I_{p,c}$ ratio and $(1-\alpha)$ at two frequencies and different ΔE_{sw} are shown in Table 1. Average values obtained for $(\overline{I_{p,a}/I_{p,c}})$, $\overline{E_f^o}$ and $(\overline{1-\alpha})$ were (1.3 ± 0.5), (0.48 ± 0.03) V and (0.43 ± 0.09), respectively.

These results allowed us to calculate the formal rate constant of the surface redox process, k_s, using the κ_{max} dependence [42,47] on both the product $n\Delta E_{sw}$ and $(\overline{1-\alpha})$. Considering $n = 1$, $n\Delta E_{sw} = 0.050$ V and $(1-\alpha) = 0.43$ a value of $\kappa_{max} = 0.89$ was obtained [47,48]. Therefore, $k_s = 81\ s^{-1}$ was calculated for the surface redox process.

3.3 Calibration Curve for OTA Commercial Reagent Samples

A calibration curve was constructed using the commercial reagent, so the matrix effects were absent. SWV was applied to OTA standard solutions in the concentration range from 4.67×10^{-11} to 1.10×10^{-9} M using 20 % ACN + 80 % pH 4.00 buffer solution as the reaction medium. SWV conditions were $f = 10$ Hz, $\Delta E_s = 0.010$ V and $\Delta E_{SW} = 0.050$ V. A plot of the net peak current of OTHQ

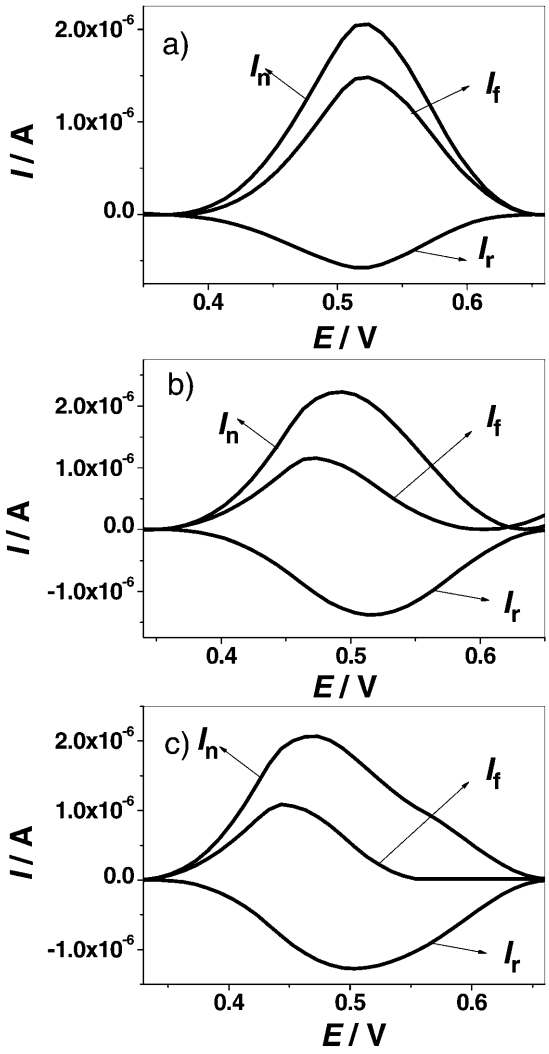


Fig. 3. The forward (I_f), reverse (I_r) and net (I_n) currents from SW voltammograms of OTHQ adsorbed at a cysteamine modified Au electrode at different SW amplitudes. ΔE_{SW}: a) 0.025 V; b) 0.075 V and c) 0.100 V. Reaction medium: 20% ACN + 80% buffer pH 4.00. $c^*_{OTA} = 6.20 \times 10^{-6}$ M. $f = 10$ Hz, $n\Delta E_s = 0.010$ V.

vs. c^*_{OTA} was linear. Results of the linear regression analysis by the method of least squares were: intercept $= (1.5 \pm 0.2) \times 10^{-7}$ A; slope $= (730 \pm 37)$ A M^{-1} and correlation coefficient, $r = 0.9947$. The detection limit (DL) for a signal/noise ratio of 3 : 1 [50] was 5.02×10^{-11} M (0.002 ppb).

3.4 Extraction Methods Comparison

The efficiency of OTA L||L partition extractive method used in this work (see experimental section) was checked against the official method [6]. Four samples of red wine spiked with different given amounts of OTA were prepared, as indicated in the experimental section. OTA was extracted by both, the IAC (Immunoaffinity Column) methodology [6] and the L||L partition. Then, solutions were evaporated and re-dissolved in methanol. The HPLC OTA determination in both groups (IAC and L|| L partition) permitted performing the correlation between the extraction methods, with the following linear regression parameters: intercept $= -0.048$ ng mL^{-1}; slope $= 0.99$; $s_{y,r=}0.032$; $r = 0.9934$, where $s_{y,r}$ is the standard deviation of the linear regression. As $r_{calculated} > r_{theoretical}$ ($0.9934 > 0.990$), the null hypothesis test is accepted [51] with a risk of 1%, for $v = 2$ and $N = 2$, where v is the freedom degree number and N the variables of the correlation. Therefore, the L||L extraction of OTA from the wine samples performed by us renders efficiency comparable to the official extraction method.

3.5 OTA Determination in Red Wine Samples

OTA indirect analysis in mycotoxin free red wines samples was carried out by spiking different amounts of pure commercial reagent in order to achieve the desired concentrations. It is well known that polyphenols can be discharged in potential regions similar to OTA oxidation, poisoning the electrode surface [40], which could produce interference with the OTA determination. Therefore, all spiked samples were pre-treated with L||L method as it was described in Experimental. The calibration curve ob-

Table 1. SWV parameters for the OTQ surface redox couple on cysteamine modified-Au electrode in 20% ACN + 80% buffer pH 4.00 OTA solution. $c^*_{OTA} = 6.20 \times 10^{-5}$ M.

f (Hz)	ΔE_{SW} (mV)	$E_{p,a}$ (V)	$I_{p,a}$ (μ)A	$E_{p,c}$ (V)	$I_{p,c}$ (μA)	$I_{p,a}/I_{p,c}$	$(1-\alpha)$[a]
10	25	0.52	1.47	0.52	0.58	2.55	0.23
	50	0.50	1.35	0.52	1.17	1.15	0.46
	75	0.47	1.15	0.52	1.35	0.85	0.55
	100	0.45	1.10	0.50	1.27	0.87	0.54
	125	0.42	1.07	0.47	1.04	1.03	0.49
	150	0.39	1.09	0.44	0.83	1.51	0.38
20	25	0.53	1.78	0.53	0.82	2.17	0.27
	50	0.51	2.60	0.53	2.10	1.24	0.44
	75	0.49	2.67	0.53	2.70	0.99	0.50
	100	0.46	2.56	0.51	2.42	1.06	0.48
	125	0.43	2.65	0.48	2.10	1.26	0.43
	150	0.41	2.64	0.45	1.70	1.55	0.37

[a] Values 0.23 and 0.27 are not rejected according to $Q_{90\%}$ criterion. $(\overline{I_{p,a}/I_{p,c}}) = (1.3 \pm 0.5)$; $\overline{E^o_f}$ and (0.48 ± 0.03) V; $(\overline{1-\alpha}) = (0.43 \pm 0.09)$.

tained for spiked samples was linear in the range from 2×10^{-11} to 1×10^{-9} M (0.008 to 0.4 ppb) with the following parameters: intercept $=2.26\times10^{-7}$ A; slope $=8760$ A M^{-1}; $s_{yr}=5.92\ 10^{-8}$; $r=0.992$. The correlation coefficient calculated is higher than the theoretical one, i.e. 0.990, so the null hypothesis is accepted [51] for 1% level of significance, $v=2$ and $N=2$, where v and N were defined in the previous section. The sensitivity measured from the slope of the linear regression is noticeably higher than that obtained from commercial reagent samples (Section 3.3), i.e., (8760 ± 776) A M^{-1} vs. (730 ± 37) A M^{-1}, respectively, probably due to a specific interaction of residual PVP with cysteamine, which in turn would favor the OTA-SAM interaction. This effect was also observed when OTA commercial reagent samples were pre-treated by the double partition extraction method. Detection limit (DL) and quantification limit (QL) were 0.004 ppb and 0.012 ppb, respectively, for a 3:1 signal/noise ratio.

3.6 Intralaboratory Testing of Method Accuracy

3.6.1 Recovery Assays

In order to evaluate the accuracy of the electroanalytical method here proposed, OTA recovery assays from spiked red wines were performed by the standard additions method and the results were matched with those obtained by the official method [6] (Identity line, section 3.6.2).

Four standard solutions were prepared by spiking the red wine samples with aliquots of 1.24×10^{-3} M OTA standard solution. The final concentrations were 0.02, 0.2, 2.0 and 4.95 ng mL^{-1} (Table 2). Then, samples were treated by both procedures of extraction at the same time. For the AOAC method [6], the samples were evaporated and re-dissolved in the mobile phase. Then, individual recoveries (R_i) for each spiked amount of OTA were determined and the mean recovery was $R=116.75\%$ for $n=4$. The comparison between the experimental $t=1.32$ with the theoretical $t_{2;0.05}=4.303$ let us to conclude that the null hypothesis test (100% of recovery) is satisfied.

The spiked versus found values plot has the following parameters: slope $=1.040$, intercept $=-0.019$, (Table 2), with residuals that fall within $\pm2s_r$, where s_r is the standard deviation of the regression. They show a reasonable normal distribution of errors when are plotted in a normal probability paper [51,52]. These facts indicate that the obtained recovery percentages are statistically acceptable [53].

Although the variation coefficients are within those expected by the Horwitz "trumpet" [52] at ppb levels, the anomalous 43.5% value at 4.95 ppb can be attributed to the incipient saturation of adsorption sites, which in turn is related to the diminution of both, sensitivity to OTA quantification (standard additions slope: $\cong183$ A M^{-1}) and correlation coefficient ($r=0.9764$).

3.6.2 Identity Line

In order to achieve another criterion to test the accuracy of the electroanalytical method for OTA quantification in red wines the results were also investigated by the AOAC 2001.01 official method [6]. Same four standard solutions of Section 3.6.1 were used to perform this analysis.

The concentrations determined by L||L-SWV methodology were plotted versus*s* those obtained from HPLC-immunoaffinity column cleanup with fluorometric detection, and the identity line renders the following parameters: intercept $=(0.037\pm0.045)$ ng mL^{-1}; slope $=(0.892\pm0.038)$ and $r=0.9991$.

Table 2. Recovery assays.

OTA spiked level (μg L^{-1})	c^*_{OTA} (μg L^{-1}) [e]	Standard additions (μg L^{-1})	% Rec	% VC [f]
0.020 [a]	0.029	0.0982 0.192 0.385	145	27.2
0.20 [b]	0.26	0.444 0.840 1.23	130	31.4
2.00 [c]	1.88	0.982 2.02 4.08	94	11.5
4.95 [d]	4.85	3.31 6.26 13.8	98	43.5

The standard additions column (column 3) shows three OTA concentrations (commercial reagent) used to perform the standard additions method at each spiked level (column 1). Linear regression parameters were: [a] slope $=(3776\pm83)$ A M^{-1} ($r=0.9995$); [b] slope $=(717\pm47)$ A M^{-1} ($r=0.9957$); [c] slope $=(603\pm31)$ A M^{-1} ($r=0.9974$); [d] slope $=(183\pm29)$ A M^{-1} ($r=0.9764$). [e] c^*_{OTA} determined by the standard additions method; average of two measurements. [f] Percentage variation coefficient.

Although the *t*-test is frequently used as a diagnosis criterion to verify the null hypothesis from the intercept and the slope, the correlation between both cannot be ignored [53]. Therefore, equation for elliptic joint confidence for the true slope ($m_0=1$) and intercept ($b_0=0$), which includes the Snedecor–Fischer statistic, *F*, was used to assess that slope and intercept are not different from the ideal values ($m_0=1$, $b_0=0$).

A way to determine the null hypothesis test, ($b_0=0$ and $m_0=1$, namely that the values are inside the ellipse) is to consider the intersections of the straight line $b_0=0$ and the ellipse [52].

The resolution of the equation for elliptic joint confidence gives, for our data, $z_1=0.557$ and $z_2=-0.597$ and so, $m_{0,2}=1.489$ and $m_{0,1}=0.335$, which satisfies the null hypothesis test.

Experimental results obtained demonstrate that L||L-SWV methodology is a very useful technique to detect and quantify OTA in wine samples. The correlation between L||L-SWV and AOAC official method [6] indicates the effectiveness of electrochemical sensor for OTA determination. The detection limit determined is lower than values in previous reports, i.e. HPLC [6,7,10,12,13,16], ELISA [8,9], capillary electrophoresis [11], surface plasmon resonance [14,17], FTIR-ATR [15], and screen printed electrodes [20,21]. Recently, Ansari et al. [27] reported a similar detection limit (0.006 ppb) for OTA using nanostructured zinc oxide platform for OTA detection. On the other hand, the extraction procedure used in this work as well as the instrumentation required is less expensive than that necessary for performing chromatographic measurements.

4 Conclusions

A very sensitive and reproducible electrochemical sensor for ochratoxin A determination in red wine samples is described. It is based on the analysis of the surface pre-peak produced by the quasi-reversible electrochemical oxidation of the product of OTA electrooxidation at cysteamine self assembled monolayer on gold electrodes. This sensor has acceptable recovery percentage (≅117 %), variation coefficient of about 27 % at 0.020 ppb level and sensitivity of 8760 A M^{-1} and it seems to be one of the most sensitive electrochemical methods reported for OTA quantification in red wines i.e. detection limit of 0.004 ppb and quantification limit of 0.012 ppb for a 3 : 1 signal/noise ratio. On the other hand, SWV was employed to perform a full thermodynamic and kinetics characterization of the surface redox couple. Results obtained with the electroanalytical method here proposed were in very good agreement with the official analytical method. Main advantage of the method here proposed lies in its simplicity, low cost and short time of performing determinations. The very low detection limit obtained for OTA determination in red wines is one of the main improvements as compared with usual methodology used for OTA quantification in a very demanding area of food industry.

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