Sensitive Voltammetric Sensor for Fast Detection of Mycolic Acids Present in *Mycobacteria*

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The present work describes a fast, simple, and economic method for electroanalytical detection of mycolic acids (MA) present in the cell envelope of *Mycobacterium*, *Corynebacterium*, *Nocardia,* and *Rhodococcus*. MA are pre-concentrated on a glassy carbon electrode modified with a poly-L-lysine film, resulting in a well-defined reduction wave at -0.73 V (vs. Ag/AgCl) and being the base for detection and quantification of bacteria, like *Mycobacterium smegmatis,* used as model in this work. The method was optimized and analytical curves were constructed for *Mycobacterium smegmatis* by using square wave voltammetry. The method offers a detection limit of 5.9×10 colony forming units (CFU) mL^{-1}, and the reproducibility for 10 measurements at 1.4×10^2 CFU mL^{-1} showed a standard deviation of 1.8%. The method was successfully applied for detection of MA in water samples as indirect measure of the presence of *Mycobacteria.*

1. INTRODUCTION

MA are α-alkyl β-hydroxylated fatty acids considered as major component of the cell wall in the genus *Corynebacterium, Gordona*, *Tsukamurella,* and *Mycobacterium* [1]. Among these genuses, problems with mycobacterium have been most often reported in the literature. Their removal from wastewater has been a challenge, since they contribute to biofilm formation which makes treatment

with chlorine and ozone difficult and also promotes deterioration of drinking water distribution systems [2].

The genus *Mycobacterium* (*M.*) includes a number of species, from highly pathogenic microorganisms such as *M. tuberculosis* and *M. leprosy*, opportunistic species such as *M. kansasii* and *M. avium*, to saprophytic species such as *M. smegmatis* [3]. The majority is ubiquitously present in soil and water [4]. They are called acid-fast bacilli, characterized by high contents of MA in their lipid-rich cell walls [5].

Rapid and sensitive detection and quantification of these clinically and/or environmentally important bacteria could be helpful for efficient and effective prevention of mycobacterial infection and safety use of water [6]. Established methods such as culture and colony counting [7-8] are time consuming and cumbersome. Polymerase chain reaction [9-10] for pathogen detection requires advanced equipment and skilled people.

In the literature a linear relationship between the concentration of MA and the number of CFU of mycobacteria is described [11-12]. Thus, the amount of MA in a microorganism suspension or clinical sample is a good indicator of the number of microorganisms present. Analytical methods based on the determination of MA by thin-layer chromatography [13], gas chromatography coupled to mass spectrometry [14], high performance liquid chromatography (HPLC) [15-17], and mass spectrometry [18-19] are currently proposed for determination and identification of mycobacteria. However, these methods require very expensive equipments and highly skilled people.

Therefore, the development of an electrochemical method as sensitive, simple, inexpensive, and user-friendly analytical method for the detection of MA is promising. However, the reduction of aliphatic chains with carboxylic groups usually requires potentials which are more negative than -1.5 V (*vs* Ag/AgCl). The reduction can take place if they are activated by the proximity of strongly electron-withdrawing groups [20]. Electrodes chemically modified with poly-L-lysine (PLL) have been described for many applications [21-22].

Therefore, the aim of the present work is to develop a voltammetric sensor for detection of MA using a PLL-modified vitreous carbon electrode. The device was applied to indirect electrochemical determination of *M. smegmatis* by quantification of MA in water collected from a drinking water treatment plant.

2. MATERIALS AND METHODS

2.1. Apparatus and reagents

The electrochemical analyses were performed using a potentiostat (PGSTAT-30, Metrohm Autolab, Utrecht, The Netherlands) controlled by general purpose electrochemical system software. A conventional electrochemical cell of 10.0 mL was used into which a glassy carbon electrode (2.0 mm diameter) as working electrode, a platinum wire as auxiliary electrode, and an Ag/AgCl (KCl 3 mol L^{-1}) reference electrode were inserted.

All pH measurements were carried out using a combined glass electrode (Corning) connected to the digital pH-meter (model pH/ion analyzer 455, Corning, New York, USA). De-ionized water was purified with a Milli-Q system (model Simplicity 185, Merck-Millipore, Billerica, MA, USA).

A liquid chromatograph HPLC Shimadzu (Model 10AVP, Shimadzu, Kyoto, Japan) equipped with photodiode array detector and auto sampler (SIL-10ADVP) was used for chromatographic analysis, injection volume 20 µL. An octadecylsilica column (250 mm x 4.6 mm, 5 µm) was used for separation at a column temperature of 35°C; the mobile phase consisted of a methanol/dichloromethane gradient with dichloromethane increasing linearly from 35 to 65% in 15 min, flow rate 2 mL min^{-1}, detection wavelength 260 nm.

A triple quadrupole mass spectrometer (LC-ESI-MS/MS 3200 Q TRAP, Applied Biosystems/ MDS Sciex) was used to obtain mass spectra of MA derived from mycobacteria extracts. Spectra were obtained of MA from the nonpathogenic bacterium *Mycobacterium smegmatis* at the following settings: negative ion mode, ion spray voltage -4500V, ion source gas nitrogen at 20 L min^{-1}, scan range 1000 a 1400 Da, declustering potential -45 V, entrance potential -10 V, ion energy -1.0 V, detector channel electron multiplier at 2200 V. The MS/MS scans of the three precursor ion m/z 339, 367, and 395 were performed in the range of m/z 1000 to 1400.

Methanol (J. T. Baker, Xalostoc, México), dichloromethane (Macron Chemistry, Phillipsburg, USA), and chloroform (J. T. Baker, Xalostoc, México) were of chromatographically pure grade; p-bromophenacyl bromide and dicyclohexyl-18-crown-6 ether were of pure grade (Sigma-Aldrich, St. Louis, USA).

The rapidly growing nonpathogenic bacterium *Mycobacterium smegmatis* strain ATCC 19420 used as model for investigating the class of mycobacteria was donated by Dr. C.Q.F. Leite. The bacteria were maintained on Lowenstein-Jensen agar slants at 37 °C [4].

2.2. Analytical Procedure

All voltammetric measurements were carried out transferring 10 mL of the supporting electrolyte solution to the electrochemical cell, where the set of conventional electrodes was inserted. After that, an aliquot of the solution of MA from *M. smegmatis* was added to the electrochemical cell, the solution was deoxygenated with nitrogen gas during 5 min before carrying out further voltammetric measurement.

The working electrode (2 mm diameter was previus polished with alumina (0.3 micrometers, BUEHLER), and then washed and dried at room temperature. So, 10 µL sample of an aqueous solution poly-l-lysine 1% w/v (MM 77300) was placed on the polished electrode surface and the inverted electrode was placed in a drying oven at 80 °C for 2 min [22]. After evaporation, the modified electrode was rinsed with water and immersed in the solution.

2.2.1. Extraction of mycolic acids from M. smegmatis

For electrochemical measurements a purified extract of the MA extracted from *M. smegmatis* was used, obtained by warming of 2 loopful of the mycobacteria grown in Lowenstein-Jensen agar slants at 37 °C with 2 mL of saponification reagent composed of 25 weight-% potassium hydroxide in a water-methanol (1:1, v/v) mixture. The suspension was vigorously mixed using a vortex and

autoclaved for 1h at 121 °C. After the mixture had cooled to room temperature, 2 mL chloroform and 1.5 mL of acidification reagent, a mixture of water and concentrated hydrochloric acid (1:1, v/v), were added slowly. This solution was mixed by vortex and the layers were separated by centrifuging at 2,500 x g for 15 min.. The lower organic layer was transferred with a Pasteur pipette to a new centrifuge tube (15 mL) and evaporated in a heat block from 85 to 105 °C with a stream of air [15]. The solid residue was dissolved in methanol. For the optimization of the methods two loopful of bacteria grown on solid medium (Lowestein Jensen) were used. The MA extracted from *M. smegmatis* obtained was confirmed by mass spectrometric analysis.

For chromatographic analysis the mycobacteria was also used a purified extract of mycolic acids and after the purification was performed the derivatization of the sample. The derivatization reagent bromophenacyl bromide (PBPA) (0.1 mol L^{-1}) and the catalyst dicyclohexyl-18-crown-6 ether (0.005 mol L^{-1}) were suspended in acetonitrile. The MA sample was derivatized to PBPA esters by adding 20 mg of potassium bicarbonate, 50 µL of derivatization reagent, and 1.0 mL of chloroform. The tubes were secured with Teflon-lined screw caps and heated from 85 to 105°C for 20 min. Samples were cooled and then clarified by filtration through a 0.45 µm pore-size filter. Derivatization of the mycolates to UV-absorbing PBPA esters resulted in a stable compound which could be stored for several months in the dark at 4°C [15].

The enumeration of *M. smegmatis* colonies was performed using the colony counting method. The mycobacterium was grown on 7H10 in several dilutions of these mother growth media and incubated for 2 days at 35 °C [7].

2.2.2. Analysis of M. smegmatis in drinking water

The method proposed was applied to the analysis of *M. smegmatis* in drinking water collected from a Drinking Water Treatment Plant, but the sample does not show any traces of mycobacteria. The results were confirmed by HPLC-DAD using the procedure described previously. Thus, 1 mL of the sample was purposely contaminated with 1.4×10^3 CFU mL^{-1} mycobacteria submitted to autoclave (T= 121 ^{0}C, 1 h), diluted to 9 mL of the supporting electrolyte and analyzed by the method proposed. The quantification of the *M. smegmatis* in the matrix was performed using the standard addition of stock solution of mycolic acids extract.

3. RESULTS AND DISCUSSION

3.1. Voltammetric reduction of mycolic acid on glass carbon electrode modified by poly-l-lysine films

Different solvents were tested to dissolve the MA from *M. smegmatis* extract, such as water, acetonitrile, methanol and ethanol. The best results were obtained for methanol, which showed the best analytic signal.

In order to confirm that MA are the main product in the extracted of *M. smegmatis*, it was submitted to mass spectrometry (MS) analysis. The full scan of electrospray ionization- mass

spectrometry ESI-MS/MS in negative ion mode obtained for MA extracted from *M. smegmatis* (Figure 1). The mass spectrum is characterized by a pattern with different peaks in the range of *m/z* 1000–1400. The different molecular weights of product ions from the single species suggested that each peak in the spectrum reflects the occurrence of MA with different α-side chain lengths. The expected fragmentation produced a pattern with derived ions at m/z= 395, m/z= 367, and/or m/z= 339, which correspond to α-alkyl side chain fragments formed after fragmentation of MA β-hydroxycarboxylic acids. The *M. smegmatis* has two types of MA: α-mycolates and epoxymycolates [23]. In terms of the Pre 367 MS/ MS scans, *M. smegmatis* produced a major precursor ion of α- mycolate at m/z = 1080 correspondent to a α- mycolate with 74 carbon atoms (C 74), 1108 (C76), 1122 (C77), 1136 (C78), 1150 (C79), 1164 (C80), 1178 (C81) and the epoxymycolates at m/z = 1082 (C73), 1110 (C75), 1124(C76), 1138 (C77), 1152 (C78), 1166 (C79) and 1180 (C80). Using Precursor ion m/z 339 this mycobacteria produced the major precursors ions of α- mycolate at m/z 1052 (C72), 1080 (C74), 1094 (C75), 1122 (C77) and 1136 (C78) and the epoxymycolates at m\z 1054 (C71), 1082 (C73), 1096 (C74), 1124 (C76) and 1138 (C80). Thus, the α-side chains of *M. smegmatis* was found to contain 22 or 20 carbons with a peak height ratio of 3.4:1 , but not 24 carbon α-side chains. Therefore, these results confirm that the main product generated in the extracted are the MA derived from *M. smegmatis*.
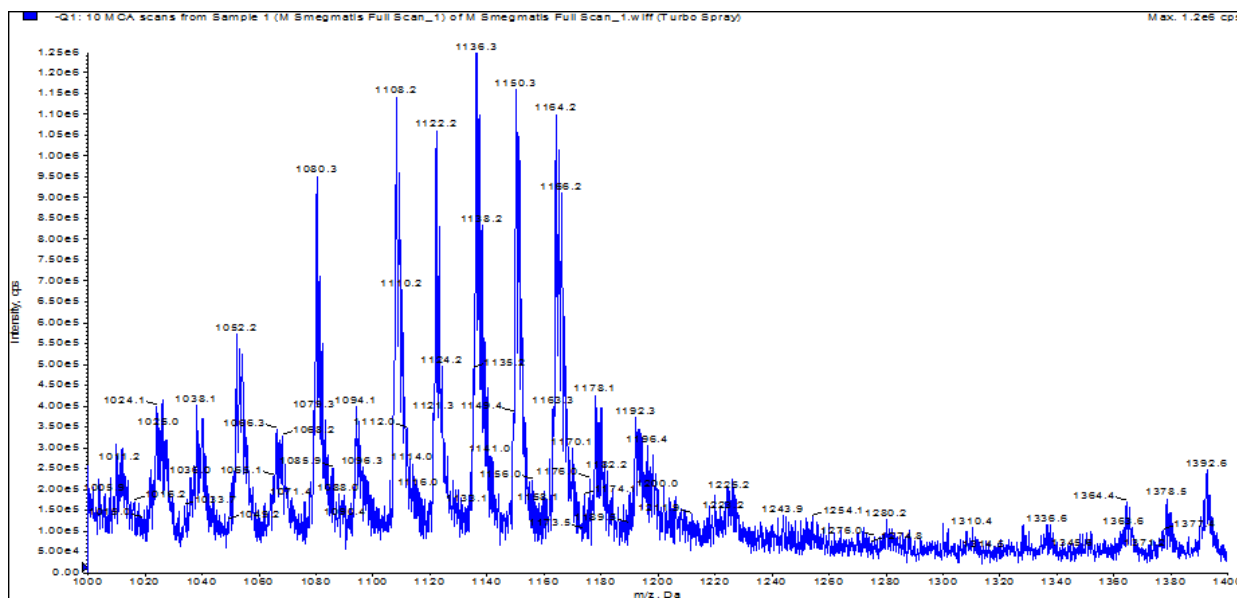

Figure 1. Mass spectrum obtained from MA extracted from 4.2×10^4 CFU mL^{-1} *M. smegmatis*.

In order to develop a simple and economic method to the MA quantification voltammetric curves were obtained for reduction of purified MA obtained from *M. smegmatis* 4.2×10^4 CFU mL^{-1} dissolved in methanol + 10 mL of Britton -Robinson (B-R) buffer (0.4 mol L^{-1} in each of acetic, o-phosphoric, and boric acids adjusted to pH 6 with 0.2 mol L^{-1} sodium hydroxide solution) on bare glassy carbon electrode. The electrochemical reduction does not show any voltammetric signal (Figure

2, dashed line, a). This is expected, since in agreement with literature [20] the reduction of long aliphatic chain with carboxylic groups usually requires potential more negative than -1.5 V (vs. Ag/AgCl). However, this reduction can take place if the group is activated by proximity with strongly electron-withdrawing group [20]. A well-defined peak at -0.73 V (vs. Ag/AgCl) is seen when they are reduced at glassy carbon electrode modified with PLL films in B-R buffer pH 6.0 (Figure 2, curve b).
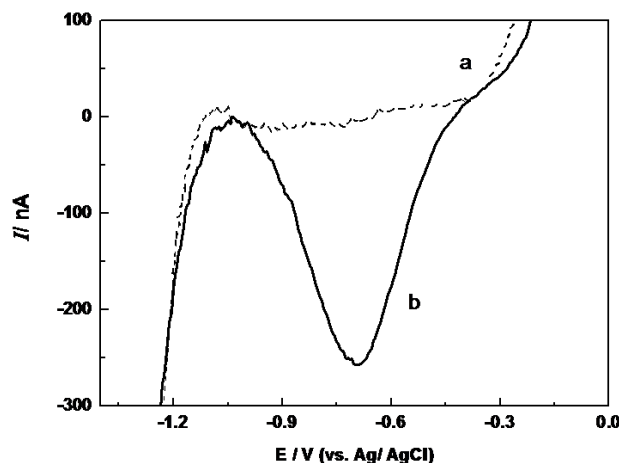

Figure 2. Linear sweep voltammograms obtained for reduction of MA (4.2×10^4 CFU mL^{-1} *M. smegmatis*) in BR buffer at pH 6.0 on glassy carbon electrode unmodified (a) and modified with poly-l-lysine (b). υ= 100 mV s^{-1}. *I*= current, E= potential.

The cathodic process is characterized as an irreversible process, since there is not current on the reverse scan at any scan rate investigated (10 to 500 mV s^{-1}).

As verified previously for cathodic reduction of amides and imides derivatives on mercury electrode [20], these results indicate that the reduction of the compound could involve 2 electrons and 2 protons. Thus, the voltammetric response for MA using glassy carbon electrode modified by PLL films (GCE/ PLL) could be due the reduction of the H-N–C=O group [20], from the peptide bond that can be formed. In agreement with the literature [5], the electrochemical reduction can be attributed to all types of MA, since carboxylic group is present as terminal group in all types of them.

In order to develop a suitable analytical procedure for MA determination, the electrochemical behavior of the reduction of MA on the modified electrode was test by linear sweep (LSV), differential pulse (DPV) and square-wave voltammetry (SWV). The respective voltammograms obtained are compared in Fig. 3. The peak current observed at   -0.75V (vs. Ag/ AgCl) is much larger on SWV, and an ill-defined shoulder is observed at less negative potential. This behavior could be attributed to the overlap of different types of MA, suggesting again that the method could be an alternative to monitor all types of mycolic acids able to form interactions with amine group of PLL. Further studies were carried out using SWV technique.
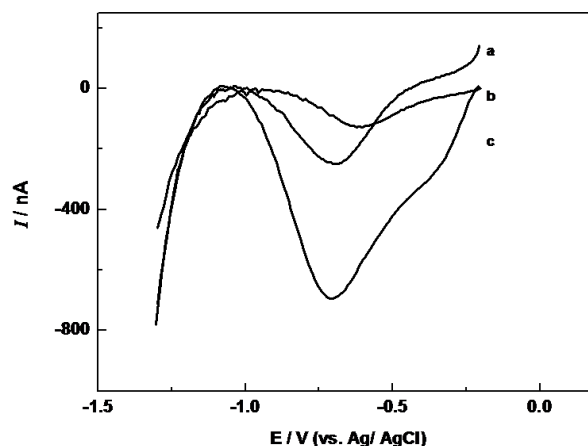
Figure 3. Voltammograms of reduction of MA from 4.2×10^4 CFU mL^{-1} *M. smegmatis* in BR buffer at pH 6.0 on glassy carbon electrode modified by PLL film using differential pulse **(a)**, linear sweep **(b)**, and square-wave voltammetry **(c)** modes. *I*= current, E= potential.

3.2. *Optimum parameters to monitor MA by square-wave voltammetry*

Experimental parameters inherent of the SWV technique were evaluated such as: frequency, f (10- 200 Hz), step potential, ΔE_s (2- 10 mV), and pulse amplitude, E_{sw} (10- 100 mV). The optimized values for the parameters taking into account the shape and intensity of the voltammetric curves was f= 50 Hz, ΔE_s = 8 mV and E_{sw} = 50 mV. It was also verified that the cathodic peak current varied linearly with the square root of the frequency according to the Equation 1, demonstrating that the MA reduction is controlled by diffusion.

$$I /\mu A= 0.180 (\pm 0.05) – 0.19\ 9 (\pm 0.006) / (f/Hz)^{1/2}\ (r= 0.993)$$

In order to check the effect of accumulation potential and accumulation time on the voltammetric response obtained for MA reduction from 4.0×10^2 CFU mL^{-1} *M. smegmatis* in B-R buffer pH 6.0 on GCE/ PLL, SWV were recorded after previous accumulation from 0 to 0.4 V (vs. Ag/AgCl) and time from 0 to 480 s, respectively. There was not modification on the voltammetric signal for both, potential and time. In addition, the peak intensity is also constant at open circuit mode showing that the reaction takes place very rapidly on the electrode surface. It was also observed that the film surface can be renewed using a subsequent step of stirring with nitrogen gas during 5 min before carrying out further voltammetric measurement. Thus, all the further studies were carried out using a nitrogen flow kept during 5 min after each measure.

The repeatability of the method was evaluated recording ten successive voltammograms for MA reduction from 1.4×10^2 CFU mL^{-1} *M. smegmatis* in B-R buffer pH 7.0. The voltammograms presented a stable signal and the obtained relative standard deviation was 1.8%.

The effect of pH on the voltammetric response was evaluated monitoring the peak current and peak potential obtained from voltammograms recorded to MA reduction from *M. smegmatis* 4.0×10^2 CFU mL^{-1} in B-R buffer at pH values changing from 2.0 to 12.0. The results are shown in Fig. 4 and 5,

respectively. Above pH ≥10 there is not peak. This behavior could be due the MA is in the form of carboxylate, which hinders the reaction or can generate an unstable product. But, at 1 < pH < 9, the peak potential shifted to more negative values following the Equation 2.

$$E/V \text{ (vs. Ag/ AgCl)} = 0.51 \ (\pm 0.01) + 0.025 \ (\pm 0.002) \ pH \ (r = 0.975) \ (2)$$

Figure 4 indicates that the slope of 25 mV per pH unit involves hydrogen ions in the electrochemical reaction. The peak current (Fig. 5) is maximum at 5< pH < 8, but decreases markedly at acidic medium. This behavior is observed for other carbonyl group of amides $(R_1C(OH)R_2CONHR_3)$, that in strongly acid medium can suffer cleavage due disproportionation reaction [20].
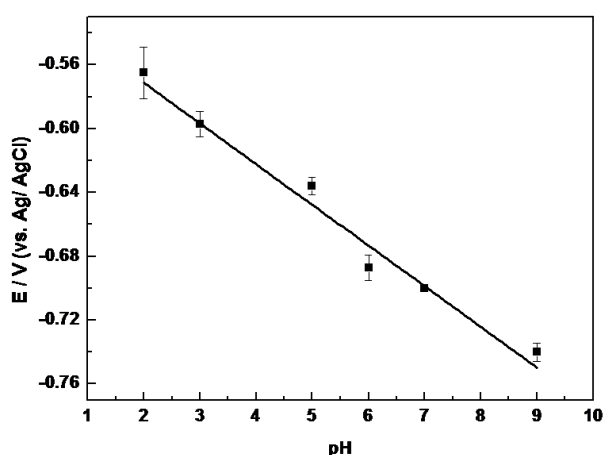

Figure 4. Effect of pH on the peak potential (E) obtained from square-wave voltammograms recorded for MA reduction from 4.2×10^4 CFU mL^{-1} *M. smegmatis* in BR buffer on glassy carbon electrode modified by PLL.

Figure 5. Effect of pH on the peak current (*I*) obtained from square-wave voltammograms recorded for MA reduction from 4.2×10^4 CFU mL^{-1} *M. smegmatis* in BR buffer on glassy carbon electrode modified by PLL.
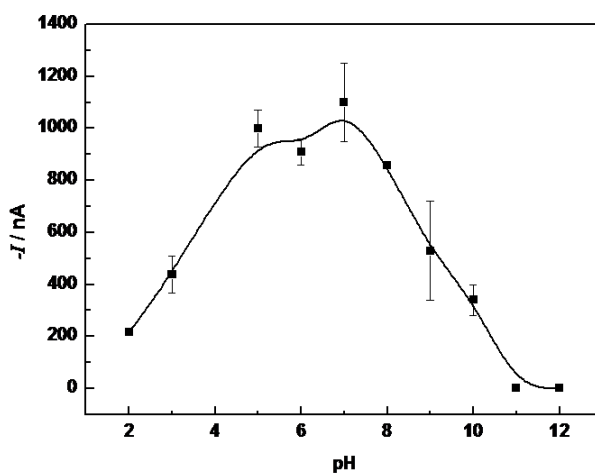
Thus, comparing the yielding peak current values, shape and reproducibility of the recorded voltammograms, the best experimental condition to detection of MA was obtained with B-R buffer at pH 7.0. Nevertheless, the use of phosphate buffer pH 7.0 was tested and showed similar behavior and more stable measures. So, this medium was chosen for further analysis.

3.3. Analytical Curve

Using the optimum experimental condition previously defined by square-wave voltammograms (f = 50 Hz, ΔE_s = 8 mV and E_{sw} = 50 mV, phosphate buffer pH 7.0) it was constructed analytical curves by measuring mycolic acids extracts from 1.4×10^2 to 1.4×10^4 CFU mL^{-1} *M. smegmatis*, the measurements were carried out in triplicate. The peak intensity increases with MA concentration up to 1.4×10^3 CFU mL^{-1} *M. smegmatis*, and it reaches a plateau above this concentration, suggesting that the active sites available on the PLL film are readily saturated at high MA concentration.

Then, it was constructed the analytical curve for MA extracted from *M. smegmatis*, the concentration of *M. smegmatis* was determined by colony counting methods [7]. The analytical curve exhibits a linear relationship from 1.4×10^2 to 1.5×10^3 CFU mL^{-1} *M. smegmatis*, following the Equation 3. The results indicate that bacteria with MA in the cell wall can be detected and quantified by SWV using its MA extract.

$$I/\mu A = 0.046 \, (\pm\, 0.001) + 5.64 \times 10^{-4} \, (\pm\, 1.17 \times 10^{-6}) \, [\textit{M. smegmatis}] \, / \, CFU \, mL^{-1} \quad (r= 0.998) \quad (3)$$

The limits of detection (LOD) and quantification (LOQ) were calculated using statistic treatment (3 Sc.D./b) and (10 Sc.D./b), respectively, where, Sc.D. is the standard deviation of the 10 voltammograms of the blank obtained in the same potential reduction of MA and b is the slope of the calibration curve. The LOD and LOQ were 5.9×10^1 CFU mL^{-1} and 1.9×10^2 CFU mL^{-1}, respectively, both values indicated satisfactory sensitivity of the proposed method. The calibration curve did not fit zero because the MA were extracted directly of the mycobacteria, it was not used a commercial reagent. The cell structure is very complex and some compounds can interfere in the measure, like other fatty acids and other compounds synthetized by the mycobacteria. So, the method of standard addition is indicated to the bacteria quantification and it was tested to obtain best and confinable results to the determination of mycobacteria concentration in water samples.

The proposed methods is more sensitivity than other techniques proposed in the literature, that shown determination at concentration level up to 10^7- 10^6 CFU mL^{-1} [9, 18-19, 24-25].

3.4. Analysis of M. smegmatis in drinking water

The optimized procedure was applied for determining *M. smegmatis* in drinking water samples using the standard addition method of purified extract of MA, as described in the experimental section. The water sample collected did not show any presence of mycobacteria, comprised by analysis of MA using the electrochemistry method proposed and the grown in medium 7 H10 by 7 days. So, water

samples were spiked to 1.4×10^3 CFU mL^{-1} *M. smegmatis* and submitted to extraction with chloroform, in order to avoid interference from others substances. After carried out the extraction the solution was added to 9 mL of supporting electrolyte and submitted to direct measurement using square-wave voltammetry with successive standard addition of MA. The peak current increased linearly and the respective values obtained for MA in the sample presented recoveries from 93.5–99.2% of *M. smegmatis* in the tap water samples (n=3 repetitions), Fig. 6. This is an evidence of the accuracy of the proposed procedure. The statistical calculations for the results suggested good precision for the voltammetric method. According to the t-test, there were not significant differences between the recovery and added values at the 95% confidence level and within an acceptable range of error, *t*= 1,83. Thus, the values obtained for the recovery and % R.S.D. (7.6%) are satisfactory and they are in agreement with the spiked value, suggesting that the proposed method is an excellent alternative for the analytical determination of *M. smegmatis* in tap water samples.
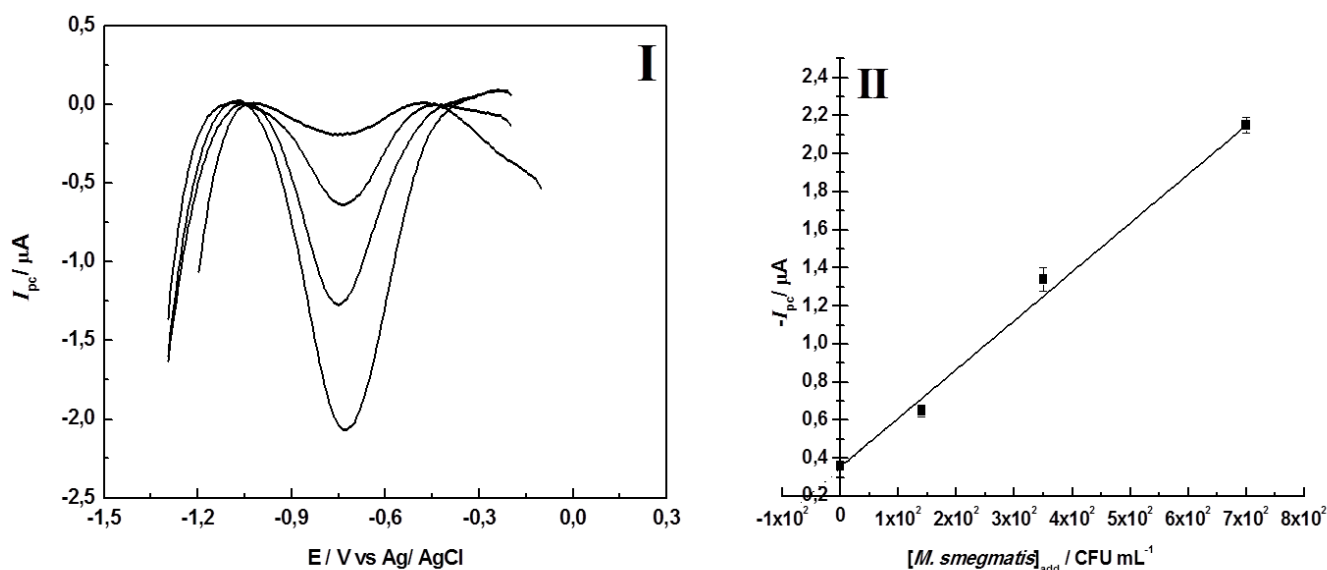

Figure 6. (I) Square-wave voltammograms obtained from water samples spiked to 1.4×10^3 CFU mL^{-1} *M. smegmatis* and with successive standard addition of MA,under optimized conditions of 0.2 mol L^{-1} phosphate buffer (pH=7.0). f = 50 Hz, ΔE_s = 8 mV, E_{sw} = 50 mV on glassy carbon electrode modified by PLL and **(II)** the curve of standard addition.

In order to test the proposed method, the results were compared with chromatographic method adapted from the methodology described by Butler [15]. It was observed that the MA was separated in the reverse-phase column by a combination of factors, including chemical functional groups, polarity, and hydrocarbon chain length. The shorter-carbon-chain fatty acids, derivatized with chemically phenyl derivatives which had demonstrated an increase in retention times with longer carbon chains but a decrease in retention times with greater unsaturation [26]. Nevertheless, the detection limit

obtained by HPLC was 2.5×10^4 CFU mL^{-1}, larger than electrochemistry method proposed in this paper.

Finally, using the spiked sample of drinking water spiked to 1.4×10^3 CFU mL^{-1} *M. smegmatis* in the chromatographic profile was not possible detect MA, indicating that this method cannot detect lower concentrations of *M. smegmatis*. Then, the proposed method designed to detect *Mycobacterium*, *Corynebacterium*, *Nocardia* and *Rhodococcus* species, based on mycolic acid detection, can also be used as a preliminary screening to detect mycobacteria's in low concentrations.

3.5. Specificity of the method proposed

The MA are characteristic lipid components of the cell envelope of mycobacteria and related actinobacteria [27], which is not present in other bacteria like *Vibrio* and *E. Coli* species. MA is fatty acids found specifically in *Mycobacterium*, *Corynebacterium*, *Nocardia* and *Rhodococcus* species [28-30]. So, the proposed method could also to detect *Corynebacterium*, *Nocardia* and *Rhodococcus* species, but it is selective in relation to others species like *E. coli*, *Pseudomonas* since they have not MA.

4. CONCLUSION

Glassy carbon electrode modified by poly-l-lysine films is a promising tool for *Mycobacterium*, *Corynebacterium*, *Nocardia* and *Rhodococcus* quantification based on the measurement of their mycolic acids reduction in low concentration, like as 5.9×10^1 CFU mL^{-1}, lower than chromatographic methods currently recommended in the literature, which are limited to concentration detection around $10^7 - 10^6$ CFU mL^{-1}. In addition, the method is simpler than other methods described to monitored mycolic acid in evaluation of antimicrobial drug susceptibility like mass spectrometry. Therefore, this method can be used as a preliminary screening to detect mycobacteria in drinking water without tedious pre-treatment and will also to be tested to available the rapid detection of drug resistance.

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