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# A Novel Voltammetric Assay of polyamine Oxidase using Polymer Modified Glassy Carbon Electrode

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**Abstract: A thin film of 4-Amino-3-Hydroxynaphthalene sulphonic acid (AHNSA) was electropolymerized on a glassy carbon electrode (GCE).Square wave voltammetry (SWV) was used to study the electrochemical property of spermine (Spm). An oxidation adsorption peak of Spm was observed at +0.74 V verse Ag/AgCl as a reference electrode in carbonate buffer (pH 9.5).This polymer modified electrode showed high sensitivity, selectivity and stability in the determination of spm. The peak current increased linearly with the concentration of spm in the range of$1.49\times10^{-6}$ – $1.96\times10^{-5}$ mol.$L^{-1}$,with a linear regression equation was expressed as y=0.8826x + $4\times10^{-7}$ and $r^2$= 0.9918.for the first time, the oxidation peak of spm was successfully directed to find the PAO activity in human maternal and cows milk.**

**Keywords: polymer modified electrode, 4-Amino-3-Hydroxynaphthalene sulphonic acid, spermine, polyamine oxidase activity, milk.**

## Introduction

Polyamine oxidase (PAO;EC 1.5.3.11) enzyme, which is present in all vertebrate tissues and in biological fluids, represent one of the key enzymes in catabolic pathways of polyamines.PAO catalyzes the oxidative deamination of Spermine (Spm) or Spermidine (Spd) producing spermidine (equation 1) or putrescine depending on the substrate nature[1,2,3](equation 2)

$$\underset{\text{Spermine}}{NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2} + O_2 + H_2O \xrightarrow{PAO}$$

$$\underset{\text{Spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + \underset{\text{3- aminopropinaldehyde}}{NH_2CH_2CH_2CHO} + H_2O_2 \quad .....(1\text{-}2)$$

$$\underset{\text{Spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + O_2 + H_2O \xrightarrow{PAO} \underset{\text{putrescine}}{NH_2(CH_2)_4NH_2} +$$

$$\underset{\text{3- aminopropinaldehyde}}{NH_2CH_2CH_2CHO} + H_2O_2 \quad .....(1\text{-}1)$$

Polyamines have important role in cell proliferation and differentiation.These aliphatic cations are essential for gastrointestinal tract, functional maturation during the neonatal period, preventing the food allergicity in sucking babies by decreasing mucosal permeability to antigenic protein [4,5]So the milk is the first source of exogenous polyamine for newborn babies and animals[6]. High level of PAO activity are present in plasma of ruminants and in plasma of pregnant women and it has been suggested that these are necessary to regulate PA levels [7]. It was proposed firstly that PAO was a constitutive enzyme. However, PAO activity was found to be increased by growth inhibition [8], in response to anticancer drug etoposide and in cancer cells when they reach high density. These results indicated that PAO may also play a role in polyamine homoeostasis [9].PAO are a good marker for the diagnosis of cerebral stroke [10].

PAO activity were detected in human sera , cerebrospinal fluid, milk, erythrocyte and leukocyte. The activity of PAO were found to be significantly increased in sera of schizophrenia and depressed patient as well as in maternal milk of schizophrenic with compared same illness [11,12,13].

Several methods have been used to assay PAO via monitoring oxygen consumption by a Clark oxygen electrode [14] or spectrophotometrically assayed as described in [11], It also could be estimation detected of $H_2O_2$ liberated (15).This enzyme has also been determined through analyzing the 1,3 diamino propane formed in the reaction using gas-liquid chromatography technique.Moreover, the activity of PAO found through the amount of Spd increase then these polyamine is measured by HPLC(16) .Usually these method demand expensive apparatus, highly skilled technicians, complicated and time consuming procedures. Since a sensitive, fast, selective and inexpensive analytical method for determining PAO is highly needed.

Electroanalytical methods have attracted more attention in the recent years for environmental and biological compounds determination due to their sensitivity, accuracy, lower cost, and simplicity[17] Polymer-modified electrode (PMEs) have received considerable attention in the recent years due to their good stability, reproducibility, homogeneity in the electro-chemical deposition and strong adherence to the electrode surface[18,19]. In thepresent work,we describe a novel procedure to find the activity of PAO through the consumption the Spm .A sensor was prepared by electropolymerizing of 4-amino-3-hydroxynaphthalene-sulfonic acid (AHNSA) at glassy carbon electrode(GCE) and investigate oxidation peak of Spm, then used for electroanalytical determination ofPAO activityin human maternal and cows' milk as highly available source of PAO.

## 2. Experimental

### 2.1. Instrumentation and chemicals

Electrochemical polymerization was carried out using eDAQ(version 2.1,Australian)potentiostat in glass cell equipped with a conventional three electrodes system with a glassy carbon electrode(1.6±0.1 mm indiameter) as the working electrode , platinum wire as an auxiliary electrode and Ag/AgCl as a reference electrode . Square Wave Voltammetry, (SWV) was performed using 797 VA Computrace stand (Metrohm AG , CH 9101 Herisau, Switzerland ) the measurements were carried out by the same equipment .During the experiments, the solutions and the electrodes were kept motionless and the solutions were thoroughly deoxygenated by a bubbled high purity nitrogen and a nitrogen atmosphere was maintained over the solutions. pH values were measured with Hanna pH 211 (Romania made) .General Laboratory Centrifuge, Sorvell type from DuPont, (USA made) was used for centrifuging of milk samples for SWV analysis. All experiments were carried out at room temperature. AHNSA was purchased from BDH while spermine tetrahydrochloride (Spm) ,spermidine(Spd), putrescindihy drochloride (Put),cadaverinedihydrochlorid(Cad) from (sigm-aldrich company). Stock amines solutions (0.01 mol/L) were freshly preparedin distilled water and stored in a dark place.All other chemicals were of analytical grade used as received without further purification.

### 2.2 Fabrication of the poly(AHNSA) modified glassy carbon

Poly(AHNSA) modified was prepared as reported in (20).Briefly, the GCE was first rinsed with distilled water, polished carefully with alumina powder ( 0.03 µm ) on a polishing cloth. Thensonicated in absolute ethanol and water for 3 minutes successively.The electrode prepared by cyclic voltammetry in the potential range ( -0.8- +2.0) V at 0.01 V/S for 15 cycle in 0.1 mol $l^{-1}$ $HNO_3$ solution containing 0.001mol $l^{-1}$ of AHNSA.The modified electrode was stabilized in 0.1 mol $l^{-1}$ H2SO4 by applied potential between -0.8 and +0.8 V untila steady cyclic voltammogram was obtained. Finally the modified electrode was stored in distilled water at 4 ºC for use.

### 2.3.Analytical procedure

#### 2.3.1. Determination of Spm

The procedure for obtaining SWV was as follow: A 10 mL aliquid of carbonate buffer as supporting electrolyte at desired pH was pipetted in a clean and dry electrochemical cell .The solution was deaerated byits passage through it a stream of nitrogen gas for 5 min,thenthe required solution of Spm was added and deoxygenated by a bubbled nitrogen gas for 30 seconds. Under the device condition ,an anodic adsorption peak of Spm was observed at +0.75 V vs Ag/AgCl. All measurements were made at room temperature.

2.3.2. Determination of PAO Activity in milk samples Via the Electrochemical oxidation peak of Spm
Localsix fresh cows' milk samples and three humane milk were collected to find PAO activity.

#### 2.3.2.1 Isolations of lipids from Milk Samples

Milk (10) mL at 4 ºC was centrifuged for 30 min at 4000 round per minute (rpm). The fatty supernatedlayer was separated and the milk was stored at(-18ºC) for further experiments.

**2.3.2.2 Electrochemical measurement for PAO activity**

After Spm oxidation peak has been optimized, PAO activity was measured as follows:In electroanalytical cell containing 10 mL of 0.1 mol $l^{-1}$ carbonate buffer(pH 9.5) ,100 µl of $10^{-3}$ mol $l^{-1}$ Spm were added ,the solution was degassed for 30 sec , The SW voltammogram was recorded(peak current = $Ip_1$ ) and the reaction was initiated by the addition of 10 µL of milk sample(diluted and incubated for 10 minutes at 35 °C). The voltammgram was recorded again (peak current = $Ip_2$ ) and the difference between the two peaks (ΔIp) was ( $Ip_1 – Ip_2$), which is equal to the amount of Spm consumed, that is related to PAO activity(21).

## 3. Results and Discussion

### 3.1. Electropolymerization of poly(4-amino – 3 hydroxynaphtaline – Sulphonic acid)

The electropolymerization of $2.0\times10^{-3}$mol $L^{-1}$AHNSA in 0.1 mol $L^{-1}$ $HNO_3$ at the polished GC is shownin the repetitive cyclic voltammograms in figure (1). As seen ,in the first scan ,anodic peak (1),cathodic peak (2) wereobserved with peak potential 0.4 V and -0.17 V respectively. From the third cycle another two anodic peak occurred at 1.68 V and 0.04 Vrespectively. As the scans continued, the peakscurrent increased until 15 scans[20]After 15 scans, athin film could be found on the electrode surface of the poly AHNSA film.

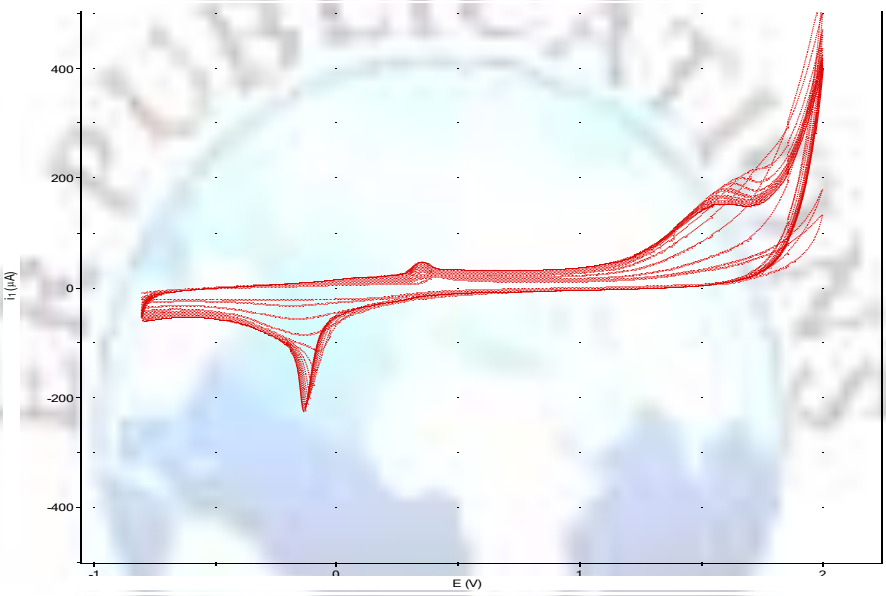


**Fig. (1): Cyclic voltammograms of bare GCE in $2.0\times10^{-3}$mol $L^{-1}$ AHNSA (0.1 mol $L^{-1}$ $HNO_3$), scanning potential: ( -0.8 - +2.0) V ;scan rate: 0.01 V/S;number of cycle:15.**

Figure (2) depicted the cyclic voltammgrams of bare GCE (curve a) and stabilized polymer modified GCE (curve b) were recorded between(-0.8 - +0.8) v in 0.1 mol $L^{-1}$ $H_2SO_4$.A three distinct peaks at the modified electrode (curve b) and the absence of peaks at the unmodified GCE (curve a) is a conformation of polymer film deposition at the GCE surface.

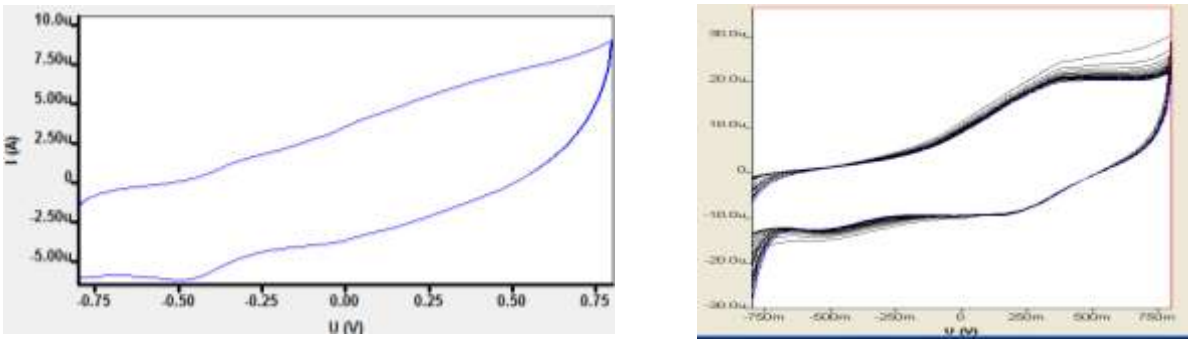


**Fig.2. (A) CV of unmodified GCE in 0.1 mol.$l^{-1}$ $H_2SO_4$.(B) CVs of stabilized poly (AHNSA) /GCE in 0.1 mol.$l^{-1}$ $H_2SO_4$.**

### 3.2 Electrochemical behavior of Spermine at the polymer modified electrode

The electrochemical behavior of Spermine at the polymer modified electrode was investigated using SWV which has a much higher sensitivity and better resolution than cyclic voltammetry[22] Figure 3 depicted the SW of bare GCE (curve a) and poly(AHNSA) modified GCE (curve b) in pH 9.0 carbonate buffer containing $49\times10^{-7}$ mol.l$^{-1}$of Spm.At the bare GCE , Spm exhibited a poor oxidation peak appeared at about 0.74V with 0.3 µA (curve a).When the polymer was introduce on the GCE (curve b) ,the current signal was shiftedto 0.75 and amplified over 10 fold compared with that of bare electrode .This could be attributed to the selective preferential accumulation of Spm on surface bound functionalities of polymer modified electrode(23).

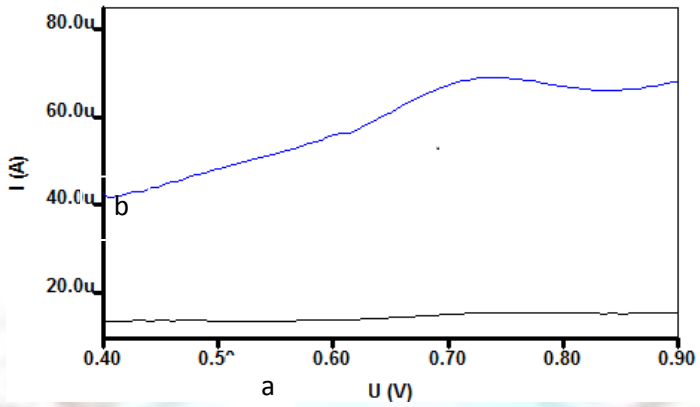


**Fig.3. Cyclic voltammograms of bare GCE (a) and poly(AHNSA)/ GCE in pH 9.0 carbonate buffer containing $49\times10^{-7}$ mol.l$^{-1}$ of spm**

### 3.3 Effect of buffer types and pH on Spm peak

In pervious study on spm [24]carbonate solution buffer at pH 10 was used as supporting electrolyte which indicate that spm exhibits better electrochemical behavior in alkaline media A work at pH 9 was suggested inorder to find the activity of polyamine oxidase enzyme .At this pH, several buffers were testedincluding potassium phosphate buffer,tris –HCl buffer, $Na_2CO_3$-$NaHCO_3$, Glycine buffer, Boric buffer, $NaHCO_3$-NaOH, $Na_2CO_3$-$NaHCO_3$+ 0.1 M $LiClO_4$,Boric buffer +0.1 M $LiClO_4$, $Na_2CO_3$-$NaHCO_4$+0.1 M $NaClO_4$, Boric buffer +0.1 M $NaClO_4$.The results indicate that the supporting electrolyte have a high effect on the spm oxidation peak .Furthermore ,no peak was observed when potassium phosphate buffer was used. On the other hand,with the use of carbonate buffer, a better electrochemical response for spm peak was observed. Moreover, the effect of pH on the peak current and peak potential was investigated in pH range 9.0-10.5 carbonate buffer (table 1).

**Table (1): Effect of pH on peak potential and current for solution ($49\times10^{-7}$)M of Spm in 0.1 M carbonate buffer**

| pH | Ep(v) | Ip correct (µA) |
|---|---|---|
| 9 | 0.770 | 3.62 |
| 9.5 | 0.750 | 4.0 |
| 10 | 0.728 | 3.7 |
| 10.5 | 0.699 | 2.98 |

The peak potential shifted slightly negative with increasing the pH indicating that the peak types is oxidation adsorption. Generally, 0.1 M carbonate buffer pH 9.5 was chosen as supporting electrolyte. In addition the SW parameters including (voltage step, amplitude ,deposition potential, deposition time, equilibrium time ,frequency) were optimized to be 0.006 V ,0.05V, -0.8 V,100 Sec,5 Sec ,60 Hz respectively and were used in subsequent analyses.

### 3.4 Analytical performance (validation of the method)

#### 3.4.1Calibration graph (Linearity) and detection limit of Spm at poly (AHNSA)/GCE

Once the optimal chemical conditions and instrumental parameters for the SW determination of Spm were established, several analytical characteristics of the proposed were evaluated. So, under the optimized conditions a successive amounts of $1\times10^{-3}$ mol.l$^{-1}$of Spm was added, the oxidation peak current was linear to the concentration of Spm from $1.49\times10^{-6}$ – $1.96\times10^{-5}$ mol.L$^{-1}$,with a linear regression equation was expressed as $y=0.8826x + 4\times10^{-7}$ and $r^2$= 0.9918.As regards to minimum substrate concentration in the analytical range 1.49 µmol/L, the current response was 1.47µA .When the concentration of Spm was more than $1.96\times10^{-5}$ mol.L$^{-1}$,the current response was stabled gradually as shown in figure (4) which could be ascribed to the saturation of the active sites of the polymer film.[20]

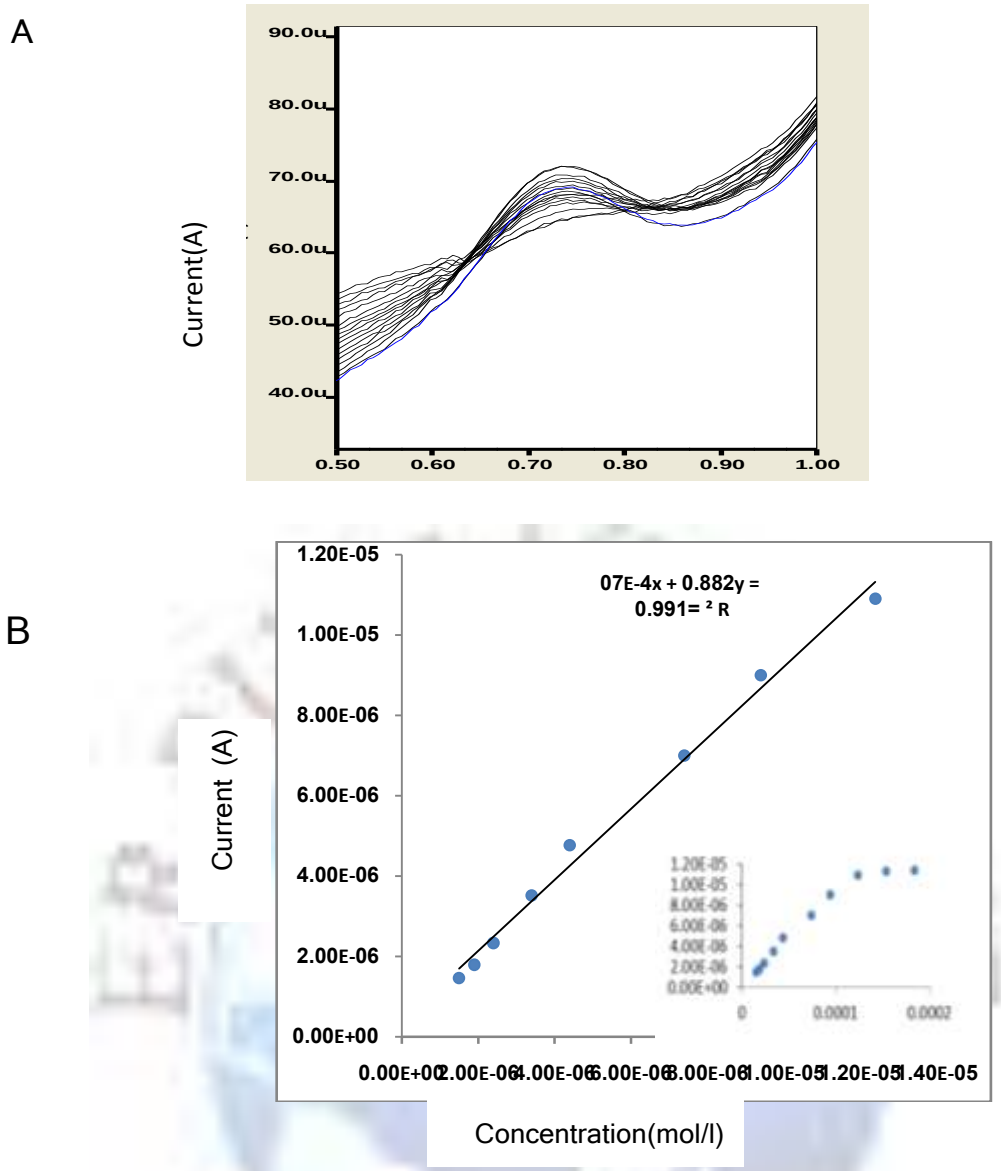


**Fig.4.A): SW voltammogramsofpoly(AHNSA)/GCE in pH 9.5 carbonate buffer containing different concentration of spm under optimum condition; B)plote of current verses different concentration of spm.**

Several approaches are given in the ICH guideline to determine LOD (limit of detection) and LOQ (limit of quantification). LOD and LOQ were calculated from theequations of LOD = 3.3 σ/S and LOQ = 10σ /S[25,26 ]using the standard deviation of response(s) and calibration curve. The limit of quantitation was calculated as 0.5 µMand limit of detection was calculated as 0.1 µM for thedesigned biosensor.

**3.3.2 precision, accuracy , stability and sensitivity of poly AHNSA/GCE to polyamine compounds**

The reproducibility of the developed procedure was evaluated from repeated measurements of 3.8 $\times 10^{-6}$mol $l^{-1}$ Spm .The precision of the method in terms of the standard deveision was 0.048.The accuracy of the electrochemical method was checked by calculating the recovery of same amount of Spm in buffer solution and analyzed by the optimized procedure and the standard deviation of the five measurementswas 0.04.When the voltammogram of Spm solution was monitored every 5.0 min , it was observed that the oxidation peak current of Spm keeps nearly unchanged for at least 40 minutes. The detection of other biogenic amines on the poly AHNSA/GCE have been investigated like spermedine(Spd),Cadavarien(Cad) and Putresien, . An oxidation peak current of $1.99\times10^{-5}$ M Spd was observed at 0.74V with $1.95\times10^{-6}$ compared with Spm current which was $9.02\times10^{-6}$ A under the same condition. On the other hand ,no oxidation peak were found for Cad and Put. So, these results proposed a method that can be used for determination of Spm and suggested to find the activity of polyamine oxidase enzyme through Spm oxidation peak.

3.4Application of the method for the determination of polyamine oxidase activity in HUMAN&cow'smilk

To our knowledge, there is no informationreported on the determination of PAO activity through Spm oxidation peak using modified electrode. Therefore, the main objective of this study was to detect the position of Spm oxidation peak

and optimizing it,then verifying the benefit of this system to find the activity of PAO. So, the proposed method has been applied todetermine of PAO activity following the decrease of Spm oxidation peak in partial purified PAO enzyme and milk samples. A preliminary investigation was to confirm that Spm peak was affected, so a partial purified PAO with activity of 108.6 unit/ ml was used in two different concentrations.
It is clear from figure (5) the occurrence of enzymatic reaction.

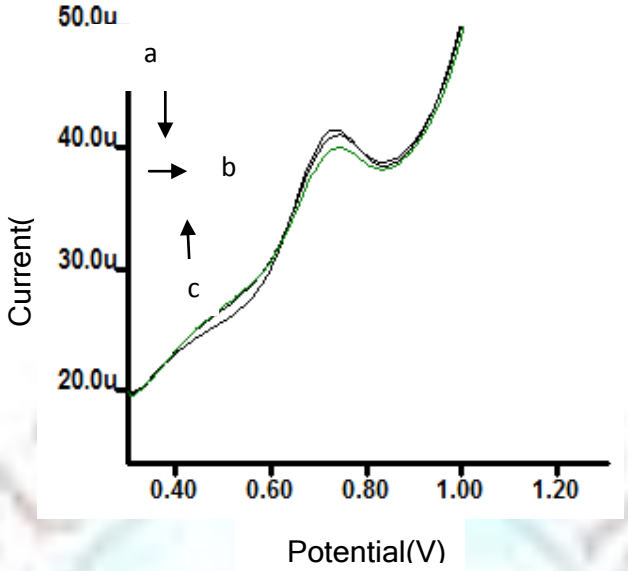


**Fig.5.: SWvoltammogramsofpoly(AHNSA)/GCE;a:9.9×10$^{-6}$ M Spm alone; b: after addition of 10 µL of partial purified PAO; c : after addition of 20 µL of partial purified PAO**

After that, two sources of milkwas used in the proposed method to determine the activity of PAO as described in experimental part. These results were depicted in Table(2) .The voltammetric results showed an agreement with previous study [22] which also directed the Spm adsorption peak on hanging mercury electrode using SW to find the activity of PAO. The suggested procedure need further exploration to enhance the applied method in term of mechanism for additional understanding.

**Table( 2): PAO activity in human maternal and cow milk usingpoly(AHNSA)/GCE**

| Sample No. | Milk Types | Dilution factor | PAO activity (U*/min. ) |
|---|---|---|---|
| 1 | Cows' milk | 5 | 3 240 |
| 2 | | 5 | 3480 |
| 3 | | 5 | 1964 |
| 4 | | 5 | 1271 |
| 5 | | 5 | 967 |
| 6 | | 5 | 813 |
| 7 | maternal milk | - | 235 |
| 8 | | - | 104 |
| 9 | | - | 375 |

*=One unit of PAO is defined as the amount of enzyme catalyzes the oxidationof one nmol of spermine per minute.

## Conclusion

The main objective of this work is to provide simple method for determination of polyamine oxidase activity through the oxidation peak of spermine on the surface of the modified glassy electrode.The proposed approach was successfully

applied to find the activity of polyamine oxidase in human and cow milk .Therefore ,the method developed can be used for detection of enzyme activity in pathological statement

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