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A Study of Voltammetric Behavior of Spermine and Its Application for Estimation of Polyamine Oxidase Activity

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ABSTRACT

Here, we present details about the voltammetric behavior of spermine (spm), showing a well-defined current peak at -0.45 V, compared to AgCl, as a reference electrode, using square wave voltammetry (SWV). Under optimal conditions, the voltammetric current is proportional to the concentration of spm, over the concentration range of 4.99 $\times 10^{-8}$ –7.98$\times 10^{-7}$ M, with a correlation coefficient of R= 0.994 and a limit of detection of 0.7 $\times 10^{-8}$ M. Repeatability was tested for 9.99$\times 10^{-7}$ M spm (n = 6), the current was 41 nA, and standard deviation (SD) was ±1.5 nA. In addition, the SD for instrument error was 1.7 nA. The proposed method was successfully applied to examine the polyamine oxidase (PAO) activity in cow and sheep's milk. A comparative study between SWV electrochemical and spectroscopic methods showed a good correlation between the two methods, indicating that the proposed method can be used as an alternative analytical technique.

Keywords: spermine, square wave voltammetry, polyamine oxidase

INTRODUCTION

Polyamine oxidase (PAO; E C 1.5.3.11) enzyme is present in all vertebrate tissues and in biological fluids, representing 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 [1-3] (equation 2).

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

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

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

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

3- aminopropinaldehyde

Polyamines have an important role in cell proliferation and differentiation. These aliphatic cations are essential for gastrointestinal tract, functional maturation during the neonatal period, preventing the food allergy in sucking

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babies by decreasing mucosal permeability to the antigenic protein [4, 5]. Therefore, the milk is the first source of exogenous polyamine for newborn babies and animals [6, 7]. PAO activity has been increased by growth inhibition and in response to anticancer drug [8]. In addition, it is considered as a good marker for the diagnosis of cerebral stroke [9]. Therefore, the determination of PAO activity may have a clinical significance, as it was detected in human sera, cerebrospinal fluid, milk, erythrocyte and leukocyte. In addition, PAO activity significantly increased in the sera of schizophrenic and depressed patients as well as in maternal milk of schizophrenic feeding mothers [10-12].

Several methods have been used to examine PAO via monitoring oxygen consumption by a Clark oxygen electrode [13], or it is spectro-photometrically assayed as described elsewhere [12], and it also could be an estimation of a H_2O_2– titanium (Ti) complex formation, as an indicator of H_2O_2 production due to polyamine oxidation [14]. Moreover, in some studies, the PAO activity was determined through the increased level of Spd, with subsequent measurement, using HPLC [15]. Usually, these methods require expensive apparatus, highly skilled technicians, complicated and time-consuming procedures. The electroanalytical methods have attracted more attention to environmental and biological compounds determination, due to their high sensitivity, selectivity and speed of the measurements. In addition, it can measure an extremely small volume of sample [16].

According to our interest in supporting the voltammetric analytical procedures, our lab has many published articles, explaining the benefit of this technique for the determination of biological substances and enzyme activity [17-20]. During several attempts made in our lab, in order to find the possibility of using voltammetric techniques to measure the activity of PAO, we observed very interesting results regarding the voltammetric behavior of spm, which can pave the road for further studies.

The current study explains the validity of voltammetric techniques to measure PAO activity, using spm substrate, which gave a special adsorption peak with SWV. To the best of our knowledge, there are no previous voltammetric studies on spm, in order to determine PAO activity.

MATERIALS AND METHODS

Instrumentation and Chemicals

All SWV measurements were performed with 797 VA Computrace stand (Metrohm AG, CH 9101 Herisau, Switzerland), in connection with a desktop computer and controlled by VA computrace 2.0, as a control software. The voltammetric measurements were carried out in a glass cell (working volume = 5─10 mL). A three-electrode system was used: the reference electrode was an Ag /Ag Cl in 3 M KCl and platinum tip, as auxiliary electrode. The working electrode is a glass capillary, mercury drops form at the end of the capillary tube with a surface of 0.15 mm^2 –0.6 mm^2. During the experiments, the solutions and the electrodes were kept motionless and thoroughly deoxygenated by bubbling high purity nitrogen for 10 min. Thermo-stating circulation water bath, HAAKE F3 (made in Germany) was used to control the temperature.

Shimadzo UV–VIS recording spectrophotometer and UV–VIS NIR Spectrophotometer Model Varian Cary 5000, made in the USA, was used to measure the PAO activity, using a spectrophotometric method. pH values were adjusted by Hanna pH 211 (made in Romania). Sorvell™ centrifuge was used for centrifuging milk samples, in order to remove the fatty layer and prepare the sample for further analysis.

Spermine (spm) was purchased from Sigm-Aldrich Company, USA. Stock amine solutions (1×10^{-2} M) was freshly prepared in deionized water and stored in a dark place. More dilute solutions (1×10^{-3} M to 1×10^{-4} M) were prepared, using deionized water. Potassium phosphate monobasic, potassium phosphate dibasic, sodium hydroxide, sodium carbonate, sodium bicarbonate, and Tris buffer were purchased from Fluka Chemicals. Hydrochloric acid and sodium hydroxide ampoules were purchased from BDH. All other chemicals were of analytical grade, used without further purification.

Deionized water was obtained from the State Company for Drugs Industry and Medical Appliances (N.D.I) Ninavah /Iraq, which provides water resistivity of ≈ 0.5 ohm.

Procedure and Analysis

Square wave Voltammetry for spermine

The procedure for obtaining SWV for spm was obtained as follows: A 10 mL liquid carbonate buffer (supporting electrolyte) at desired pH was pipetted in a clean and dry electrochemical cell. The solution was deoxygenated by passing through a stream of nitrogen gas for 5 min. A certain amount of spm solution was added to the supporting electrolyte, and deoxygenated again for 30 seconds. Under device condition, a special adsorption peak of spm was observed at -0.44 V vs Ag/AgCl.

A calibration graph is produced under the optimized conditions of the assay.

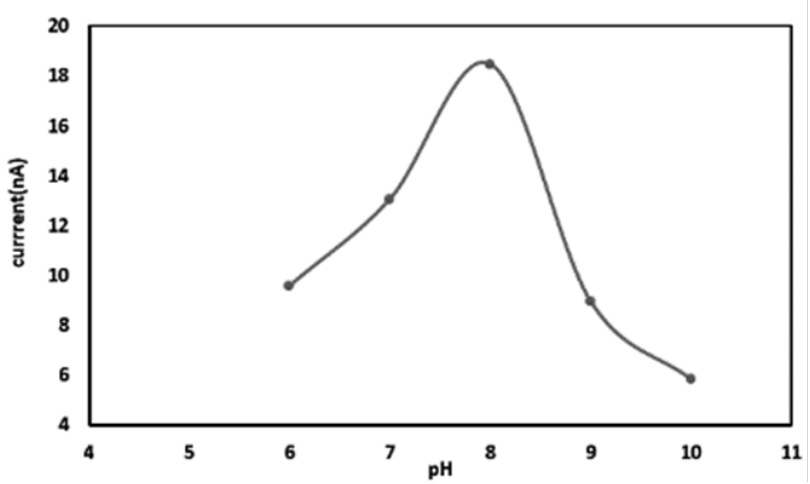

Figure 1. Effect of pH on the voltammetric current for 9.99×10^{-7} M spm peak at 0.04 M $NaHCO_3$-HCl buffer

Determination of polyamine oxidase activity in milk of cows and sheep

Local fifteen animals' (cows and sheep) milk samples were collected for determination of PAO activity. The Ethics Committee of Mosul University approved the study.

***Isolations of lipids from Milk Samples*:** Ten milliliters of milk is centrifuged for 30 min at 4 ^{o}C and 4000 rounds per minute (rpm). The fatty layer was separated and the milk was stored at -18^{o}C freezer, for further experiments.

***Electrochemical measurement for PAO activity*:** After spm adsorption peak has been optimized, the PAO activity was measured as follows: In polarographic cell containing 10 mL of 0.1 M carbonate buffer (pH 8.0), 100 µL of 10^{-3} M spm were added, the solution was degassed for 30 sec, and the SW voltammogram was recorded (peak current = Ip_1). Subsequently, the reaction was initiated by the addition of 10 µL of milk sample (diluted and incubated for 10 minutes at 35C ^{o}). The voltammgram was recorded again (peak current = Ip_2) and the difference between the two peaks (ΔIp) was reported as Ip_1–Ip_2, which is equal to the amount of spm consumed, correlating with the PAO activity [18].The results obtained were compared with that taken by the spectroscopic method.

***Spectrophotometric measurement for PAO activity*:** PAO activity in milk samples were spectrophotometrically assayed with minor modifications, as described elsewhere [21], which clearly explained in our previous work [22].

RESULTS AND DISCUSSION

Electrochemical Behavior of spm at Mercury Electrode (HMDE)

Unexpected result was observed by SWV, during an attempt to find a procedure to determine PAO activity, as a well-defined peak appeared when spm was added at -0.446V, as shown in **Figure 1**. This interesting result urged us to further explore the reason. Accordingly, one of the species to be determined at mercury electrodes is an organic compound, which can be adsorbed on mercury [23]. Therefore, spm peak is probably due to non-faradic processes that may cause a physical change in the structure of the electrode surface leading to creation of the peak, which may be resulted from adsorption. For further confirmation, the following experiments were performed.

Effect of supporting electrolyte type

Different types of supporting electrolyte (0.1 M, pH 8.0) were used to find out the influence of the supporting electrolyte on the adsorption peak of spm at room temperature, using SWV. The results depicted in **Table 1** shows the adsorption peak for spm was highly affected by buffer type. On the other hand, the peak current (Ip) is also dependent on the buffer used in the experiment. The result also indicated that Na_2CO_3-$NaHCO_3$, $NaHCO_3$-NaOH and $NaHCO_3$ – HCl buffer solutions gave the most promising results. Therefore, it was recommended in this study.

Table 1. Effect of the supporting electrolyte type on the peak current of 9.99×10^{-7}M spm

Types of supporting electrolyte (0.1M, pH 8.0)	Ep (V)	Ip correct (nA)
Potassium phosphate buffer	No peak	-
Tris-HCl	No peak	-
Na_2CO_3-$NaHCO_3$	-0.464	8.00
$NaHCO_3$-NaOH	-0.446	15.70
$NaHCO_3$-HCl	-0.446	5.21

Table 2. Study of spm peak 9.99×10^{-7} M in different concentrations of $NaHCO_3$ at pH 8.0

Conc. Of support electrolyte (M)	Ip correct (nA)
0.1	5.21
0.08	9.38
0.06	13.59
0.04	18.47

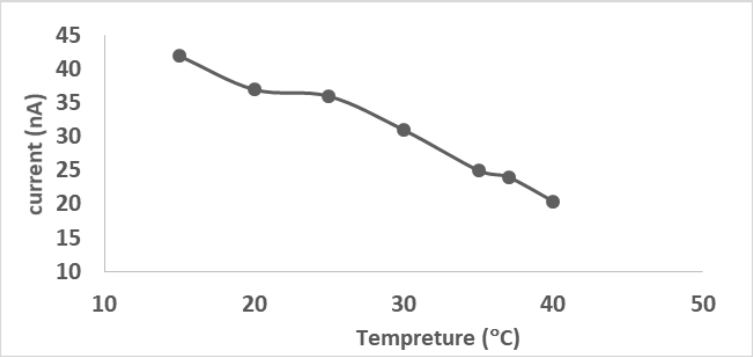

Figure 2. Influence of temperature on voltammetric peak current for 9.99×10^{-7} M spm at 0.04 M $NaHCO_3$-HCl buffer, pH=8

Effect of Time on the Stability of Peak Current of spm in Different Media

The effect of time on the voltammetric behavior of spm had been studied in Na_2CO_3-$NaHCO_3$, $NaHCO_3$-NaOH and $NaHCO_3$-HCl buffer solution (0.1 M, pH 8.0). All measurements were carried out at room temperature (24±2) ^oC, in the presence of 9.99×10^{-7} M spm. The results showed that the spm peak is time dependent in Na_2CO_3 - $NaHCO_3$ buffers, as it was stable only about five minutes. Nevertheless, the spm peak current in $NaHCO_3$-NaOH buffer was not stable at all. On the other hand, further study was carried out on $NaHCO_3$-HCl, although the peak current was less than the other buffer solutions, the results showed that the spm peak was more stable (about 12 min), therefore it was chosen as supporting electrolyte.

Effect of supporting electrolyte concentration and pH

Among different concentration of bicarbonate buffer, the best electroanalytical signal in term of the SWV peak current was obtained with 0.04 M, as shown in **Table 2**, and this buffer was chosen for further experiments. This result was due to a decrease in solubility of the organic compound, meaning an increase of the hydrophobic – hydrophobic interactions at the surface of the electrode [24]. Generally, the adsorption peak was mainly pH independent, since the monitored voltammetric signal for spm was only changed by the current. The influence of pH factor on the SWV signal is illustrated in **Figure 1**. Due to the observation that the voltammetric peak potential of spm did not shift when pH was changed over the studied pH range, these results gave further evidence that this voltammetric peak belongs to SWV adsorption. Fortunately, the maximum current was found at pH 8.0, because spm is fully protonated beyond pH 7.4, so this may be the reason behind the increase in the peak current at pH 8, which do not affect the optimal enzyme activity measurement. After fixing the peak position, pH and the suitable supporting electrolyte, the device condition for SWV was optimized to Voltage step 0.01(V), Amplitude 0.05(V), Deposition Potential -1.0 (V), Deposition time 60(sec), Equilibrium time 10 (sec) and Frequency 50 (Hz). Under this condition, the peak current of spm reached to 40 nA.

Effect of temperature

The influence of temperature is considered as a diagnostic tool for the presence of adsorption and the adsorption species in favor to desorbed [24], as an increase in temperature leads to decrease in the quantity adsorbed. This fact was proved (**Figure 2**) and the efficiency of spm adsorption behavior was checked, under the optimum condition over the temperature range of 15–40°C.

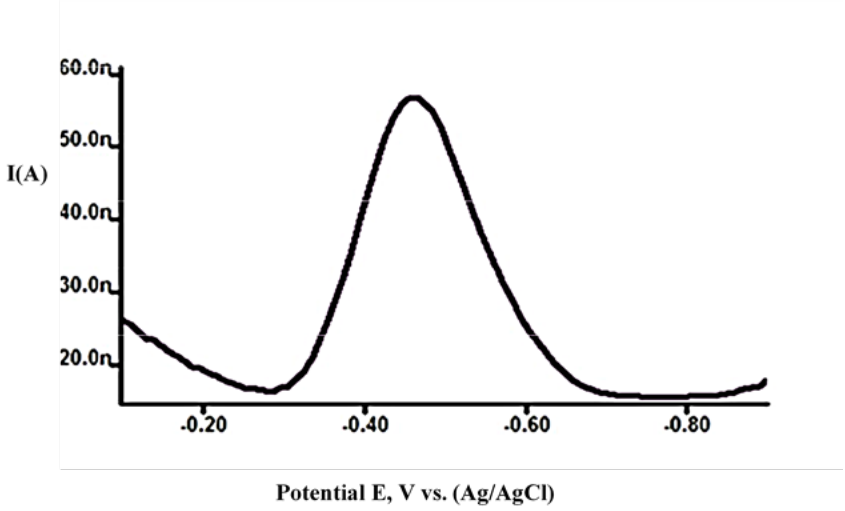

Figure 3. Square wave voltammogram of 9.99×10^{-7}M spm at 0.04 M $NaHCO_3$-HCl buffer, pH=8, T=25 ºC

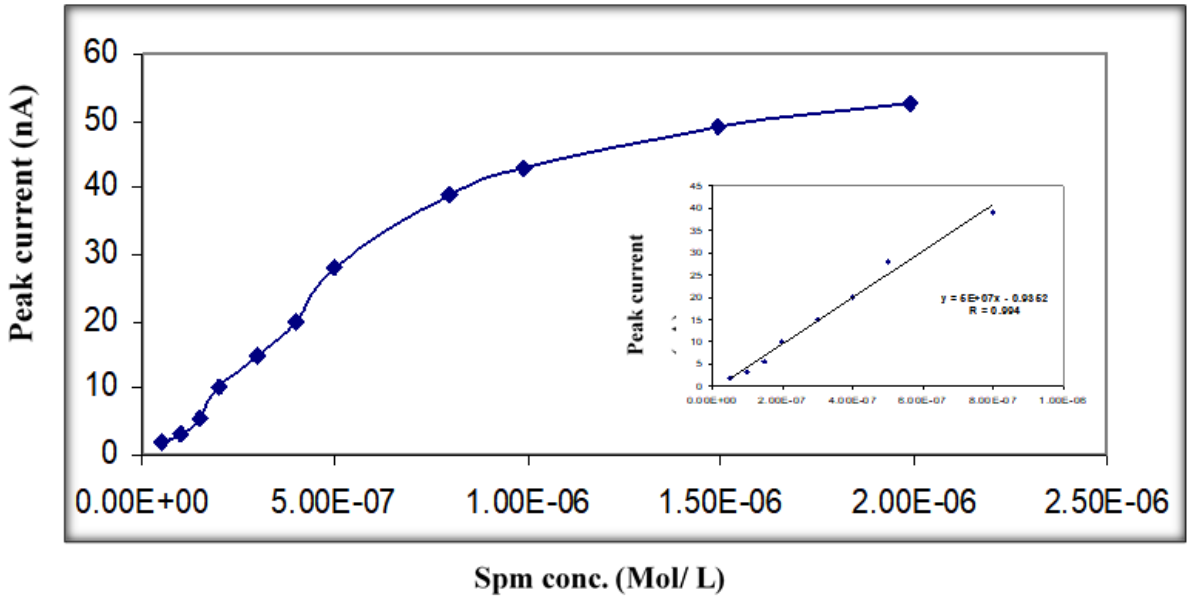

Figure 4. The effect of concentration on the voltammetric peak current of spm

The 25ºC temperature was chosen for further experiments, as it is suitable for measuring the enzyme activity, also it gives a good peak current as shown in **Figure 3**, a SWV for 9.99×10^{-7} M of spm. After experimental conditions were optimized (The optimal electrochemical parameters of SWV were set at 60 sec, 10 sec equilibrium time, and 50 Hz frequency), illustrating a well-defined adsorption peak at -0.45 V vs Ag/AgCl, as a reference electrode.

Analytical performance (validation of the method)

After establishment of the optimal chemical conditions and instrumental parameters for the SWV determination of spm, under the optimized conditions, the validity of the SWV technique as an analytical method for the determination of bulk spm was evaluated, as a function of its concentration as observed in **Figure 4**. Linear response was obtained over the concentration ranges of 4.99×10^{-8}–7.98×10^{-7} M with a correlation coefficient R= 0.994. However, at a high concentration, the relation between concentration and peak current was no longer a straight-line relation, suggesting the spm saturation on the mercury electrode surface. In regard to minimum substrate concentration in the analytical range 4.99×10^{-8} M, the peak current was 1.38 nA.

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 the equations of LOD=3.3_{ϱ} /S and LOQ=10_{ϱ} /S [25, 26], using

Table 3. Comparison of the analytical performance for determination of spm by SWV method with other methods

Technique used	Linearity(μM)	R [a]	References
X D- FIA [b]	0.005–0.05	N.R [c]	[26]
MEKC–MPEF [d]	1.25–205	0.9905	[25]
HPLC-BCD [e]	Up to 650	0.9881	[27]
SWV	0.04–0.79	0.9940	Present work

[a] Correlation coefficient ; [b] spectorphotomtric determination of Spm by forming a complex with xanthene dye followed by flow injection analysis ; [c] Not reported ; [d] micellar electrokinetic chromatography scheme based on multiphoton excitation fluorescence detection ; [e] high performance liquid chromatography depends on benzoyl chloride derivatization

the standard deviation of response (S) and calibration curve. The LOQ was calculated as 2.1×10^{-8}M and LOD was detected as 0.7×10^{-8} M for the proposed method. Repeatability was tested for 9.99×10^{-7}M spm (n=6) and standard deviation (SD) was ±1.5 nA. In addition, the (SD) for instrument error was 1.7 nA.

Table 3 shows the comparison between SWV and other methods. The proposed method is simple, very fast and the spm can be measured with low experimental concentration and gave a better correlation coefficient than other methods. However, when the concentration of spm is high, no detection can be done due to saturation.

Analytical Applications

After spm adsorption peak being optimized using SWV, in order to assess the validity of this technique, cow and sheep milk samples have been used for determination of PAO activity, due to availability and high activity of PAO in milk [27, 28, 29, 30], as drugs or other substances containing spm cannot be supplied.

Determination of activity of partially purified cow's milk PAO

Partial purification of PAO from cow's milk was described in the previous study [22]. Thus, it was used to determine its activity by the SWV method and it is illustrated in section "*Spectrophotometric measurement for PAO activity*".

In general, enzyme activity= moles of substrate converted per unit time (the amount of substrate consumed = the amount of product produced). Here, the current observed after the addition of the sample (Ip) was subtracted from the magnitude of spm (Ip_0) and the activity of PAO was evaluated by estimation of the difference between the two currents (Δ I).

$$\Delta I = Ip_0 - Ip$$

The calibration graph of spm was utilized to find the concentration of spm consumed, which is equivalent to the PAO activity. Then, a comparison was carried out between the results obtained by SWV vs. spectroscopic method. As explained in section "*Electrochemical measurement for PAO activity*", the PAO activity by electrochemical method was found to be 705 U/min, while in spectrophotometric method, it was 670 U/min. The data suggest that our proposed method is potent for measuring the enzyme activity by SWV.

Determination of PAO activity in milk samples

Upon obtaining promising result, the SWV method was applied to determine the activity of PAO for fifteen animals' (cows and sheep) milk samples. The results obtained were compared with that taken by the spectroscopic method ("*Determination of activity of partially purified cow's milk PAO*"), which repeated two times. The results obtained are shown in **Table 4**.

The comparison study between SWV electrochemical method and spectroscopic method was achieved for this fifteen milk samples, a good linear relationship between the two methods was found. No significant result also was obtained between these two methods, using student t- test.

The equation of straight line in the graph presented in **Figure 5** is: y = 1.1119x – 39.883; where x: is the PAO activity in milk samples, obtained from the SWV electrochemical method application, U/min; and y is the PAO activity in milk samples, obtained from the spectroscopic method application, with a correlation coefficient of (R) = 0.9881.

According to the results obtained in this study, it was clear that the new method, which depends on the SWV of spm, is more valid, as no toxic compound was used and low concentration of material and sample were required, as compared to the other methods.

Table 4. A comparison between spectroscopic and SWV methods for estimation of PAO activity (U/ min)

Sample No.	Milk Types	Spectroscopic method (U*/ min)	Dilution factor	Ep(V)	ΔIp (nA)	SWV method (U/min.)
1	Cows' milk	1706	10	-0.464	8.8	1817
2		1300	10	-0.464	9.7	1384
3		853	10	-0.464	6.6	936
4		853	10	-0.464	6.9	979
5		1950	10	-0.464	13.9	1990
6		1584	10	-0.464	10.9	1557
7		934	10	-0.464	8.4	1183
8		1029	10	-0.464	7.6	1081
9	Sheep's milk	1950	20	-0.464	6.9	1959
10		2113	20	-0.464	8.4	2392
11		5038	10	-0.464	17.0	5216
12		4063	14	-0.464	25.1	5051
13		2166	20	-0.464	8.4	2392
14		1463	20	-0.464	5.4	1529

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

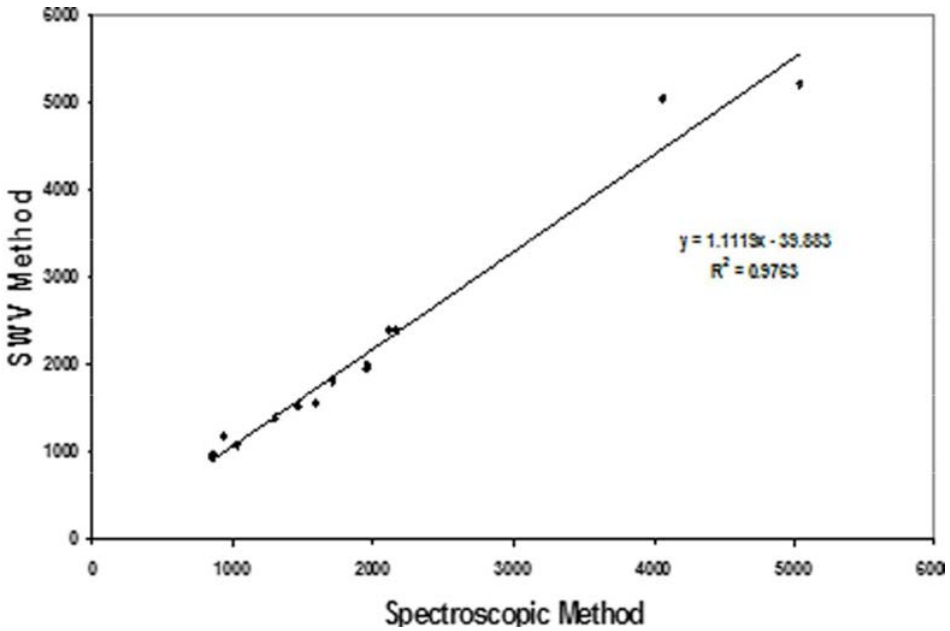

Figure 5. The linear relationship between spectroscopic and SWV method for determination of PAO activity (U/ min)

CONCLUSION

In this work, a well-defined adsorption peak for one of the most important polyamine compounds (spermine) can be detected, using SWV for the first time, which was clearly elucidated and discussed. Moreover, the new voltammetric peak current has potential to be used in the determination of PAO activity. Certainly, a simple, fast and low cost SWV procedure for determination of the activity of PAO was compared with spectrophotometric method. However, the application of the proposed method for determination of PAO activity in other biological fluids requires validation, using a larger sample size, and more extended future study.

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