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Simultaneous Electrochemical Determination of Hypoxanthine and Xanthine by Poly(Threonine) Film-Modified Electrode

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Abstract-Poly-(threonine) film modified carbon paste electrode was successfully fabricated through electropolymerization technique and was applied to investigate the electrochemical behaviors of hypoxanthine (HX) and xanthine (Xa) using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The oxidation peak currents for HX and Xa were strong, well resolved, and linear over the range of 8×10^{-6} M to 1×10^{-3} M. The limits of detection for HX and Xa by DPV were found to be 0.10mM and 0.08mM, respectively.

Keywords- Poly-(threonine) film, Modified carbon paste electrode, Hypoxanthine, Xanthine, Electrochemical, Voltammetry

1. INTRODUCTION

Hypoxanthine (HX) and xanthine (Xa) are among the degradation products of purine metabolism in humans and other higher animals [1-3]. HX and Xa can pass through cell membrane and accumulate in extracellular fluids. Extracellular levels of HX and Xa are indicators of clinical illnesses such as gout, hyperuricemia, xanthinuria, and renal failure [3-

5]. Moreover, determination of the levels of HX and Xa in meat and fish products was established to be an important and convenient indicator of freshness [3,6-9]. Previous methods of determination of HX and Xa that include high-performance liquid chromatography [10-12], capillary electrophoresis [1-2], spectrophotometry [7] and electrochemiluminescence [13-16] are costly, laborious, and require complicated sample pretreatment methods and longer analysis time [1-3].

Electrochemical methods of analysis, on the other hand, offer several advantages such as relatively simple and cheap instrumentation, rapid response time, high selectivity and sensitivity, and high stability [1-4]. Moreover, modifications on the working electrode may be done in order to further improve the analytical capability of the electrode. Carbon paste electrodes are generally used for analysis of organic compounds owing to its biocompatibility with tissue [17]. The use of conducting polymers as a modification for working electrodes provides numerous advantages such as hastened electrode processes (*i.e.* electron transfer), reduced overvoltage, and less prone to surface fouling (compared to bare electrodes) [18].

This study aims to develop a simple and highly sensitive electrochemical method for the simultaneous determination of Xa and HX. Specifically, this study investigates the electrochemical activity of Xa and HX on a poly(threonine) film-modified carbon paste electrode by CV and DPV techniques. The effects of experimental conditions such as solution pH, scan rate, pulse amplitude and width, and presence of interfering species on the determination of Xa and HX using DPV were also examined. The analytical parameters such as the limit of detection (LOD), limit of quantification (LOQ), reproducibility, stability, and dynamic range were also evaluated.

2. EXPERIMENTAL

2.1. Chemicals and Apparatus

All reagents were obtained as analytical grade and used without further purification. Phosphate buffer solutions were prepared by mixing stock solutions of KH_2PO_4 and K_2HPO_4 with pH adjustments using 0.10 M NaOH. All solutions were prepared using deionized water. All experiments were performed at room temperature. All solutions were freshly prepared and purged with nitrogen gas for at least 20 minutes prior to measurements.

All electrochemical measurements were carried out using the eDAQ EChem Startup System unit using a three-electrode system (*i.e.* reference electrode: Ag/AgCl and counter electrode: Pt/Ti titanium wire anode) in a 30-mL electrochemical cell. The working electrode was a carbon paste packed into the cavity of a Teflon tube electrode with 2-mm diameter. Bare carbon paste electrode was prepared by mixing 70% graphite powder and 30% paraffin oil for 30 min to obtain homogeneous carbon paste. Before measurement, the electrode surface was smoothened using a piece of transparent paper.

2.2. Preparation and Characterization of Working Electrode

The polymer film-modified electrode was prepared by electrochemical polymerization of threonine on a carbon paste electrode in 0.10 M phosphate buffer solution (pH 7.0) containing 0.5 mM threonine. The potential in CV runs was swept in the range -500 to 1500 mV at a scan rate of 50 mV/s. After 10 cycles, the electrode surface was washed with deionized water to remove the physically adsorbed material.

The characterization of the modified electrode in CV was performed in phosphate buffer solution (pH 7.0) in the potential range of 0 to 1500 mV at 100 mV/s.

2.3. Optimization of Experimental Conditions

Cyclic voltammetry was performed between the potential of 0 to 1400 mV at a scan rate of 100 mV/s with 0.1 mM HX and 0.1 mM Xa in phosphate buffer solutions of varying pH (5.0 to 10.0). Similar experiments were also conducted using different scan rates (from 20 mV/s to 250 mV/s with increments of 20 mV/s) in a solution with 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0).

2.4. Voltammetry of HX and Xa

CV measurements were performed at the potential range of 0 to 1400 mV at a scan rate of 100 mV/s. DPV measurements were performed using the following optimized conditions: potential window of 0 to 1400 mV, scan rate of 20 mV/s, pulse width of 35 ms, pulse height of 50 mV, and sampling time of 30 ms. Measurements were carried out using the bare CPE, and the poly-(threonine) modified electrode with 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0).

2.5. Simultaneous Determination of Hypoxanthine and Xanthine

Under the optimized conditions, DPV were performed on solutions with a fixed concentration of one analyte and varying concentration of the other in a supporting electrolyte solution.

2.6. Evaluation of Analytical Parameters

To determine the effect of potential interfering species on the determination of Xa and HX, DPV was performed in the presence of uric acid under optimum conditions and constant concentrations of the analytes. Calibration curves for the two analytes were constructed from the collected data. From the calibration curves obtained, the linear range, limit of detection (LOD), and limit of quantification (LOQ) for the analytes were determined. Specifically, a particular modified electrode was used in the simultaneous determination of HX and Xa six times. To regenerate the modified electrode, the electrode was cycled five times at the

optimal potential window. Another modified electrode was stored in phosphate buffer solution (pH 7.0) at room temperature for 1 week to determine its stability. This electrode was used in the determination of the analytes under optimum conditions.

3. RESULTS AND DISCUSSION

3.1. Electropolymerization of *L*-threonine

Following the procedure of Chitravathi and coworkers [17], the electropolymerization of *L*-threonine was performed between the electrode potential of -500 to 1500 mV at a scan rate of 50 mV/s for 10 cycles. The corresponding voltammogram (Figure 1) displays two waves: the anodic peak at ~+1400 mV and the cathodic peak at ~-400 mV. The increasing peak currents, as the number of CV scans was increased, correspond to the growth of the film on the electrode surface [17], albeit the current response of the poly(threonine) film decreased after the 10th cycle.

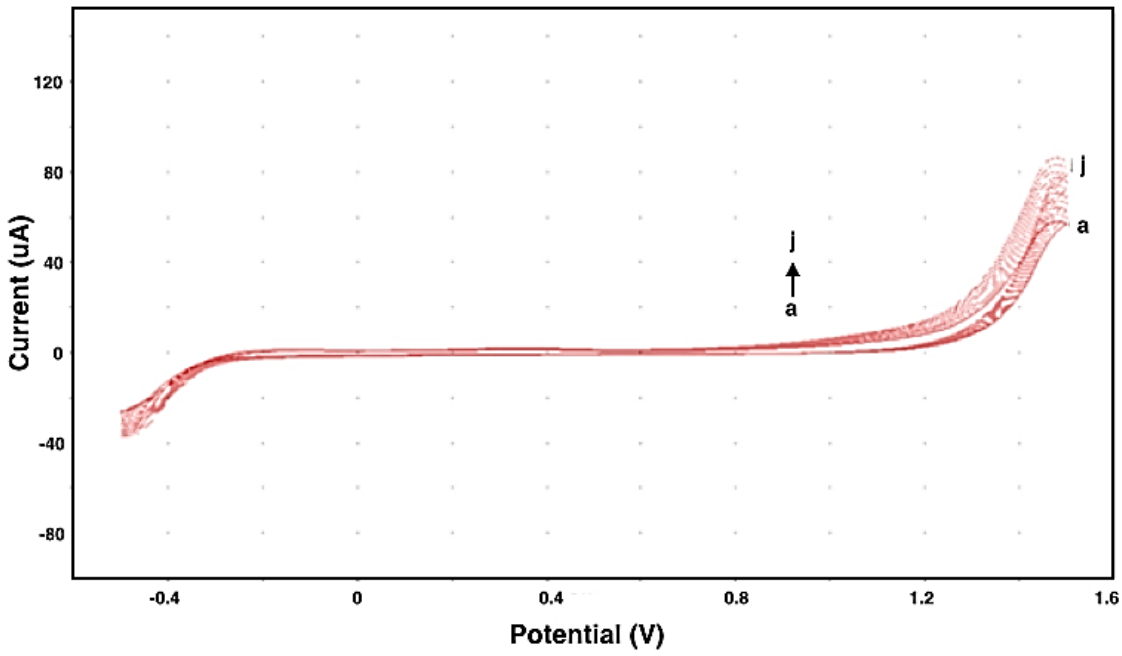

Fig. 1. Electropolymerization of 0.mM threonine on carbon paste electrode in phosphate buffer (pH 7.0) at scan rate of 50mV/s. (a → j: cycles 1 to 10)

It is evident from Figure 2 that there was a marked difference between the responses of the pre-modified and post-modified electrodes on the blank. This shows that the poly-(threonine) film was indeed formed on the surface of the electrode.

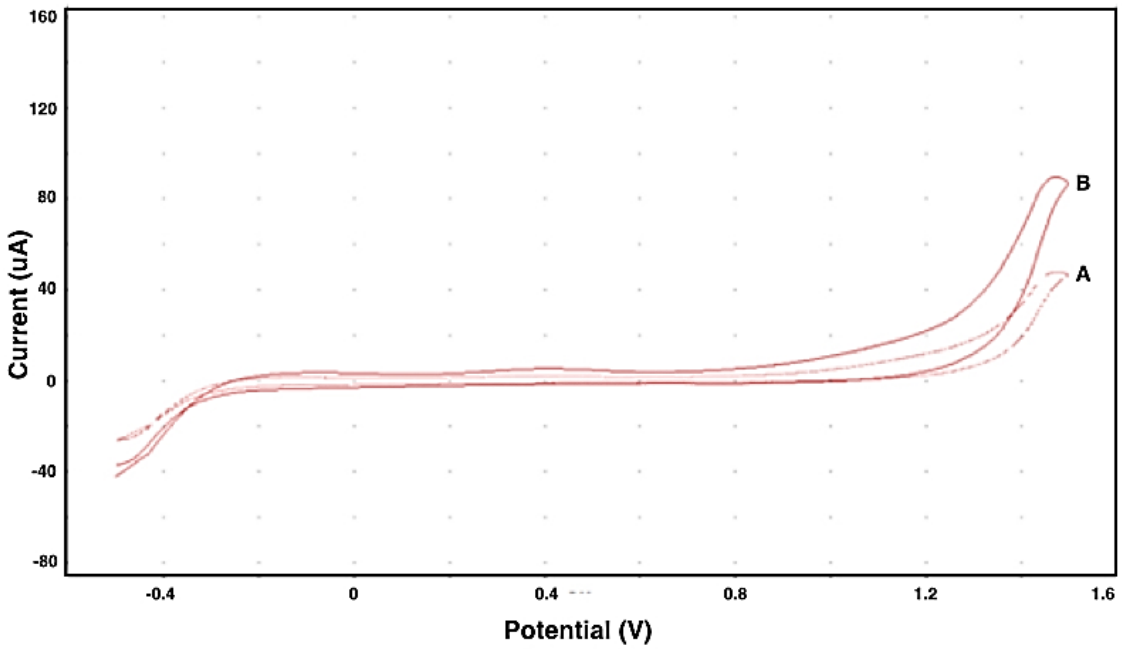

Fig. 2. Cyclic voltammogram of phosphate buffer(blank; pH 7.0) on carbon paste electrode B) before and A) after electropolymerization at scan rate of 100 mV/s.

3.2. Electrochemical behavior of HX and Xa at the poly-(threonine) modified electrode

The electrochemical activity of the analytes HX and Xa was examined using CV and DPV. Figures 3 and 4 show the cyclic voltammogram and the differential pulse voltammogram, respectively, of 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0) on both bare CPE and poly-(threonine) modified CPE. The CV shows that the electro-oxidation of Xa and HX using the modified electrode was irreversible, the anodic peaks in the forward scan having no corresponding discernible reduction peaks on the reverse scan (Figure 3).

The irreversibility of analyte oxidations was also evident in DPV studies (Figure 4). Obviously, the signals were asymmetric, indicative of an irreversible process [1,2]. Table 1 summarizes the anodic peak currents (i_{pa}) and potentials (E_{pa}) observed from CV and DPV measurements for the analyte solution that is 0.1 mM HX and in 0.1 mM Xa in phosphate buffer (pH 7.0) at both the bare and modified electrodes. Clearly, there was a significant increase in the current response of the analytes in both CV and DPV using the modified electrode compared to that of the bare electrode. The formation of the poly-(threonine) film on the electrode surface increased the surface area of the electrode, allowing more analyte molecules to be oxidized. The amplification of the current responses of the analytes indicates that the coated polymer provides much greater area for the target electrode processes to occur. The peaks for the analytes were found to be well resolved at the optimized conditions, with the difference of the oxidation peaks of approximately 400 mV. The modified electrode is therefore suitable for the simultaneous determination of HX and Xa.

Nevertheless, the succeeding scans (not shown) in CV yielded lower anodic peak currents (i_{pa}), possibly due to the adsorption at the surface of the modified CPE of either the analytes or their oxidation products.

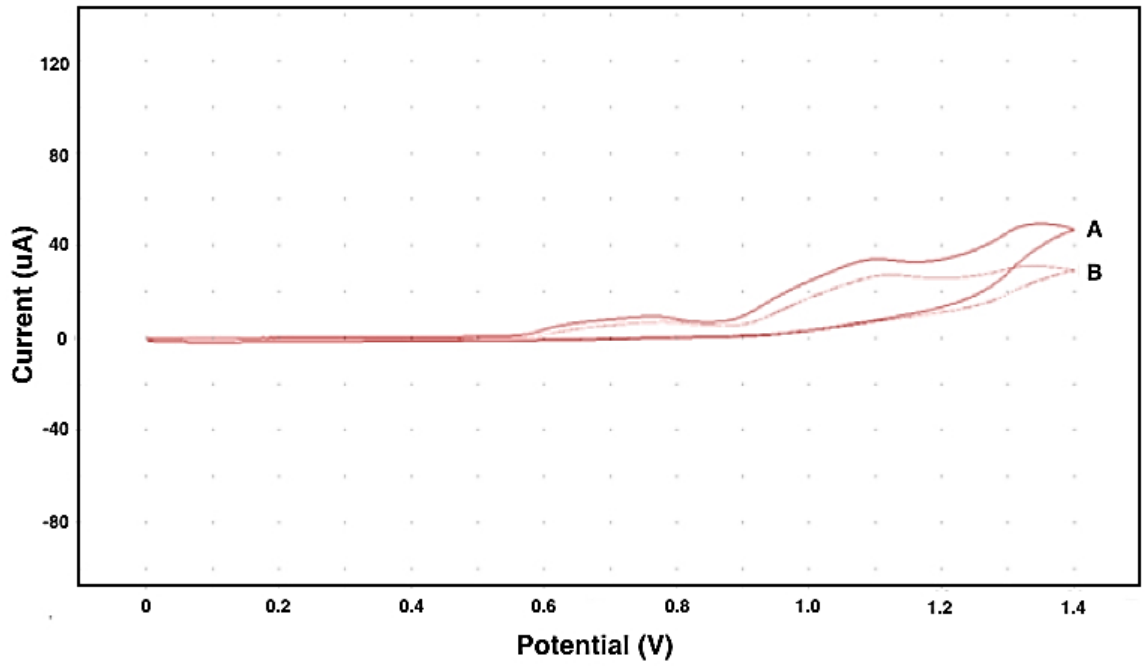

Fig. 3. Cyclic voltammograms of 0.1 mM HX and 0.1 mM Xa in PB (pH 7.0) at B) bare CPE and A) poly-(threonine) modified CPE at scan rate 100 mV/s

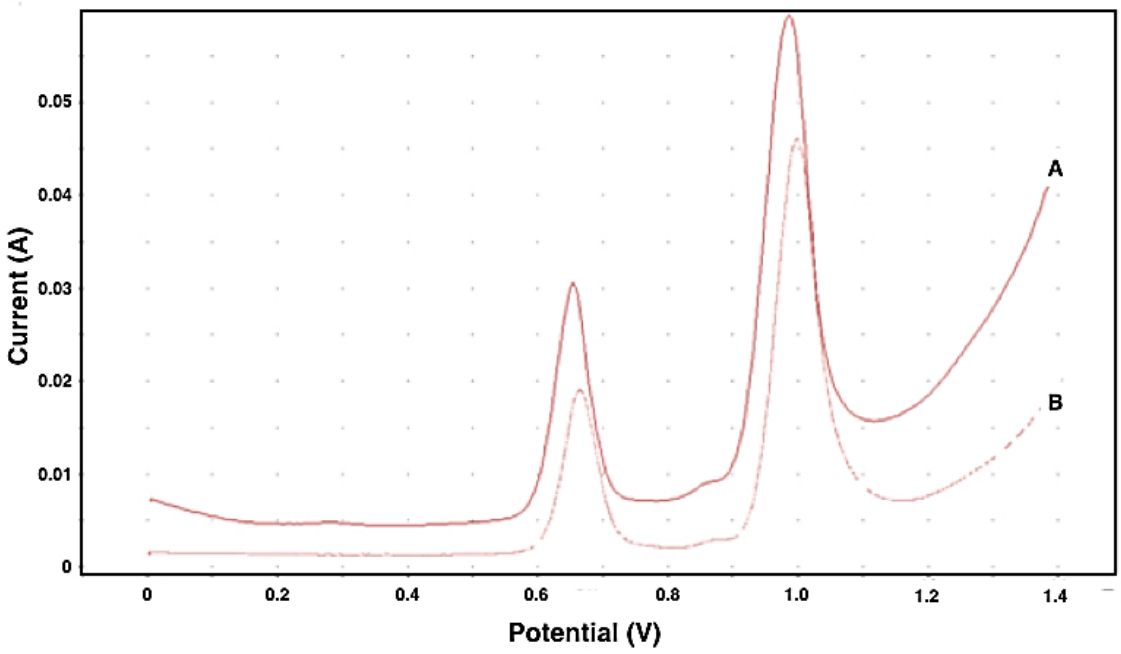

Fig. 4. Differential pulse voltammograms of 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0) at B) bare CPE and A) poly-(threonine) modified CPE at scan rate 20 mV/s

Table 1. Peak currents and peak potentials of HX and Xa at bare CPE and poly-(threonine) modified CPE

Technique	HX		Xa		Electrode
	I_{pa} (µA)	E_{pa} (V)	I_{pa} (µA)	E_{pa} (V)	
DPV	40.7	1.00	16.7	0.66	Bare
	46.3	0.99	23.8	0.66	Modified
CV	31.15	1.05	11.70	0.71	Bare
	33.76	1.03	16.31	0.70	Modified

3.3. Optimization of CV and DPV parameters

To optimize the experimental parameters for CV, a solution containing 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0) was used. The electrochemical oxidation of the analytes was expected to be observed at ~700 mV for Xa and ~1000 mV for HX [17]. The potential window of 0 to 1400 mV was found to give the best CV for the mixture of Xa and HX, and was chosen as the potential window for the subsequent experiments.

To optimize the experimental parameters for DPV, solutions of the same composition as in optimization of CV were subjected to DPV measurements at different pulse widths, pulse heights, and sampling time. The scan rates used in DPV were from 5 to 20 mV/s. In particular, a particular parameter was varied while the other two were kept constant. Wang and coworkers [1,2] observed that higher pulse amplitude (or height) yields larger, broader peaks, and recommended that the range used in measurements be at 25 to 50 mV at low scan rates. The conditions in which the peaks for the analytes were observed to be well resolved and with minimal background noise were chosen as the optimal parameters for DPV. The conditions so determined were as follows: pulse height=50 mV, pulse width=35 ms, and sampling time=30 ms.

3.4. Effect of pH

CV measurements were performed on a mixture of known concentrations of the analytes in a solution of the supporting electrolyte at different pH. The peak currents of the analytes at different pH were determined. Figure 5 shows the relationship of i_{pa} of HX and Xa with pH. For HX, a maximum was observed at pH 8, at which the deprotonation of hypoxanthine could have occurred. The maximum current for Xa was observed at pH 6, albeit the deprotonation of Xa is predicted to occur at pH 7.5 [19]. Like in HX, the deprotonation of the analyte is responsible for the decrease in the peak current beyond that point. Nevertheless, in

order to simulate the biological environment and obtain moderately high current responses, pH 7.0 was used in subsequent experiments as solution pH.

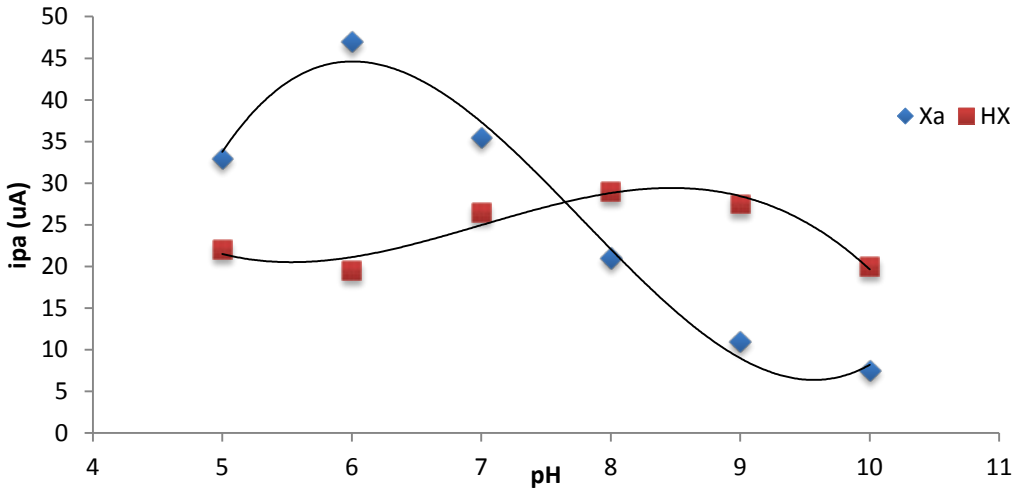

Fig. 5. Effect of pH on the i_{pa} of 0.1 mM HX (blue) and 0.1 mM Xa (red) in phosphate buffer

The shift towards negative potential indicates that the protons participated in the electro-oxidation of the analytes [1,2]. Moreover, the slopes of the linear segments (for HX=0.053 V/pH and for Xa=0.055 V/pH) are close to the theoretical value of 59 mV/pH. These suggest that there are equal number of protons and electrons involved in the oxidation of the analytes [1,2,20].

3.5. Effect of scan rate

The effect of scan rate on the electrochemical response of the analytes was investigated using 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0). Figure 6 shows the overlay of the voltammograms at different scan rates from 20 mV/s to 250 mV/s. Evidently, the increase in scan rate was accompanied by an increase in $i_{pa}$for both analytes. The peak currents were corrected for the drifting baseline and the logarithm of corrected current is plotted against the logarithm of scan rate. Figure 7 shows the practically linear dependence of log i_{pa} on the log scan rate.

More specifically, the observed linear plot of log i_{pa} versus log v, which actually depicts the linear relationship between the square root of scan rate and the anodic peak current (i_{pa}) for both analytes HX and Xa, is in accord with Randles-Sevcik equation and is indicative of a diffusion-controlled process [21].

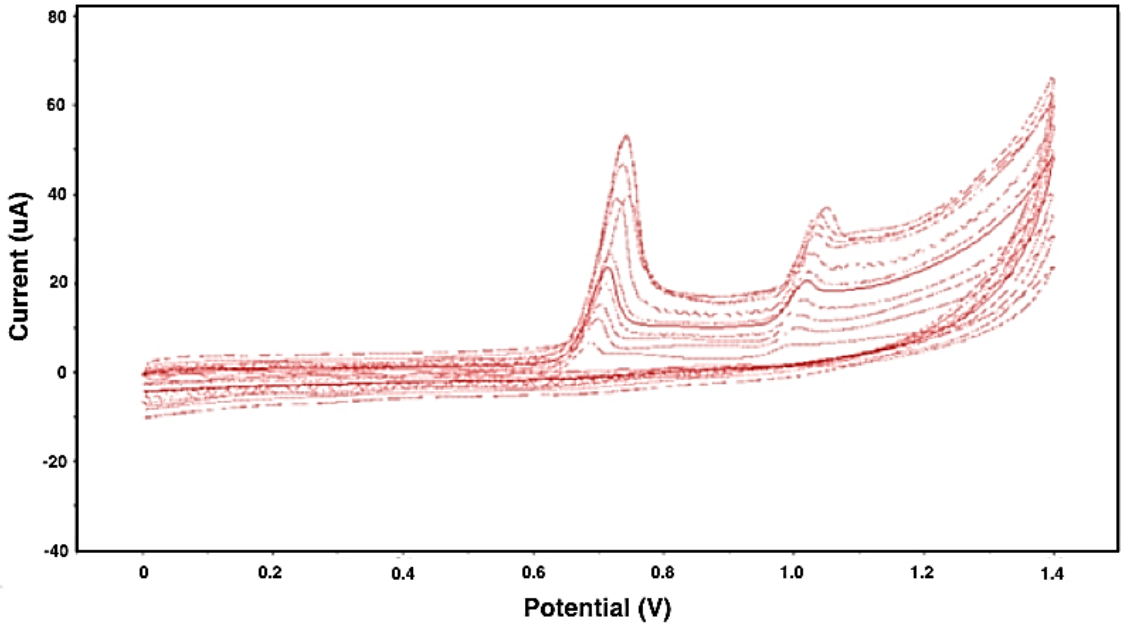

Fig. 6. Cyclic voltammograms of 0.1 mM HX and 0.1 mM Xa in phosphate buffer (pH 7.0) at poly-(threonine) modified CPE at varying scan rates

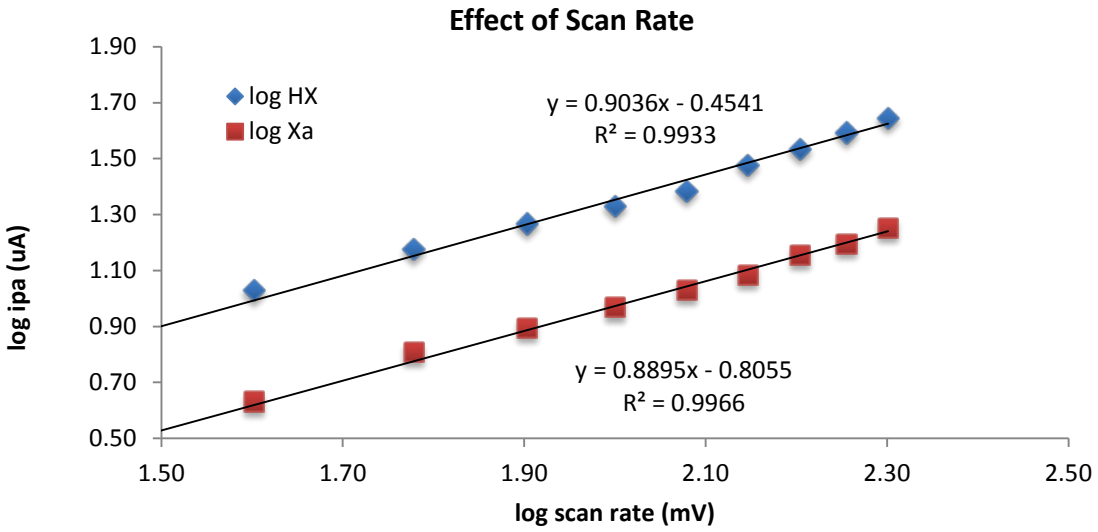

Fig. 7. Effect of scan rate (in mV/s) on the anodic peak current, i_{pa}, (in uA) of 0.1 mM HX (blue) and 0.1 mM Xa (red) in phosphate buffer (pH 7) solution

3.6. Evaluation of analytical parameters

The effect of the presence of interfering species like uric acid (UA), an analog of the analytes, on the current responses was determined using DPV. Table 2 shows the relative standard deviation of the current responses of the analytes at a particular concentration of the

interfering species. The spread in current readings is minimal at low (micromolar) concentrations of UA but becomes remarkable at higher (millimolar) concentrations due to the possible adsorption of UA (or its oxidative products) on the surface of the electrode. UA is expected to be oxidized first at around ~400 mV.

Table 1. Relative standard deviation of the current responses of HX and Xa in the presence of uric acid (UA)

[UA] added (M)	RSD % HX	RSD % Xa
0.001	13.68	16.42
0.00001	7.75	2.23

More importantly, the dependence of the anodic peak current on the concentration of the analytes was also studied by DPV. Figure 8 shows the distinctly linear dependence of peak current with the concentrations of HX and Xa. In particular, the anodic peak currents vary with concentration according to the following equations:

$i_{pa} = 0.051C_{HX} + 0.010$ (R^2=0.992) (Equation 1)

$i_{pa} = 0.043C_{Xa} + 0.013$ (R^2=0.992) (Equation 2)

Using the generated linear equation, the limit of detection (LOD) and limit of quantitation (LOQ) of the method using the modified electrode were computed using the formulas: LOD=3.3S/M and LOQ=10 S/M (*n*=3), where S is the residual standard deviation and M is the slope obtained from three different calibration curves [22]. Accordingly, the LOD was found to be 1.05×10^{-4} M for HX and 8.53×10^{-5} M for Xa and the LOQ was 3.17×10^{-4} M for HX and 2.58×10^{-4} M for Xa. The low detection limits demonstrate the suitability of the modified electrode for the simultaneous determination of Xa and HX even at low concentrations.

Moreover, the stability and reproducibility of the poly-(threonine) modified electrode were investigated using DPV to determine any deterioration of signals with time and repeated measurements. One electrode was used in the determination of 0.1 mM of the analytes for 6 times. The coefficient of variation of the current signals was found to be 3.12% and 6.60% for HX and Xa, respectively. Meanwhile, another modified electrode was kept at room temperature and was used in the determination of the analytes after standing for one week. The relative standard deviations of the current responses widened to 10.36% and 21.16% for HX and Xa, respectively.

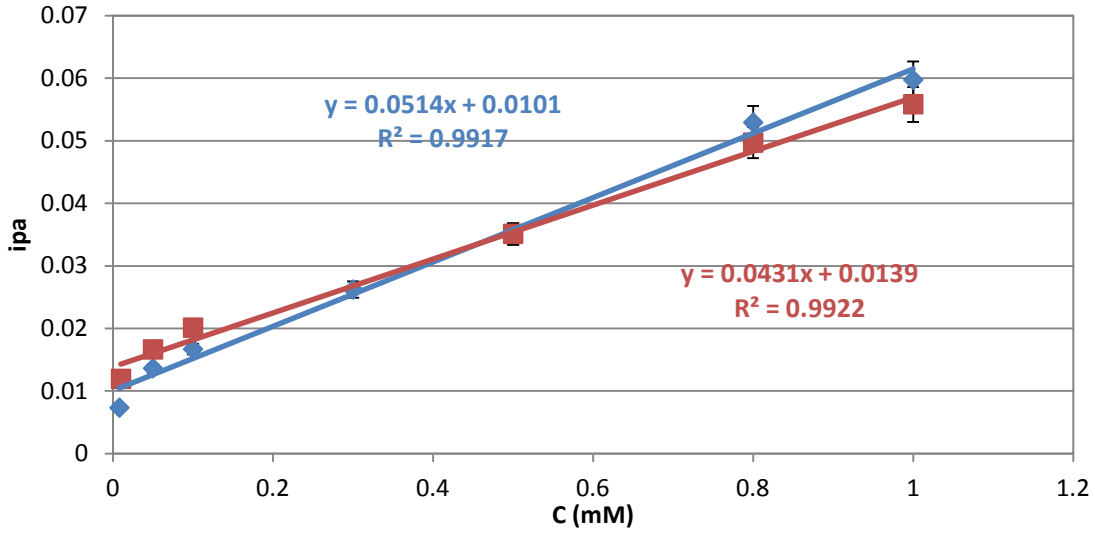

Fig. 8. Variation of anodic peak current at various concentrations (mM) of HX (blue) and Xa (red)

4. CONCLUSION

Poly-(threonine) film modified carbon paste electrode was successfully fabricated and applied in the simultaneous determination of xanthine (Xa) and hypoxanthine (HX) by both CV and DPV methods. The modified electrode was found to tolerate the presence of interfering species (*e.g.* uric acid) at micromolar concentrations. The linear range, and the low LOD and LOQ obtained make this modified electrode suitable for the simultaneous determination of Xa and HX.

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