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Determination of Xanthine in the Presence of Hypoxanthine by Adsorptive Stripping Voltammetry at the Mercury Film Electrode

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ABSTRACT: A stripping method for the determination of xanthine in the presence of hypoxanthine at the submicromolar concentration levels is described. The method is based on controlled adsorptive accumulation at the thin-film mercury electrode followed by a fast linear scan voltammetric measurement of the surface species. Optimum experimental conditions were found to be the use of 1.0×10^{-3} mol L^{-1} NaOH solution as supporting electrolyte, an accumulation potential of 0.00 V for xanthine and −0.50 V for hypoxanthine–copper, and a linear scan rate of 200 mV $second^{-1}$. The response of xanthine is linear over the concentration ranges of 20–140 ppb. For an accumulation time of 30 minutes, the detection limit was found to be 36 ppt (2.3×10^{-10} mol L^{-1}). Adequate conditions for measuring the xanthine in the presence of hypoxanthine, copper and other metals, uric acid, and other nitrogenated bases were also investigated. The utility of the method is demonstrated by the presence of xanthine associated with hypoxanthine, uric acid, nitrogenated bases, ATP, and ssDNA.

KEYWORDS: xanthine determination, hypoxanthine, copper, ATP, ssDNA, mercury electrode, stripping voltammetry

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Introduction

Oxypurines, xanthine, hypoxanthine, and uric acid are formed during purine metabolism and are found in tissues and body fluids such as blood and urine. When adenosine-5′-triphosphate (ATP) converts to adenosine-5′-monophosphate (AMP), and then subsequently converts to hypoxanthine and xanthine through inosine-5′-monophosphate or adenosine pathways, hypoxanthine and xanthine are created.[1] Determinations of extracellular concentrations of purines are of considerable significance, because changes in their concentrations can indicate several dysfunctions or diseases. For example, excessive accumulation of uric acid, produced as the end product from hypoxanthine and xanthine metabolism, may result in gout. Higher levels of purines in dialysates and heart perfusates can be an indicator of a heart attack since extracellular purines are also markers of the level of cellular energy metabolism.[2] Electroanalytical methods have been developed for the determination of xanthine.[3–10] Adsorptive stripping analysis has been shown to be an important method in ultratrace organic analysis because of its broad scope of applications and relatively simple instrumentation. The associated analytical costs are lower by orders of magnitude in comparison to HPLC.[11,12] In addition, electrochemical detection easily allows the development of in situ and continuous sensoring techniques. With the recent advancements in properties of the adsorptive stripping voltammetry, new methodologies were developed for adenine, thymine, guanine, ATP, and DNA using alkaline solution with lower ionic strength as supporting electrolyte.[13–16] Using this alkaline electrolyte, this work reports a new stripping voltammetric procedure for the measurement of xanthine in the presence of hypoxanthine based on its adsorption at the thin-film mercury electrode.

The advantages, instrumental parameters, and possible limitations of this procedure are also explained in this study. Furthermore, the effect of a wide range of potentially interfering compounds such as purine compounds, uric acid, ATP, ssDNA, and some metal ions were examined.

Experimental

Reagents. Water purified in a Milli-Q purification system (Millipore, Billerica, MA, USA) was used for all dilutions and sample preparations. All chemicals were of analytical reagent grade. Stock solutions of 1000 ppm were prepared from Sigma Chemicals (Sigma-Aldrich Brasil Ltda., São Paulo-SP, Brasil) by dissolving 25 mg of the target reagent xanthine or hypoxanthine plus solid NaOH with 25 mL of water (to achieve a final concentration of 0.1 mol L^{-1} NaOH). Stock solutions of uric acid and other bases were similarly prepared. Solutions were stored in the dark at 4°C. A 1000 ppm stock solution (atomic absorption standard solution; Sigma-Aldrich Brasil Ltda) was used for metals and diluted as required for standard additions. Stock solutions of 1000 ppm of ATP were prepared by dissolving 10 mg of the target reagent in 2 mL of diluted perchloric acid (10^{-1} mol L^{-1}). The subsequent solution was heated at 70°C for 30 seconds. Thereafter, the sample was cooled down and diluted to 10 mL with water. A single-stranded calf thymus DNA (Cat. No. D-8899; Lot 43H67951) was used as received from Sigma. A 500 µg DNA/mL stock solution (around 5 mg/10 mL; lyophilized powder containing 63% DNA) was prepared according to the procedure described for ATP. ATP and DNA were first treated with acid to obtain purine and pyrimidine bases and other degradation products. The final solution was stored at 4°C.

Apparatus. Linear cyclic voltammograms were obtained with an EG&G PAR model 384-B Polarographic Analyser (Princeton Applied Research, Princeton, NJ, USA), equipped with an external cell and a Houston Ametek-DMP-40 series digital plotter. The electrochemical cell was formed by a glassy carbon electrode (GCE) with thin-film mercury as a working electrode, an Ag/AgCl reference electrode with vicor tip and a platinum auxiliary electrode. A magnetic stirrer and a stirring bar (Nalgene Cat. No. 6600-0010, Rochester, NY, USA) provided convective transport during the accumulation step.

Forming the thin-film mercury electrode. In the formation of the mercury film on the surface of the electrode, a solution of 10^{-2} mol L^{-1} Hg (NO_3) was used. This solution was prepared by dissolution 0.4 g of mercury (II) nitrate in 100 mL of acidified Milli-Q water (5% of HNO_3). For the formation of the mercury film, the GCE (3.0 mm diameter, BAS-Bioanalytical Systems, Inc., West Lafayette, Indiana 47906, USA) was first polished with alumina (BAS, PK-4) and then mounted with the help of a Teflon holder in a voltammetric cell containing 1 mL of mercury (II) nitrate solution, 1 mL of 10^{-1} mol L^{-1} potassium nitrate solution, and 8 mL of purified water. This solution in the voltammetric cell was then purged with nitrogen for 240 seconds to eliminate the oxygen initially present. Mercury plating in the GCE surface was carried over for five minutes at −0.9 V. After the time of plating, it is necessary to visually check if the mercury film was well formed. The electrode array should then be rinsed with pure water. After this step, the electrodes are suitable for the implementation of other measures of this work.

Procedure. A known volume (10 mL) of the supporting electrolyte solution (1.0×10^{-3} mol L^{-1} sodium hydroxide) was added to the voltammetric cell and degassed with nitrogen for eight minutes (and for 60 seconds before each adsorptive stripping cycle). Initially, the condition potential (−0.9 V) was applied to the electrode for a selected time (usually 60 seconds). Then the initial potential (usually 0.05 V for xanthine and −0.50 V for hypoxanthine-copper) was applied to the electrode over a selected time interval (usually 60 seconds), while the solution was stirred slowly. The stirring was then stopped, and after 30 seconds, the voltammogram was recorded by applying a negative-going potential scan. The scan (at 200 mV $second^{-1}$; calculated drop time, 0.050 seconds; scan increment, 10 mV) was terminated at −0.60 V for xanthine and at −1.10 V for hypoxanthine–copper. The adsorptive stripping cycle was repeated using the same thin-film mercury. After obtaining the background stripping voltammograms, aliquots of the xanthine standards were introduced. To allow for the simultaneous xanthine and hypoxanthine determination, copper was usually added to a final concentration of 0.05 ppm. A similar procedure was followed to test the interference of cations, purine and pyrimidine bases, and ATP or DNA degradation products. The entire procedure was automated and controlled by a 384-B Polarographic Analyzer. Throughout this operation, nitrogen was passed over the solutions surface. All data were obtained at room temperature (25°C).

Results and Discussion

Parameters affecting the adsorptive stripping behavior. Until now, few studies on the mechanism of electrode processes of xanthine for adsorption using cathodic stripping voltammetry at thin-film mercury electrode have been reported. Using a mechanism similar to that proposed by Chen and co-workers, we suggest that the following electrode reaction processes take place during the accumulation and stripping steps[17] according to the following equations:

$$X_{sol} \rightarrow X_{ads}$$

$$X_{ads} + Hg \rightarrow (Hg(II)X_2)_{Film} + 2e^-$$

$$(Hg(II)X_2)_{Film} + e^- \rightarrow (Hg(I)X) + X$$

$$(Hg(I)X) + e^- \rightarrow Hg + X$$

The copper ion in the presence of xanthine fails to form a complex, but it can complex in the presence of hypoxanthine, which is verified by the formation of a new peak at a more negative potential and far from the peak potential of xanthine. The copper (II) ion in the presence of hypoxanthine may be

reduced to copper (I). The copper (I) thus developed can form a complex with hypoxanthine which can then be adsorbed on the surface of mercury electrode. Thus, the copper (I) present in the complex adsorbed may then be reduced to copper (0) using cathodic stripping voltammetry or oxidized to copper (II) using anodic stripping voltammetry. A possible mechanism is proposed below.[18–21]

$$Cu(II) + e^- \rightarrow Cu(I) \quad (1)$$

$$Cu(I) + HX \rightarrow (Cu(I)\ HX)_{ads}\ \{\text{sparingly soluble}\} \quad (2)$$

$$(Cu(I)\ HX)_{ads} + e^- \rightarrow (Cu(0)\ HX)_{ads} \quad (3)$$

Preliminary studies of the determination of xanthine by cathodic stripping voltammetry were conducted and published recently.[5] These results served as a starting point to verify the possibility to determine xanthine in the presence of hypoxanthine as well determining these simultaneously bases. The following experiments were then performed. The effect of the accumulation potential on the xanthine stripping peak current was examined over the 0.10 to −0.10 V range. The xanthine stripping peak current decreases between 0.05 and −0.1 V. A well-defined xanthine peak appears at −0.180 V (using 0.05 V as accumulation potential). A pre-peak appears at approximately 0.07 V (using 0.10 as accumulation potential) and the post-peak appears at −0.470 V (using −0.10 V as accumulation potential). This suggests strong xanthine adsorption on the mercury film at the studied accumulation potentials. Because of the appearance of complex multiple peaks, a special care in choosing the accumulation peak potential is needed. The effect of scan rate (υ) in the xanthine voltammograms was also tested (experimental conditions for xanthine (0.20 ppm): accumulation time, 30 seconds at 0.05 V; final potential, −0.6 V; scan rate, 20–200 mV second^{-1}; other conditions as in Fig. 1) in 1.0×10^{-3} mol L^{-1} NaOH. As expected, the xanthine peak current (I_p) depends strongly on the scan rate. A plot of log I_p vs log υ was linear (correlation coefficient, 0.975), with a slope of 1.3. The potential shifts negatively from −0.150 to −0.200 V when the scan rate increases from 20 to 200 mV second^{-1}, and the $b_{1/2}$ of xanthine peak increases from 50 to 87 mV. Thus, a higher scan rate of 200 mV second^{-1}, which also developed a higher current, was chosen as one of the parameters for all subsequent works. The shift of peak potential, with the accumulation rate shifts, confirms the possible non-Faradaic nature of the pre-concentration step. Figure 1 shows the dependence of the xanthine linear cyclic voltammetry (CV) peak current with the pre-concentration time. Adsorption increases with accumulation times of up to 60 seconds (resulting in higher peak currents), and then level off at longer accumulation times.

To verify the possibility of determining xanthine and hypoxanthine simultaneously, preliminary studies of complexation of hypoxanthine with the copper ion were made. A detailed study of the variation of the scan rate was then required and is shown below. The effect of the accumulation potential on the hypoxanthine–copper stripping peak was examined over the 0.0 to −0.5 V range (Fig. 2). A gradual increase in the hypoxanthine–copper current is observed as the pre-concentration potential is changed from 0.0 to −0.3 V. At higher accumulation potentials (greater than −0.3 V), two processes were observed: a peak associated with the reduction and oxidation of copper disappears and the hypoxanthine–copper stripping peak is maintained with signal constant. Thus, using more negative accumulation potentials, the stripping measurements that yielded a hypoxanthine–copper peak of higher analytical interest were verified (extremely low detection limits). Note that the peak potential of hypoxanthine complexed with copper is far enough away from xanthine. This difference between the values of the peaks (potential) tells us the great possibility of these compounds to be measured simultaneously.

Quantitative utility. The linear CV adsorptive stripping response of 0.20 ppm xanthine is highly reproducible and is adequate for an analytical procedure. Ten successive measurements (using 30 seconds accumulation times at 0.05 V; final potential at −0.6 V; 60 seconds condition time at −0.9 V; scan rate, 200 mV second^{-1} and 1.0×10^{-3} mol L^{-1} of NaOH as supporting electrolyte) yielded a mean peak current of 3084 ± 58 nA and a range of 3000–3125 nA. The effective pre-concentration provided by the adsorption process results in significant lowering of the detection limit of 36 ppt (2.3×10^{-10} mol L^{-1}). This was estimated by the quantitation of 1 ppb after a 30-minute accumulation (S/N = 2). Thus, 0.36 ng could be detected in the 10 mL of solution. Figure 3 shows voltammograms with the increase of peak current when the xanthine was added at increasing aliquots of 20 ppb (at accumulation time of 60 seconds at 0.05 V). Figure 4 also shows the resulting linear calibration plots. A least-squares analysis of the standard addition data yields a slope of 45 nA/ppb and a correlation coefficient of 0.997. Another experiment for concentrations increments of 100 ppb and using accumulation time of 15 seconds at 0.05 V was also realized. A least-squares analysis of the standard addition data yields a slope of 12 nA/ppb and a correlation coefficient of 0.993. In both experiments, well-defined stripping peaks were observed. Such precision compares favorably with that reported for other compounds measured by adsorptive stripping analysis.[22,23]

Potential interferents such as some metal ions, uric acid, and certain nitrogenated bases were tested in the presence of xanthine. Measurements of 1.0 ppm xanthine were not affected by the addition of up to 1.0 ppm of iron (III), cadmium (II), and mercury (II); up to 0.40 ppm of cobalt (II); and up to 0.10 ppm of zinc (II) and nickel (II). Copper (II) at high concentrations (above 1.0 ppm) is a serious interferent in the xanthine determination; the current peak xanthine is depreciated. No interference was observed in the concentration range of 0.02 to 0.10 ppm for thymine or methyl cytosine and up to 0.5 ppm for uric acid. Other data obtained from these experiments are shown in Table 1.

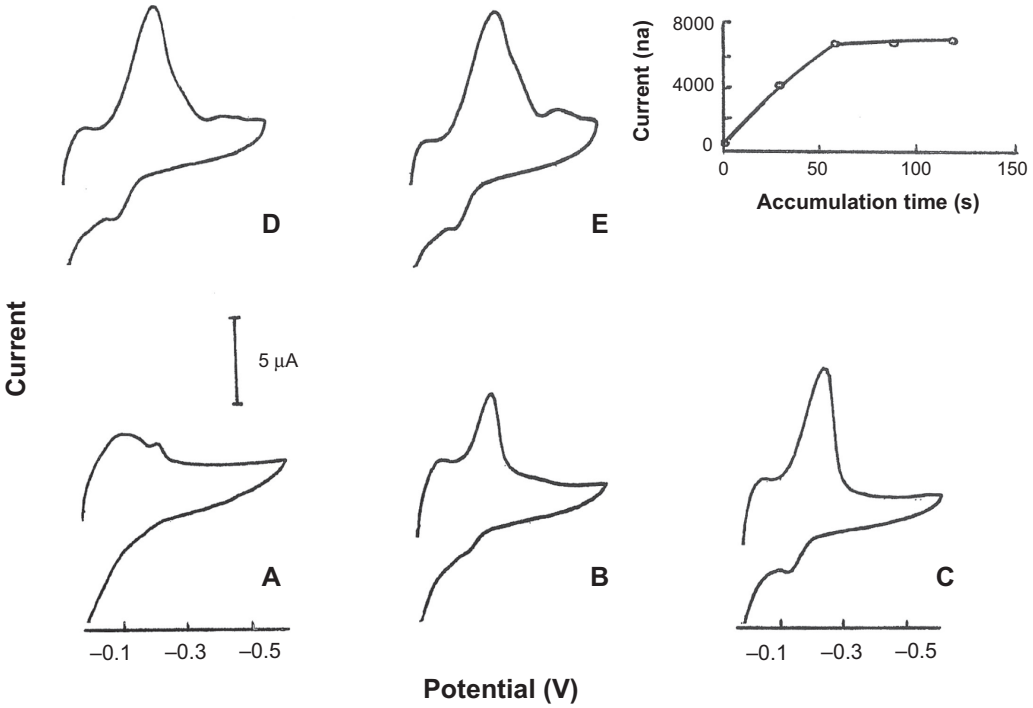

Figure 1. Effect of accumulation time on the linear CV adsorptive stripping voltammograms of the xanthine (0.20 ppm) in a solution of 1.0×10^{-3} mol L^{-1} NaOH. Accumulation time, **A** (0), B (30), **C** (60), **D** (90), **E** (120), at 0.05 V at −0.0 V. Final potential, −0.6 V. Condition time, 60 seconds at −0.9 V. Equilibrium time, 30 seconds. Scan rate, 200 mV $second^{-1}$. Thin-film mercury electrode (5 min at −0.9 V) is shown. Also shown is the resulting accumulation time plot.

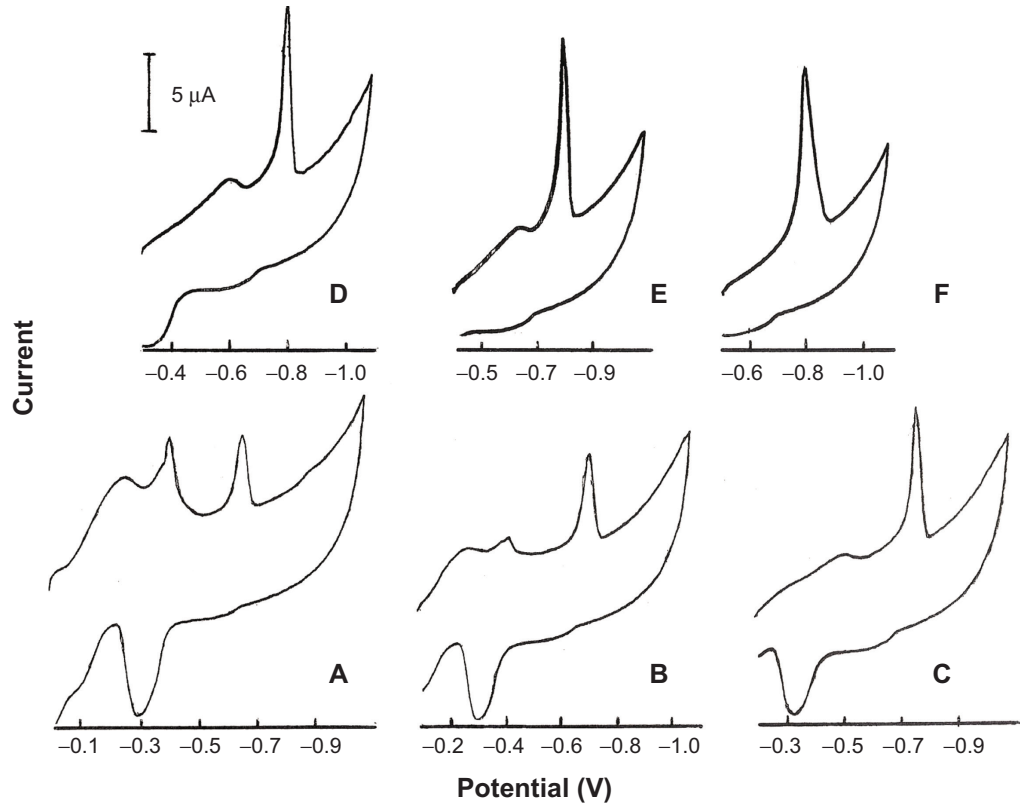

Figure 2. Effect of accumulation potential on the linear CV adsorptive stripping voltammograms of hypoxanthine (0.50 ppm) in presence of copper (0.50 ppm) in a solution of 1.0×10^{-3} mol L^{-1} NaOH. Accumulation time, 60 seconds at 0.0 (**A**), −0.1 (**B**), −0.2 (**C**), −0.3 (**D**), −0.4 (**E**) and −0.5 V (**F**). Scan rate, 200 mV $second^{-1}$. Other conditions as in Figure 1.

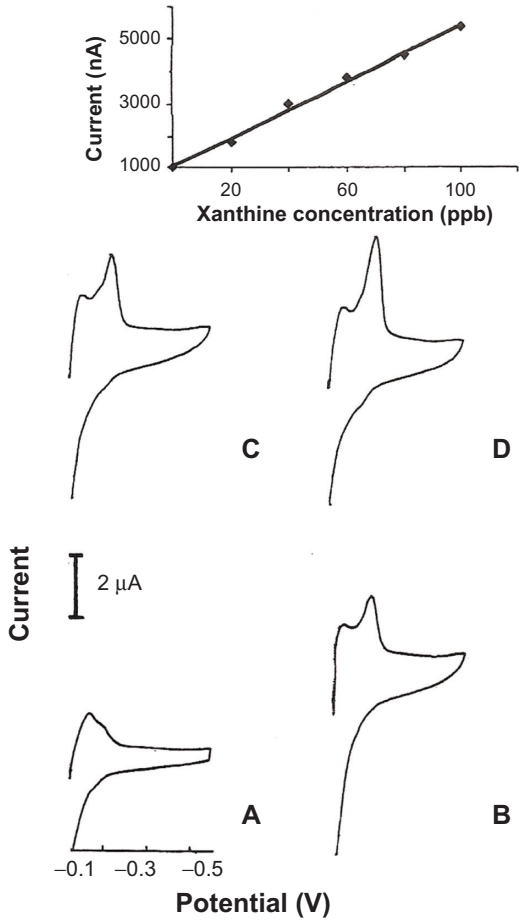

Figure 3. Linear CV adsorptive stripping voltammograms obtained after increasing the xanthine concentration in 20 ppb steps (**B**–**D**) in a solution of 1.0×10^{-3} mol L^{-1} NaOH. (**A**) Blank; without xanthine. Accumulation time, 60 seconds at 0.05 V. Final potential, −0.6 V. Other conditions as in Figure 1. The resulting calibration plots are also shown.

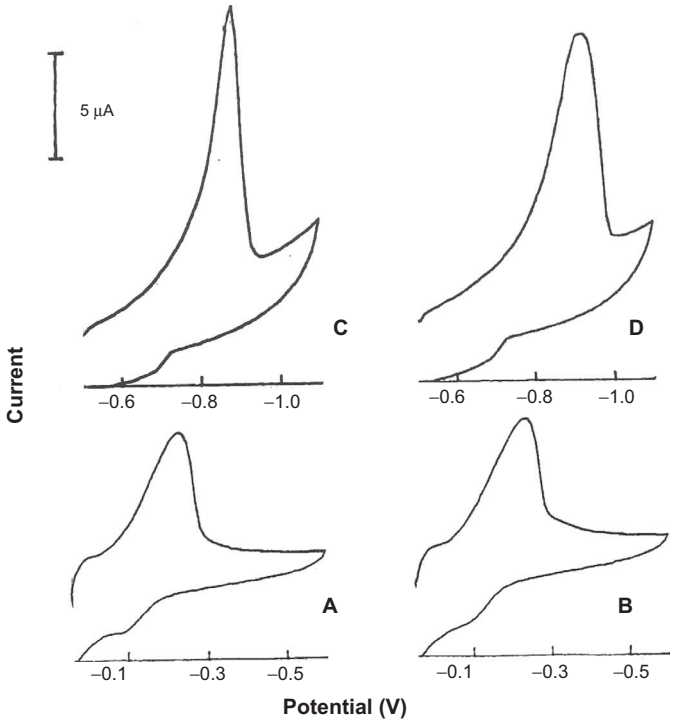

Figure 4. Linear CV adsorptive stripping voltammograms of the xanthine (**A**, 0.50 ppm) in presence of hypoxanthine (**B**, 0.50 ppm); hypoxanthine (**C**, 0.50 ppm) in presence of xanthine (**D**, 0.50 ppm); and copper (0.35 ppm) in a solution of 1.0×10^{-3} mol L^{-1} NaOH. Accumulation time of (**A**,**B**) 15 and (**C**,**D**) 60 seconds at 0.05 V (**A**,**B**) and −0.5 V (**C**,**D**), respectively. Other conditions as in Figure 3.

Several preliminary tests were performed to verify the possibility of determining xanthine in the presence of hypoxanthine. In these studies, it was verified that copper ion (II) developed complex only in the presence of hypoxanthine and the presence of hypoxanthine and uric acid does not change the xanthine peak. Figure 4 shows the xanthine peak in the presence of hypoxanthine (a and b: accumulation time, 15 seconds at 0.05 V) and the hypoxanthine peak in the presence of xanthine (c and d: accumulation time, 60 seconds at –0.5 V) at 0.35 ppm of copper. These results confirm a substantial potential for simultaneous determination of xanthine with the hypoxanthine. In such case, when using the same voltammetric cell, the determination of xanthine must be carried out first with the determination of subsequent hypoxanthine in the presence of copper. In preliminary studies, it was found that the response of hypoxanthine–copper is linear over the concentration ranges of 10–60 ppb. For an accumulation time of 30 minutes, the detection limit was found to be 250 ppt (1.8×10^{-9} mol L^{-1}).

To check the efficiency of the new method developed for determining xanthine in the presence of hypoxanthine, synthetic samples with significant amounts of possible interferences were developed. Figure 5 illustrates the method for the determination of xanthine in a synthetic sample containing hypoxanthine, uric acid, nitrogenated bases (guanine, thymine, cytosine, methyl cytosine, adenine, and uracil), ATP and DNA (all at 1 ppm concentration), using linear cyclic adsorptive stripping voltammetry. Four successive standard additions to the sample resulted in well-shaped adsorptive stripping peaks. Thus, the xanthine peak in the original sample (curve a) was quantified based on the resulting standard addition plot (also show in Fig. 5). Because of the inherent sensitivity of the method, short (30 seconds) accumulation times can be used. Five consecutive analyses of samples yielded an average value of 1.1 ppm with a standard deviation of 0.1 ppm. This works well with the original xanthine concentration (1.0 ppm).

Conclusions

An effective means for the determination of ultra-trace levels of xanthine in the presence of hypoxanthine has been described. The use of the simple and diluted alkaline electrolyte provided a sensitive and selective adsorptive stripping voltammetric method. The NaOH medium (E_p xanthine = −0.150 V, and E_p hypoxanthine–copper = −0.800 V) seems to be advantageous when compared with the borate buffer medium[24] (E_p xanthine = −0.07 V, and E_p hypoxanthine = +0.04 V) for

Table 1. Effect of guanine, thymine, cytosine, methyl cytosine, adenine, and uracil concentration on the linear adsorptive stripping peak for 0.10 ppm of xanthine in a solution of 1.0×10^{-3} mol L^{-1} NaOH.

ANALYTES (CONCENTRATIONS IN ppm)		% DEVIATION OF CURRENT PEAK XANTHINE	VARIATIONS OF XANTHINE POTENTIAL (mV)
Guanine*	0.02	0	<20
	0.04	0	<20
	0.06	0	<20
	0.08	0	<20
	0.10	0	<30
Cytosine	0.02	0	0
	0.04	0	0
	0.06	>22	<10
	0.08	>22	<10
	0.10	>22	<10
Adenine	0.02	0	0
	0.04	>7	0
	0.06	>21	0
	0.08	>21	0
	0.10	>21	<10
Uracil	0.02	0	0
	0.04	0	<10
	0.06	0	<10
	0.08	0	<10
	0.10	0	<10

Notes: Xanthine peak (approximately at –150 mV). For uric acid, effect was 0.50 ppm of xanthine. Conditions: Accumulation time, 30 seconds at +0.05 V. Potential final, –0.6 V. Equilibrium time, 35 seconds. Other conditions as in Figure 1. For uric acid study, accumulation time, 60 seconds at +0.05 V. *The half-width, $b_{1/2}$, of the xanthine peak increases (55 mV) in the presence of guanine.

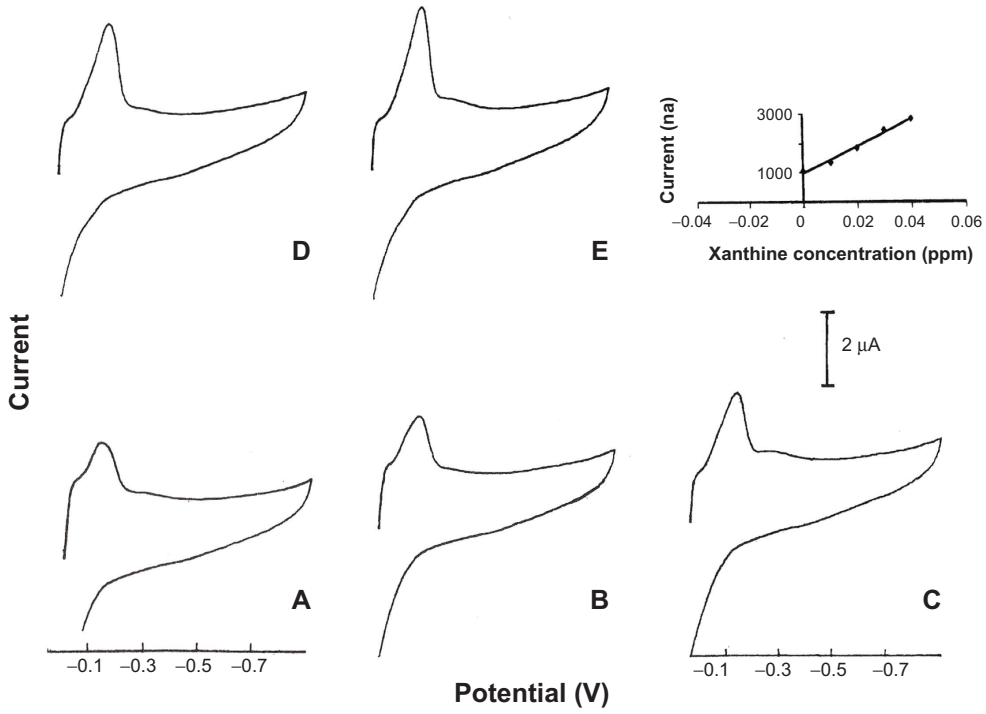

Figure 5. Illustration of xanthine determination in a synthetic sample by linear cyclic adsorptive stripping voltammetry. Electrolyte support, 10 mL of 1.0×10^{-3} mol L^{-1} NaOH. (**A**) Addition of 2.0×10^{-1} mL of synthetic sample; (**B**–**E**) steps of addition of 0.01 ppm of standard xanthine. Accumulation time, 30 seconds at 0.05 V. Final potential final, –0.9 V. Other conditions as in Figure 3. The resulting calibration curve for the sample is also shown.

studies of xanthine and hypoxanthine behavior at the electrode because of the peak potential shift further away from the mercury wave. Using pyrolytic graphite electrode, Dryhurst and co-workers showed that the peaks of hypoxanthine and xanthine could be separated by 0.50 V[25] while the newly proposed method is of 0.65 V. In addition, it was confirmed that supporting electrolyte solutions diluted with a low ionic strength are effective for the cathodic stripping voltammetry.[26] There are strong indications that the xanthine may yield better results in the absence of copper while optimal sensitivity and selectivity for hypoxanthine are verified when copper is present. In relation to the sensitivity and selectivity, the use of mercury films in the presence of copper (II) offers an attractive alternative to carbon electrodes.[27–29] The uric acid, which is the main metabolite of purine metabolism, does not modifies the xanthine and hypoxanthine–copper peaks. The detection limit of 2.3×10^{-10} for xanthine is also comparable with those found for HPLC methods.[11,12] Further studies using alkaline solutions as supporting electrolytes in the presence of copper can be conducted for the detection of HIV drugs, DNA-intercalating dyes, amino acids, peptides, and proteins determinations.

Author Contributions

Conceived and designed the experiments: PAMF, AAC. Analyzed the data: PAMF. Wrote the first draft of the manuscript: PAMF. Contributed to the writing of the manuscript: PAMF. Agree with manuscript results and conclusions: PAMF, AAC. Jointly developed the structure and arguments for the paper: PAMF. Made critical revisions and approved final version: PAMF. All authors reviewed and approved of the final manuscript.

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