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The Determination of Ammeline by Differential Pulse Polarography /Application to Milk

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ABSTRACT

An electrochemical approach has been proposed for the determination of ammeline. Electrochemical behavior of ammeline was investigated by differential pulse polarography and cyclic voltammetry in a Britton–Robinson buffer. Optimum conditions for the analytical determination were found to be pH 9.5 at a reduction potential of –30 mV. The calibration graph was linear in the concentration range of ammeline from 0,5 µM - 39,65 µM (0.06 µg mL^{-1} – 4.76 µg mL^{-1}) with a correlation coefficient of 0.998. Based on this use and under optimized conditions, with the DPP method for ammeline analysis had detection of limit (LOD) and the limit of quantification (LOQ) were obtained as 0.15 µM and 0.5 µM (0.02 µg mL^{-1} - 0.06 µg mL^{-1}), respectively. This offered method has been used for the determination of ammeline in spiked milk. The result demonstrated that this method is a simple, rapid, sensitive, stable and low-cost method for ammeline detection.

Keywords: *ammeline, determination, polarography, milk, cyclic voltammetry*

1. INTRODUCTION

Ammeline (4,6-Diamino-2-hydroxy-1,3,5-triazine) is a product of the degradation of s-triazine herbicides. Ammeline gives the alkaline hydrolysis of melamine [1]. There are various uses of ammeline. Ammeline is used in lubricating greases [2] the properties of ammeline greases, including their thermo-oxidative stability (TOS), have been studied. It was determined the influence of the ammeline concentration on the TOS. Another use of is Ammeline-melamine-formaldehyde resins (AMFR). Melamine-based resins represent an important class of aminoplastic resins and there are most applications as wood adhesives. AMFR systems containing from 5-10% ammeline are more flexible than MFR resins and exhibit different adhesion properties than MFR resins [3]. The aqueous ozone treatment of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] in the presence of hydrogen peroxide was reinvestigated using a new tandem solid-phase extraction procedure. In the experimental conditions ammeline was found as the major end-product (20% at pH 8) [4].

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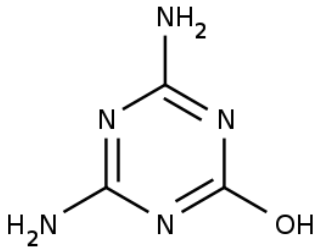
In March 2007, the illness and deaths of cats and dogs were reported to the United States FDA [5] (Food and Drug Administration). The FDA's investigation revealed that they had consumed melamine-contaminated pet food. This incidence caused a dozen deaths and hundreds of cases of kidney-related illness to cats and dogs, and led to the largest pet food recall in FDA history. Further investigation found that the melamine-tainted pet foods were also contaminated with structurally similar compounds such as cyanuric acid, ammeline and ammelide. It was revealed that some ingredients used to make pet food, such as wheat gluten and rice protein, had been intentionally adulterated with melamine and related compounds. It is assumed that these nitrogen-rich chemicals were added to make the final product appear to be protein rich, resulting in higher commercial value [2]. Thousands of pigs died after being fed diets containing melamine at 3026 mg/kg, ammeline at 958 mg/kg and cyanuric acid at 69 031 mg/kg [6] Sheep given ammeline or ammelide with various concentrations of melamine developed crystalluria and renal failure and died [7]. Data from experimental animals and pets have shown that co-exposure, in particular to melamine and analogues, results in renal crystal formation and subsequent kidney damage. Currently, there are insufficient data on which to develop a hazard characterization for these combined exposure scenarios, but it is anticipated that they would be more toxic than separate exposures. Chinese infant formula reportedly contained levels of cyanuric acid, ammeline and ammelide that were only about 0.1% of the melamine levels. They were also much lower than levels present in contaminated wheat gluten and rice protein concentrate ingredients that were used in the production of pet foods during the 2007 melamine contamination incident in the USA, Canada and South Africa [8] This was confirmed by [9] who conducted a rat study that tested ingestion of melamine alone, ammeline or ammelide alone (both analogs of melamine), a mixture of melamine and cyanuric acid and a mixture of all four compounds. Neither ammeline nor ammelide alone produced any renal effects, but the mixtures produced significant renal damage and crystals in nephrons. Analysis confirmed the presence of melamine and cyanuric acid in the kidney [8]. These human data are different from what has been described for the outbreaks in pets in 2004 and 2007. From all data reported to date, infants were exposed primarily to melamine alone or to very low levels of cyanuric acid when melamine was present at very high concentrations, whereas pets were exposed to melamine and cyanuric acid and possibly to ammeline and ammelide. Affected infants appear to have developed stones primarily in the urinary tract, which sometimes led to obstructive renal failure [10]. Pets, however, exposed to the combination of melamine and cyanuric acid, formed crystals in renal tubules, developing an intra-tubular obstructive nephropathy. Pets developed acute renal failure within 2 days of exposure in severe exposures, whereas most infants with stones reportedly did not have overt clinical symptoms [11]. Regarding the toxicity, Stratton [4] determined the toxic effects of atrazine and four of its degradation products. Point out that the hydroxytriazines as the ammeline are less toxic than the chlorotriazines as the atrazine.

Testing of the wheat glutens showed they contained significant levels of melamine and/or several related compounds including cyanuric acid, ammeline, and ammelide [12]. These compounds are structurally similar, containing a 1,3,5-triazine ring (see Scheme 1). Quantitative analysis was needed as a partial basis for health hazard evaluations, to understand and track the sources of the problem, and to help assess cross contamination in the manufacturing chain. Consequently, there has been growing concern about the significance and toxicity of melamine and analogues; this fact encouraged the development of methods for its trace determination in various sample materials.



Scheme 1. Structure of ammeline

Chromatographic methods have been usually used for ammeline detection. A variety of chromatographic detection methods, including high performance liquid chromatography [13], development of a high performance liquid chromatography method and a liquid chromatography–tandem mass spectrometry method with pressurized liquid extraction for simultaneous quantification and confirmation of cyromazine, melamine and its metabolites in foods of animal origin [14-16]. Micellar electro kinetic chromatography (MEKC) [17] liquid chromatography-tandem mass spectrometry [18, 19] hydrophilic interaction liquid chromatography [20] Matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry [21] and isotope dilution gas chromatography-mass spectrometry (ID-GC-MS) [22] are the preferred methods of choice for ammeline analysis. Ammeline and ammelide are also produced during the production of melamine as by-products and their HPCEC analysis has been reported by Debowski and Wilde [23].

Only a few studies are available for the electrochemical investigation of melamine in general [24-26] and to the best of our knowledge, no publications dealing with the electroanalytical or polarographic determination of ammeline have appeared so far. Compared to chromatography, voltammetric techniques have several advantages such as low cost and possibility of analysis without the need of pre-treatments, as well as the short time required for the analysis [27].

The purpose of the present work is to examine the polarographic determination of ammeline, find out

optimum analysis conditions and apply the method for the determination of melamine in milk. Also, electrochemical behaviors of melamine are investigated with cyclic voltammetry.

2. EXPERIMENTAL

2.1. Reagent

The mercury used in the dropping mercury electrode was purchased from Merck (Darmstadt, Germany). Ammeline was purchased from Sigma. Other all chemicals used analytical grade, was purchased from Merck and Sigma. Working standard solutions were prepared by dilution of stock solution with water. Distilled water was used in the preparation of all solutions.

2.2. Apparatus

All differential pulse polarography (DPP) and cyclic voltammetry (CV) measurements were performed on a BAS model (Bioanalytical Systems, Epsilon Basic Plus Potentiostat/ Galvanostat, USA) electrochemical analyzer with a conventional three electrode system comprising platinium wire as the counter electrode, as an Ag / AgCl (3 mol L^{-1} NaCl) reference electrode and a a dropping mercury electrode (DME) as a working electrode. pH values were measured with a WTW pH/ION 735 (WTW Instruments, Germany) pH meter.

2.3. Electrochemistry

Contaminated mercury was cleaned by passing it successively through dilute HNO_3 (3.0 mole L^{-1}) and water columns in the form of fine droplets by using a platinum sieve. The collected mercury was dried between sheets of filter paper. Whether there is of impurities before use in mercury was checked by DPP.

Ten milliliters (10 mL) of supporting electrolyte solution was put into the polarographic cell. The supporting electrolyte was used Britton-Robinson (B-R) buffer solution. NaOH and HCl was used to adjust pH. At the start of all experiments, pure N_2 gas about 5 min was passed from all of the prepared solutions for extract oxygen. All the experiments were done at room temperature. The background polarograms were obtained by scanning the potential from 0.0 V to about -1400 to -2200mV (vs. Ag/AgCl) depending on the pH of the solution. Polarograms were recorded by applying 5 mV / s pulse amplitude and 50 mV/s potential scan rate.

3. RESULTS AND DISCUSSION

3.1. Effect of pH

B-R buffer that can be used in a wide pH range was chosen as support electrolyte. In order to determine the electrochemical behavior of 30 μM ammeline, differential pulse polarographic responses were examined over the pH range of 1.0 to 13.0. One or two well defined peaks to the reduction of ammeline were recorded in the pH range of 9.5 -12.5 (Figure 1).

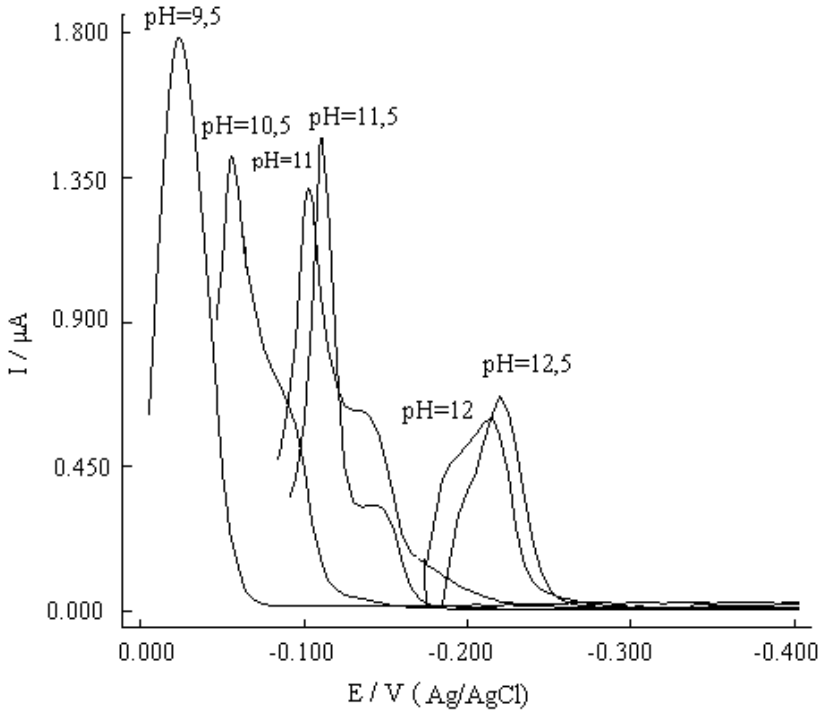


Figure 1. Differential pulse polarograms of 3.81 μg mL^{-1} (30μM) ammeline in pH 9.5 - 12.5 B-R buffer solution.

As a result of the pH screening, reduction of ammeline was found to be pH dependent. As seen in Figure 1, peak potentials with increasing pH values has shifted to negative. In addition, depending on pH of the solution are also differences in peak currents (Figure 2). The maximum peak current to for the ammeline was recorded

at pH 9.5. In addition, peak shape is appropriate for determination of ammeline. As a result, optimum conditions for analytical determination of the 30µM ammeline by DPP were found to be, pH 9.5 B-R buffers, at a reduction potential of -30 mV, 2 s drop time, 50 mV pulse amplitude. The polarographic behavior in the Britton-Robinson buffer of Ammeline is given in Table 1.

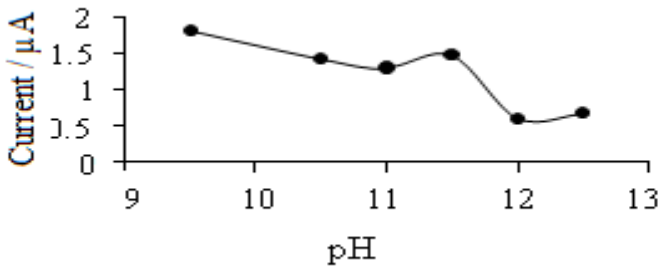


Figure 2. Dependence of pH on the DPP peak currents of 3.81 µg mL^{-1} (30µM) ammeline

Table 1. Polarographic behavior of 30 µM ammeline Britton-Robinson buffer electrolytes

	Electrolyte	E_{peak}(mV)	I(µA)	Peak shape	C(µM)
Ion	pH=9.5	-30	1.780	sharp	30
	pH=10.5	-55	1.415	sharp	
	pH=11	-102	1.305	sharp	
	pH=11.5	-110	1.500	sharp	
	pH=12	-213	0.595	broad	
	pH=12.5	-220	0.665	broad	

3.2. Analytical characteristics

The peak current-potential curve is the most useful analytical signal for DPP technique. The polarographic responses of ammeline at the dropping mercury electrode (DME) were analyzed in B-R buffer solutions (pH 9.5). Determination of ammeline at supporting electrolyte was performed with calibration graph and standard addition methods. The analytical curves for ammeline were obtained by standard addition of ammeline. The experiment wasrepeated5 times. Concentration of 1×10^{-5} M ammeline in the B-R buffer was found as (1,01 ±0,050) $\times 10^{-5}$ M at 95% confidence level. The results are given in Table 2.

Calibration studies for ammeline have been done under all the optimized experimental conditions and determination ranges have been determined (Figure 3).

The DPP responses at a potential of -30 mV showed that the dependence of peak currents on the ammeline concentration was linear, in the range of concentration from 0.5 µM (0.06 µg mL^{-1}) to 39.65 µM (4.76 µg mL^{-1}) with the linear regression equations given by;

I_p (µA) = 0,095 C (µM) + 0,072 (R^2 = 0,998) (n=5) (1)

The limits of detection (LOD) and quantification (LOQ) were calculated using the relations k x S_b/b (k=3 for LOD and k=10 for LOQ, respectively, where Sb is the standard deviation of intercept and b is the slope of the calibration curve. LOD and LOQ were obtained as 0.15 µM and 0.5 µM (0.02 µg mL^{-1} - 0.06 µg mL^{-1}), respectively. The method developed for the determination of ammeline by DPP has high accuracy and repeatability is good.

Table 2. Determination of 10 µM ammeline (in polarography cell) in pH 9.5 B-R Britton-Robinson buffer electrolyte

Presented ammeline (M)	Calculated ammeline $\overline{X}$ (M)	s	* $\overline{X}$ ± ts/√N
1 x 10^{-5}	1,01 x 10^{-5}	0,04 x 10^{-5}	(1,01 ± 0,05) x 10^{-5}

* N=5 %95 Confidence interval,

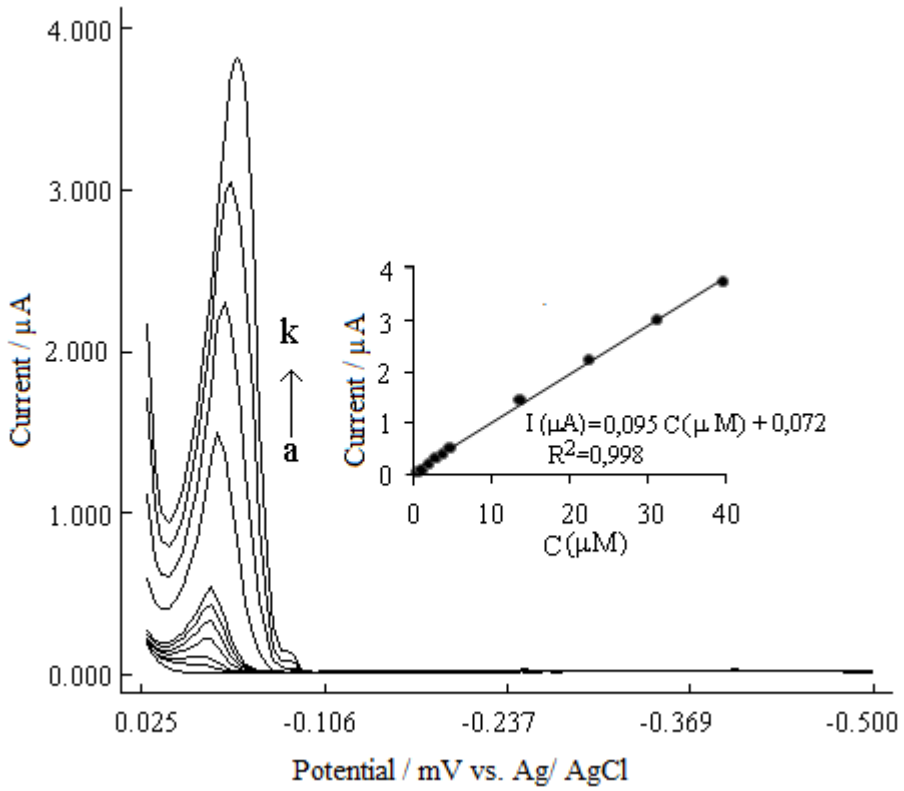


Figure 3. DPP responses for several concentrations of ammeline: a) 9.5 mL pH 11.2 B–R buffer solution b) a + 0,5 mL 1 x 10-5 M ammeline; c) b + 0,05 mL 1 x 10-4 M ammeline; d) c + 0,1 mL 1 x 10-4 M ammeline; e) d + 0,1 mL 1 x 10-4 M ammeline; f) e + 0,1 mL 1 x 10-4 M ammeline; g)f+ 0,1 mL 1 x 10-4 M ammeline; h) g + 0,1 mL 1 x 10-3 M ammeline ; i) h + 0,1 mL 1 x 10-3 M ammeline; j) i + 0,1 mL 1 x 10-3 M ammeline; k) j + 0,1 mL 1 x 10-3 M ammeline.

3.3. Interference Studies

To determine the selectivity of the developed method, the interference effects of some ions which are commonly found in milk has been examined and their recoveries has been calculated. In order to determine the selectivity of the developed method for the determination of ammeline, (Ca^{+2}, Mg^{+2}, K^{+}, Fe^{+3} and Cu^{+2}), Co^{+2}, Pb^{+2}, Zn^{+2}, Cd^{+2}, Mn^{+2}, Se^{+4}, NO_2^{-}, Ba^{+2}, NO_3^{2-}, SO_4^{2-}, Cl^{-} ions in, examined the effect of interference on the determination of ammeline. For this purpose, recovery of ammeline was calculated in the presence of ions (Table 3). Fe^{+3}, Ni^{+2}, Co^{+2}, Pb^{+2}, Zn^{+2}, Cd^{+2}, Mn^{+2}, Se^{+4}, NO_2^{-} ions are electro-active, Ca^{+2}, Mg^{+2}, K^{+}, Ba^{+2}, NO_3^{2-}, $SO_4^{2-}$and Cl^{-} ions are inactive.

First, polarograms were recorded in the range of 0-2200 mV ions to determine whether the peaks of the ions. While concentration of ammeline is 1 x 10^{-5} mole L^{-1}, concentration of these ions are 1 x 10^{-4} mole L^{-1} (Table 3). The peaks of ions electro-active Co^{+2}, Mn^{+2}, Se^{+4} and $Pb^{+2}$was not shown. Therefore, there are not interference effect of these ions. The peaks of Cu^{+2} (-182 mV), Fe^{+3} (-1440 mV), Cd^{+2} (-660 mV), Zn^{+2} (-1351 mV) ions were recorded at the indicated potentials. The peak potentials of these ions does not overlap with the peak potential of

the ammeline (-30 mV). K^+, Ba^{+2}, Ca^{+2}, Mg^{+2}, NO_3^-, SO_4^{2-} and Cl^- are polarographically inactive species and therefore, had no serious effect on the polarographic determination of ammeline. For added ions didn't cause a change in peak height of ammeline, determination of ammeline was made with standard additions. The results of the recovery ranged from 91.7 to 101.5% for the determination of ammeline in B-R buffer that was attained by standard addition method (Table 3).

Table 3. Influence of interfering species on the recovery of 1.27 μg mL^{-1} (10 μM) ammeline

Interfering species	C (μM)	Recoveries (%) of ammeline	Interfering species	C(μM)	Recoveries (%) of ammeline
Pb^{2+}		97.6	K^+		101.5
Ni^{2+}		95.0	Ba^{2+}		95.2
Cd^{2+}		95.8	Mg^{2+}		96.2
Fe^{3+}		91.7	Ca^{2+}		98.0
Zn^{2+}	100	96.2	NO_3^-	100	101.5
Se^{4+}		98.1	SO_4^{2-}		96.6
Co^{2+}		100.0	Cl^-		99.0
Cu^{2+}		98.0	NO_2		97.6
Mn^{2+}		96.6			

3.4. Cyclic Voltammetry

Cyclic voltammograms recordings were obtained within the range 0-(-0.6) V at a scan rate of 200 mV s^{-1} for 20 μM ammeline in B-R buffer pH 9.5 using hanging mercury drop electrode (Figure 4). On the electrode the ammeline exhibits an anodic peak at -41mV and two cathodic peak at -88 mV and -323 mV (Figure 4).

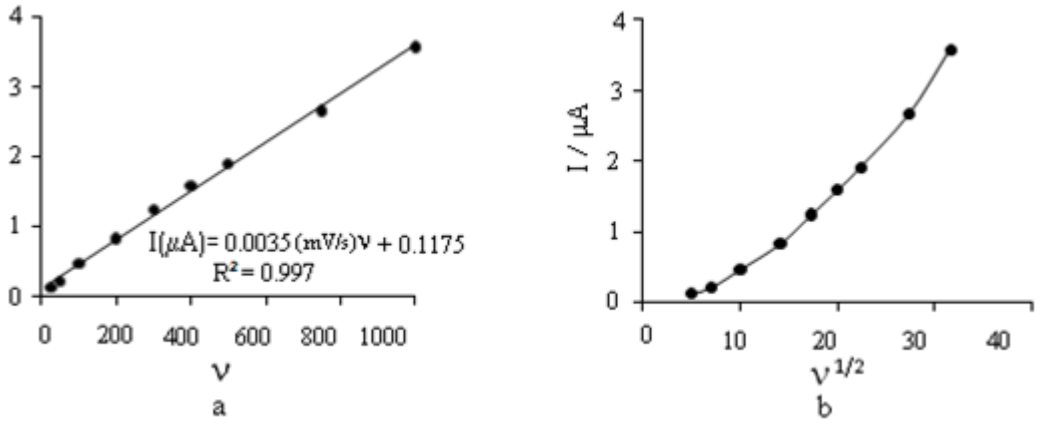


Figure 4. A cyclic voltammogram of 20 μM for ammeline at a hanging mercury drop electrode (scan rate 100 mV s−1).

The effect of potential scan rate on the voltammetric response at a concentration of 30 μM ammeline reduction in the B-R buffer was investigated between 25 and 1000 mV s^{-1}. The cathodic peak current varied linearly with the square of the scan (Figure 5a) (Eq.2) suggesting that ammeline reduction follows a adsoption-controlled mechanism [29]. Reduction peak of ammeline at -88 mV seem to semi-reversible. I_p with $\upsilon^{1/2}$ increases in quasi-reversible reactions at cyclic voltammetry, but is not linear [28] (Figure 5b). E_{pc} shifts to negative value with

the increase υ in quasi-reversible reactions. E_{pc} shifted up to -105 mV (at 1000 mV/s) from -79 mV (at 25 mV/s) at CV of ammeline. ΔE_p, at low scan rate approximates to 59 / n mV. ΔE_p increases with υ. While ΔE_p was recorded 28 mV (at 25 mV/s), ΔE_p was recorded 68 (at 1000 mV/s).

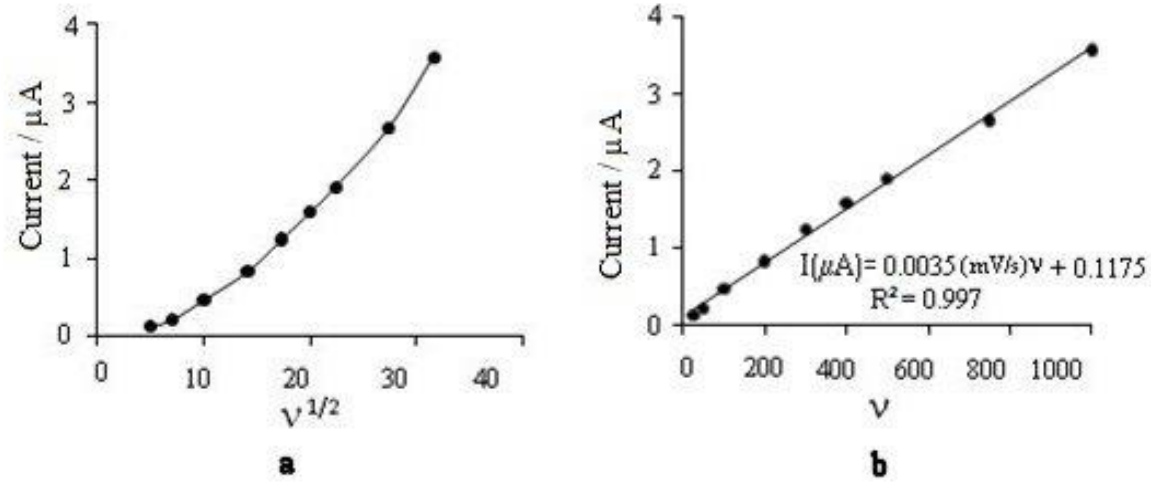


Figure 5 a. Peak current (Ip;µA) versus the square root of the sweeping rate, $v^{1/2}$ for 2.54µg mL^{-1} (20µM) ammeline in B-R buffer pH=9.5 b. Peak current (Ip;µA) versus scan rate (ν; mV s^{-1}) for 2.54 µg mL^{-1} (20µM) ammeline in B-R buffer pH=9.5

A plot of logarithm of peak currents (log I_p) versus logarithm of scan rate (log ν) gave a straight line (Figure 6) (Eq.3) with a slope of 0.910 (greater than 0.5) this value being close to that theoretically expected (1.0) for adsorption controlled systems [30].

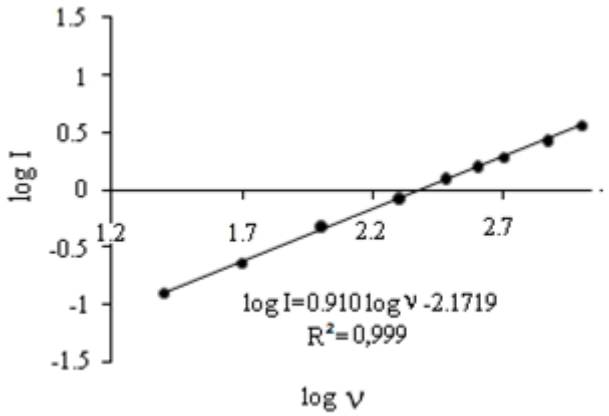


Figure 6. logIp (µA) versus log ν (mVs^{-1}) for 2.54µg mL^{-1} (20µM) ammeline in B-R buffer pH=9.5

$$I\ (\mu A) = 0{,}0035\ \nu\ (mV/s) + 0{,}1175\ (R^2 = 0{,}997) \quad (2)$$

$$\log I\ (\mu A) = 0{,}910 \log \nu - 2{,}1719\ (R^2 = 0{,}999) \quad (3)$$

CV works showed that the reduction mechanisms of ammeline are adsorption controlled. When the product or the reactant is strongly adsorbed, a prepeak or a postpeak shows in cyclic voltammogram [31]. These peaks are called as adsorption peaks. If adsorption is weak, these peaks are not observed. Since adsorption peaks haven't observed in this study, weak adsorption tests were applied.

3.5. Application to milk

The utility of proposed method was proven by spiking milk samples with ammeline. In order to eliminate matrix effects, the standard addition method was used. For this purpose, 1.0 mL milk samples were spiked with stock ammeline solution resulting to 0.51µg mL^{-1} -2.54 µg mL^{-1} (2.0 µM-10.0 µM). Acetonitrile removes serum proteins

more effectively, as the addition of 3.0 volume to 1.0 volumes of milk in sufficient to remove of the proteins [32]. The same procedure was also followed in parallel for ammeline-free milk sample. After homogenizing the samples 20 min, centrifuged for 20 min at 9000 rpm to get rid of milk protein residues. From the supernatant, 0.1 mL of ammeline-free aliquots were collected, transferred to the polarographic cell containing 9.9 mL of B-R buffer solutions at pH 9.5 supporting electrolyte solutions. Differential pulse polarogram was recorded and then were added two times from the milk sample that contains 2.0 µM-10.0 µM of ammeline. And ammeline level in spiked milk samples was determined by standard additions.

Recovery works have been done for the determination of the ammeline added to the milk and the recovery values of the 2 µM and 10 µM ammeline have been calculated as 98,75% and 87,70 % (Table 4). Proposed differential pulse polarographic method provided good recovery values with low relative error, demonstrating that the procedures were sufficiently effective in determining ammeline in spiked milk samples.

Table 4. Determination of spiked ammeline in milk samples (in polarography cell)

Added (µM)	Found (µM) [a] $\bar{X} \pm ts/\sqrt{N}$	RSD%	Recovery%
-	[b]N.D.	-	-
2	1.97±0.12	3.55	%98.75
10	8.77±0.85	6.09	%87.70

[a]95 % confidence interval (N=4)

[b]N.D.: not detected

4. CONCLUSIONS

From the literature review, there are no reports on the differential pulse polarographic (DPP) determination of ammeline. This study, electrochemical behavior of ammeline investigated using DPP and DV techniques. A simple electrochemical method was successfully applied for the determination of ammeline. This compound was detected with differential pulse voltammetry a reduction potential of -30 mV at hanging mercury electrode in the B-R buffer (pH 9.5).

Under the optimized parameters, the limit of detection (LOD) and limit of quantification (LOQ) was 0.15 µM and 0.5 µM (0.02 µg mL^{-1} - 0.06 µg mL^{-1}), respectively and the peak current was linear in the concentration range 0.5 µM – 39.65 µM total ammeline in the B-R buffer. The proposed method was applied to milk samples that ammeline added. The developed method has high selectivity and sensitivity for ammeline. In order to determine the selectivity of the developing methodology was investigated interference effects of various ions on the determination of ammeline. With the help of cyclic voltammetry studies, the reduction of ammeline was determined to be effective of adsorption.

The results obtained in proposed method are very reproducible since with the use of dropping mercury electrode the surface of the electrode is always new and the behavior of the electrode is independent of its past history. The main advantage of such a procedure is the possibility to determine the trace quantities of ammeline directly from natural samples (milk) without any previous treatment, such as extraction, clean-up and derivatization or pre-concentration which are tedious, time consuming and also polluting.

Additionally, the other advantages of this voltammetric methodology such as simplicity, cheapness and rapidity were demonstrated by the successful application in the samples of ammeline content in ammeline spiked milk after a simple preparation of samples.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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