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# Using Cysteine-CdSe Nanoparticles for Quantification of Ovalbumin Thermal Denaturation by Cyclic and Differential Pulse Voltammetry

Leon Nejem[1,2], Maha Mosallb[1*]

[1] *Department of Chemistry, college of Sciences, University of Aleppo, Aleppo, Syria.*
[2] *E-mail: nejem@scs-net.org*
[*] ***corresponding author**: E-mail: maha.am.bc@hotmail.com*

**ABSTRACT:** *A rapid electrochemical characterization of water soluble cysteine-CdSe nanoparticles (Cys-CdSe NPs) using cyclic (CV) and differential pulse (DPV) voltammetry was studied. The obtained peaks were identified, and the stability was determined. The Cys-CdSe NPs as electrochemical probe for determination of Ovalbumin (OVA) as a model protein, with a new highly sensitive electrochemical method was investigated, and applied by DPV technique. Proposed method was easy with good results in determination of protein in the concentration range 0.1-25 µg.mL$^{-1}$ ($r^2$ = 0.998), the recoveries were within the range of 97.00-100.02 %, with percent relative standard deviations ranging from 0.84 % to 3.10 %.*

***Keywords*** *- Cyclic Voltammetry, Cysteine-CdSe, Differential Pulse Voltammetry, Nanoparticles, Ovalbumin.*

## I. INTRODUCTION

Colloidal inorganic nanoparticles (NPs) have become the most explored nanomaterial, since the 1990, due it have several unique optical, chemical, and electronic characteristics [1,2]. NPs physicochemical characteristics have been extensively studied by different techniques such as: inductively coupled plasma mass spectrometry (ICP-MS), photoluminescence, X-ray photoelectron spectroscopy (XPS), X-ray diffraction (XRD) or transmission electron microscopy (TEM) [3]. These techniques have several advantages and disadvantages, but their use can be laborious, time consuming and/or requiring expensive instrumentation.

Electrochemical techniques have been also used for the characterization of NPs[4, providing valuable information about the NPs, such as energy bands [5] or surface defects [ 6,7]. For instance, cyclic voltammetry (CV) has been used to study the quantum confinement of nanoparticles dispersions [8].

Voltammetry measurements have been previously used for the quantification and stability studies of NPs [9,10,11]. For analytical applications, since beginning of 21$^{st}$ century, a great attention has been paid to apply of NPs as probes for inorganic ions, organic compounds, and biological molecules. These applications offer advantages in sensitivity, speed, and cost. Electrochemical applications offer a great advantage in trace analysis because the analytes can be measured easily without addition of mediators. Direct electrochemical reactions based on NPs have been reported in many applications [12]. The amperometric response of NPs with good biocompatibility and conductivity has been observed [13]. It was remarkable that the most development of NPs-based analysis has only occurred within almost two decades. Yet with continued development of new synthetic strategies it can be expected that NPs will find a wider range of applications in the near future [12]. However, among the types of NPs, the II – VI type NPs were the largest group of NPs, especially monodisperse CdX NPs (X = S, Se, or Te) that have become the prototypical semiconductor NPs.

The protein analysis was an important area as a reference for its measurement in food [14], biochemistry [15], technology [16], medical and clinical applications [17,18]. However, there were several methods to identify and determine protein, such as HPLC analysis of amino acid mass after acid hydrolysis [19,20], KJELDAHL nitrogen determination [21], Vis.-spectrophotometry as Biuret reaction [22,23,24,25], Lowry method [26,27,28,39], Bradford method [30,31,32,33], and bicinchoninic acid assay [34,35,36], UV-spectrophotometry [37,38,39,40], fluorescence [41,42], and capillary electrophoresis [43].

However, the electrochemical methods were also used in protein analysis [44,45,46], Cyt c was one of the most studied analytes in protein analysis.

In the present work, a new voltammetric method for Ovalbumin (OVA) (hen), as a model protein, by utilization of electrochemical characterization of Cys coated CdSe NPs, as electrochemical probe was developed. The experimental conditions were optimized, and the electrochemical determinations were calculated. The determination of OVA at the ultra-trace graphite (UTG) were carried out according to the changes of electrochemical responses of the measured solution contain Cys-CdSe NPs in absence and presence OVA in the optimum conditions.

Cyclic voltammetry (CV) was the most widely used electrochemical technique, frequently used for the characterization of redox systems, and often the first experiment performed in an electro analytical study. In other hand, it can provide rapid information about the number of redox states of the electroactive species, as well as qualitative information about the stability of these oxidation states and the electron transfer kinetics [47,48].

## II. MATERIAL AND METHODS

### 1.1. Instruments and tools:

The electrochemical measurements were operated on 1.746 VA Trace Analyzer with 1.747 VA stand (Metrohm Herisau-Switzerland). The traditional three-electrode system were done using various electrodes: Hanging mercury drop electrode (HMDE) with multi-mode electrode (Metrohm, 6.1246.020) and ultra-trace graphite (UTG) (Metrohm, 6.1204.100) as working electrodes; Ag/AgCl/3 mol.$L^{-1}$ KCl (Metrohm, 6.0728.020) as the reference electrode (RE); graphite electrode (Metrohm, 6.1204.100) and platinum wire (Metrohm, 6.1204.120) as the auxiliary electrodes. The pH measurements were performed on an inoLab pH Level 1 (WTW, Weilheim, Germany), using glass pH probe (WTW, glass pH precision electrode Plus SenTix® 81 with temperature sensor 0-100°C and DIN cable, Weilheim, Germany). The conductivity measurments were done by MP513 laboratory conductivity meter (0-~200mS/cm, ±1.0% FS, Shanghai, SanXin, China), using conductivity cell (Cell constant 1 $cm^{-1}$, Shanghai, SanXin, China). The spectroscopic measurments were done by F-2700 fluorescence spectrophotometer (light source 150w xenon lamp, 220-730 ±3.0 nm, Hitachi, Tokyo, Japan), and UV/Vis. spectrophotometer measurements T90+ (PG Instruments, China). Other instrument and tools were: Kern ALS 220-4 analytical balance ± 1mg, max. 220 g (Kern & Sohn GmbH, Germany), EM 0500 mantel for 500mL round flask (Electrothermal, England), MSH-20A Hot-plate stirrer (Daihan Scientific, Korea), EBA 20, 6000 rpm centrifuge (Hettich®, Tuttlingen, Germany) and 10-100±1µL and 100-1000±5µL mechanical single channel adjustable volume micropipettes (BioHit-ProLine®, Germany). All the experiments were carried out at 25±1°C, otherwise mentioned at text.

### 1.2. Reagents and chemicals:

The grade of reagents and chemicals used were of the highest purity available from their sources and need no further purification. The chemicals used are: Ovalbumin (≥99.0%, flake, Himedia, Ref), l,d-cysteine (97%, Aldrich, BioR), l(+)-tartaric acid crystals (>99%, Merck, extra pure), d-glucose (99.5%, Himedia, Reagent grade), biuret (J. T. Baker Chem. Co.), citric acid monohydrate (99.5%, SRL, extra pure), tri-sodium citrate (>98.0%, Himedia, Ref), ammonium tartrate (99%, Merck, AR), selenium metalloid powder black (99.5%, Merck, Lab), 2-propanol (99.5%, Panreac Quimica SA, PRS), ethanol absolute (>99.2%, Eurolab, GR), phosphoric acid (85-88%, Merck, GR), sulfuric acid (95.0-98.0%, Panreac Quimica SA, PRS), acetic acid (99.5-100.5%, Panreac Quimica SAU, PRS), boric acid (>99.5%, Himedia, RM), ammonium acetate (96%, Merck, extra pure), sodium acetate anhydrous (99.5%, Panreac Quimica SA, PRS), sodium dihydrogen orthophosphate dihydrate (96%, Loba Chemi, extra pure), cadmium nitrate tetrahydrate (99%, Merck, pure), calcium chloride (>96.0%, Himedia, RM), iron(III) nitrate nonahydrate (>99%, Merck, analytical grade), potassium chloride (99-100.5%, Merck, extra pure), sodium sulfide hydrate (32-38%, flake, Himedia, Ref), zinc nitrate hexahydrate (>99.0%, Himedia, RM), sodium borohydride (98%, Himedia, Ref), sodium hydroxide (96%, pellats, Loba Chemi, extra pure), ammonium solution (30-33%, Merck, extra pure), $N_2$ gas (95%, local market, trade grade), coated abrasives sheet for polishing 1, 0.3 and 0.05 µm $Al_2O_3$ (Extec®, USA). Fresh bi-distilled water (Conductivity <6 µS.cm-1, Janat instruments Co., Syria), was used at reagent preparation and assay,

other lab-works mono-distilled water (Conductivity <25 µS.cm$^{-1}$, WSA-08, Human Lab, Koria).

Many buffer solutions [49] were used, the phosphate buffer solution (PBS) at pH 6.8 as supporting electrolyte, $NaH_2PO_4/H_3PO_4$ for pH 5.5-7.2, Britton-Robinson universal buffers for pH 3.50-8.00, sodium citrate /citric acid for pH 3.5-4.2, $CH_3COONa$ /$CH_3COOH$ for pH 4.2-5.5, and ammonium tartrate/ tartaric acid for pH 7.20-8.0.

**1.3. Synthesis of CdSe core nanoparticles:**

The water soluble core Cys-CdSe NPs were synthesized hydrothermally according to the published method [50] with minor modifications. Simply, 2 mmol of Se metalloid powder and 4 mmol of the $NaBH_4$ were mixed with 25 mL of ethanol in a 100 mL triple-necked flask. The reaction system was degassed and purged with $N_2$ protection for 30 min. Then, the reaction mixture was reflexed until Se powered almost disappear, so there was no evolution of $H_2$ gas. It allows the rapid and easy preparation of NaHSe without the necessity of generating dangerously toxic $H_2Se$. Then, 25 mL of N2-saturated bi-distilled water were added. The reaction mass cooled to room temperature under $N_2$ protection to prevent NaHSe oxidation by air [51]. Thus, dark pink ~40 mM hydroethanolic solution of NaHSe were obtained. In another 250 mL three-neck flask, a mixture of 0.6 mmol $Cd(NO_3)_2$ and 3.0 mmol Cys were mixed in 120 mL bi-distilled water. The pH of the mixture was adjusted to 12.0 by adding 1.0 mol.L$^{-1}$ NaOH solution drop-wise under $N_2$ protection. Then, 5 mL (~0.2 mmol) of NaHSe solution prepared earlier was added into the mixture drop-wise at room temperature. The mixture was reflexed until the fluorescence emission of Cys-capped CdSe (Cys-CdSe) nanoparticles was observed under UV lamp, the temperature was suddenly lowered to room temperature [52]. After adding about 75mL the cold 2-propanol that facilitate breaking off the turbidity, the turbid system was centrifuged (10000 rpm for 5 min). The final isolated and deposited product, Cys-CdSe NPs, was washed and dried overnight through the drying oven at 40 °C and stored in amber will-closed bottle at a refrigerator for the further experiments [53].

**1.4. Cyclic Voltammetry (CV)**

Cyclic voltammetry (CV) measurements common parameters for Cys-CdSe NPs and Ova protein were: scan rate 100 mV.s$^{-1}$, the reference electrode was Ag/AgCl/3 mol.L$^{-1}$ KCl, and scan rate 100 mV.s$^{-1}$. The reaction system was degassed and purged with $N_2$ protection for 3 min. All experiments were carried out at room temperature 25 °C.

1.4.1. Cys-CdSe NPs

CV measurements parameters for Cys-CdSe NPs were as follows: HMDE as working electrode vs. reference electrode and graphite rod as auxiliary electrode, and initial and end potentials were +1800 mV, and -1600 mV, respectively. In addition, 0.2 mol.L$^{-1}$ acetate buffer at pH 5 was used as the supporting electrolyte. The studied solutions was stirred.

1.4.2. Ova determination

CV measurements parameters for Ova protein were as follows: UTG as working electrode vs. reference electrode and platinum as auxiliary electrode, initial and end potentials were -800 mV, and +200 mV, respectively. Unstirred 0.2 mol.L$^{-1}$ phosphate buffer solution (PBS) at pH 6.8 as supporting electrolyte.

**1.5. Differential pulse voltammetry (DPV):**

Differential pulse voltammetry (DPV) measurements common parameters for Cys-CdSe NPs characterization and Ova protein determination were as follows: reference electrode was Ag/AgCl/3 mol.L$^{-1}$ KCl, amplitude 0.5 mV, pulse width 0.05 s, sample width 0.05 s, pulse period 0.2 s, quiet time 2 s, and scan rate 100 mV.s$^{-1}$. The studied solutions was unstirred.

1.5.1. Electrochemical characterization of Cys-CdSe NPs:

DPV measurements parameters for Cys-CdSe NPs were as follows: HMDE as working electrode vs. reference electrode and graphite rod as auxiliary electrode. Start and end potentials were determine depended on position of the studied peak at cyclic voltammogram (CVg). All experiments were carried out at room temperature 25 °C. The reaction system was degassed and purged with $N_2$ protection for 3 min.

1.5.2. Ova determination:

DPV measurements parameters for Ova protein were as follows: UTG as working electrode vs. reference electrode

and platinum as auxiliary electrode. The DPV was carried out from 0.0 to -600 mV. In a typical process, 1 mL of Cys-CdSe NPs solution 0.3 mmol.$L^{-1}$ and 10 mL of PBS solution was transferred into a clean glass electrochemistry cell. Then, the required volume of Ova or sample solution was micro-pipetted. The UTG was polished with 1, 0.3 and 0.05 µm $Al_2O_3$ abrasive sheets successively washed with bi-distilled water, ultrasonically cleaned with ethanol and bi-distilled water and blown dry with the $N_2$ gas.

## III. RESULTS AND DISCUSSION

### 2.1. Electrochemical characteristics of Cys-CdSe NPs:

2.1.1. Cyclic voltammetry:

Based on the fact, chemicals used for synthesis of Cys-CdSe NPs were electroactive, so it should be interested in the issue what a CVg of Cys-CdSe NPs looked like. Typical CVg of Cys-CdSe NPs were shown in Fig. 1.

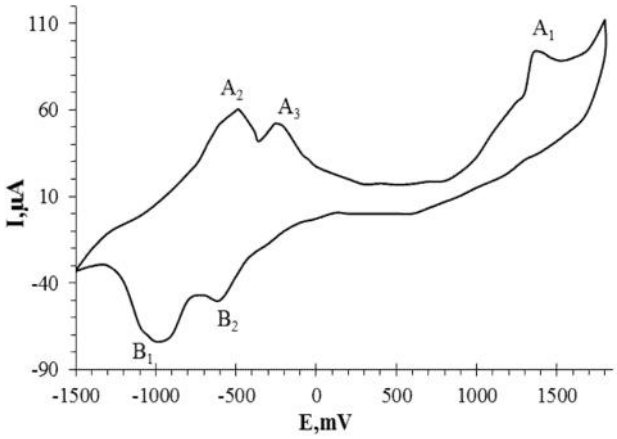


**Fig. 1: Typical CVg of Cys-CdSe NPs measured at HMDE vs. Ag/AgCl/3 mol.$L^{-1}$ KCl. Acetate buffer (0.20 mol.$L^{-1}$, pH 5) as the supporting electrolyte. CV parameters were as follows: initial and end potentials were +1800 mV, and -1600 mV, respectively; at fixed scan rate 100 mV.$s^{-1}$. The experiment were carried out at room temperature 23-25 °C. The solution was unstirred**

Due to the presence of Cys, Cd, and Se, the CVg have obtained five peaks, $A_1$ (1350 mV, 92.6µA, Cys-CdSe NPs), $A_2$ (-480 mV, 60 µA, Cd(II)), $A_3$ (-200 mV, 50µA, Cyc), $B_1$ (-994 mV, -73.9 µA, Electrode/Cys-CdSe NPs) and $B_2$ (-600 mV, -50 µA, Cd(II)), which correspond to the redox reactions of the Cys-CdSe NPs.

These results indicate that Cys-CdSe NPs were electroactive; however, CV was a method useful for redox characterization only. Quantification of any component was doubtful due to poor sensitivity to NPs signals. Therefore, to determine each peak due to any electroactive species, it should be aiming the attention on the quantity studying of synthesis Cys-CdSe NPs and the basic electrochemical behavior of Cys, Cd, and Se as the components of synthesis Cys-CdSe NPs, by using DPV, which was more sensitive to electroactive species compared to CV.

2.1.2. Differential pulse voltammetry:

The DVP characterization of Cys-CdSe NPs was performed by CVgs of synthesis Cys-CdSe NPs that shown in Fig. 1. Clearly, all the five detected peaks enhanced with the increasing concentration of Cys-CdSe NPs. Initially, to identify peaks, the CVgs scanned for many solutions prepared by mixing a fixed concentration 0.10 mmol.$L^{-1}$ of Cys-CdSe NPs and successive define different concentrations of Cd(II) ions between 0.03-0.45 mmol.$L^{-1}$. Then, the differential pulse voltammograms (DPVgs) scanned for oxidation and reduction electrochemical peaks. The $A_2$ and $B_2$ electrochemical peaks enhanced with the increasing concentration of Cd(II), which means these electrochemical peaks were due to oxidation and reduction of Cd(II), respectively. The DPVgs shown at Fig. 2a and 2c. Later, as done initially, the CVgs scanned for many solutions prepared by mixing a fixed concentration 0.10 mmol.$L^{-1}$ of Cys-CdSe NPs and successive define different concentrations of Cys between 0.30-4.50 mmol.$L^{-1}$. Then, the DPVgs scanned for oxidation and reduction electrochemical peaks. The $A_3$ electrochemical peak enhanced with the increasing concentration of Cys, which means this electrochemical peak was due to oxidation of Cys.

The DPVgs shown at Fig. 3a.

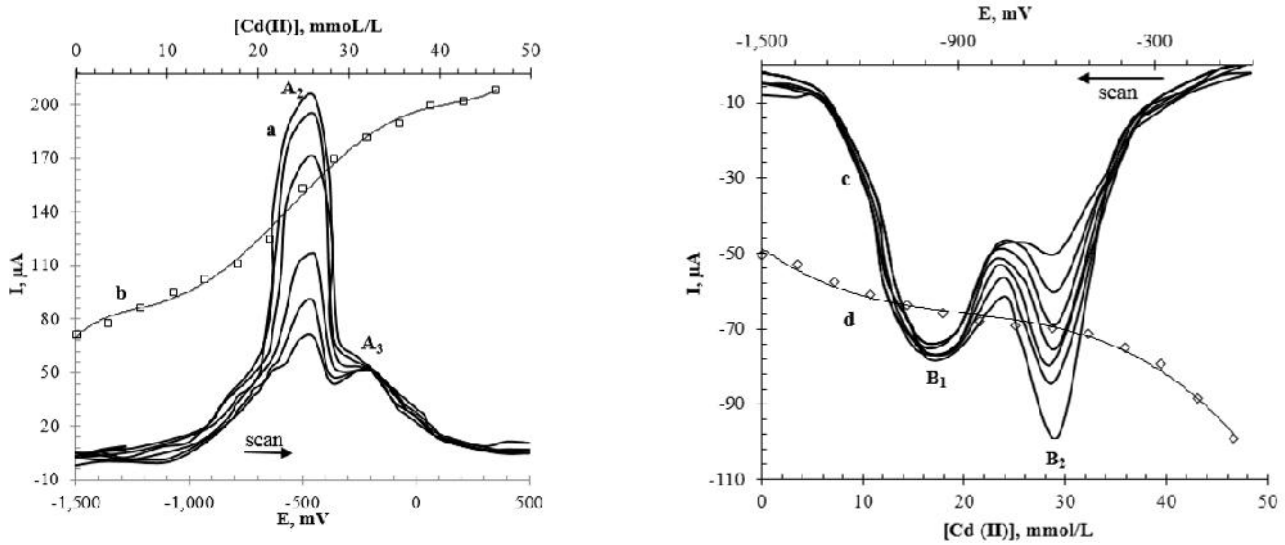


**Fig. 2: (a & c) Typical DPVgs of Cd(II) measured at HMDE vs. Ag/AgCl/3 mol.L$^{-1}$ KCl; in inset: dependences of Cd(II) "(a) oxidation (c) reduction" peak height on Cd(II) concentration; (b & d) Calibration plot of Cd(II) "(b) oxidation ($A_2$ peak) at -480 mV (d) reduction ($B_2$ peak) at -600 mV" electrochemical peaks vs. Cd(II) concentration.**

While, no considering noticeable changes appeared when CVgs was scanned for solutions prepared by mixing a fixed concentration 0.10 mmol.L$^{-1}$ of Cys-CdSe NPs and successive define different concentrations of Se(II). Its concluded that, Se(II) was not electroactive component in the applied experimental conditions. From the previous experiments, the irreversible oxidation electrochemical peak $A_1$ and board irreversible reduction electrochemical peak $B_1$ were not change with changing the concentration of Cd(II), Se(II) or Cys. The broad reductive peak $B_1$ attributed predominantly to slow kinetics of electron transfer between the electrode / Cys-CdSe NPs. It was followed by products produced from irreversible electrochemical decomposition reaction of Cys-CdSe NPs to their initial composed elements, which was in their turn entered in the electrochemical reactions and the Cd(II) and Cys peaks ($B_2$, $A_2$ and $A_3$) were observed.

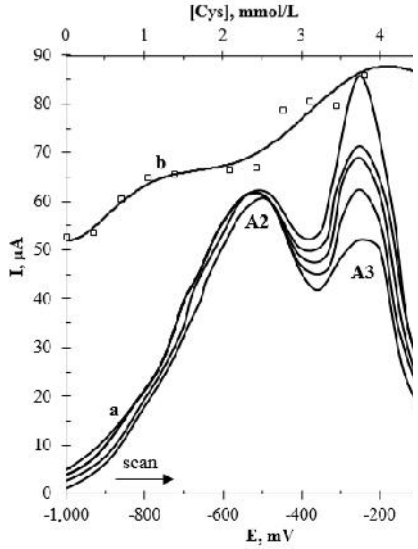


**Fig. 3: (a) Typical DPVgs of Cys measured at HMDE vs. Ag/AgCl/3 mol.L$^{-1}$ KCl; in inset: dependences of Cys peak height on its concentration; (b) Calibration plot of Cys oxidation electrochemical peak vs. its concentration.**

Quantification study of Cys-CdSe NPs was performed on the peak $A_1$. The results show, as seen in Fig. 4, that the electrochemical peaks increased linearly at 1366 mV vs. the concentration of Cys-CdSe NPs in the two ranges. The first range was between 15-50 µmol.L$^{-1}$ ($I = 1.5061\ C + 13.8851$, $R^2 = 0.9930$), and the second range was between 50-150 µmol.L$^{-1}$ ($I = 0.1987\ C + 80.8380$, $R^2 = 0.9915$). The reason of breaking of the first linear line at 50 µmol.L$^{-1}$ may due to saturation of

the compact layer known as the "inner Helmholtz" layer on electrode surface [54].

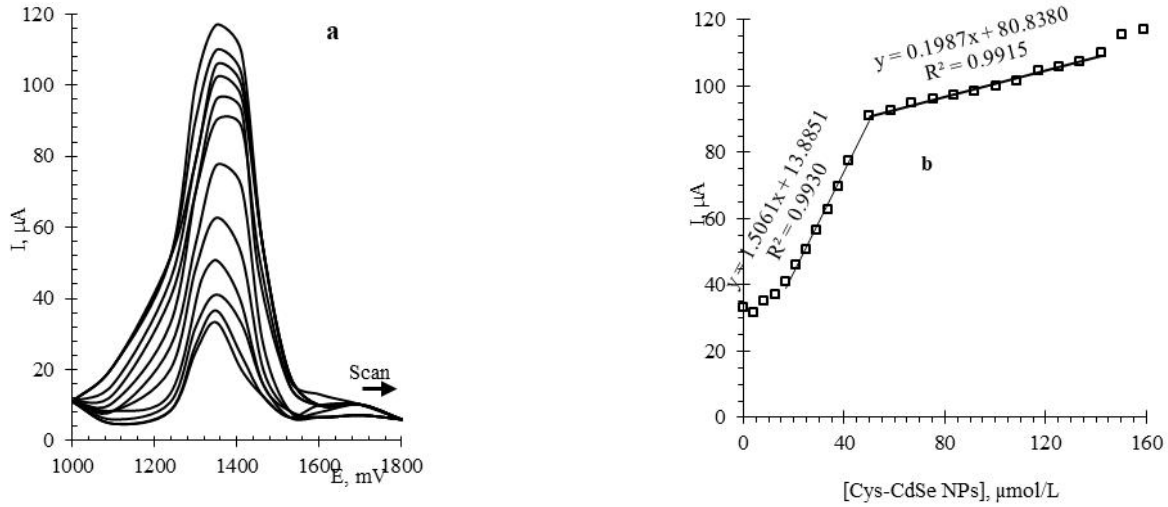


**Fig. 4: (a) Typical DPVgs of Cys-CdSe NPs measured at HMDE vs. Ag/AgCl/3 mol.L$^{-1}$ KCl; in inset: dependences of peak height on Cys-CdSe NPs concentration; (b) Calibration plot of oxidation electrochemical peak vs. Cys-CdSe NPs concentration at 1366 mV.**

### 2.2. Stability of Cys-CdSe NPs:

The electrochemical studying of Cys-CdSe NPs behavior gave a tool for detected peaks. On the other hand, it could be show the possibility to monitor their stability. Therefore, it should be focusing on studying the time of stability of the used Cys-CdSe NPs. The decaying of Cys-CdSe NPs can be characterized by the detection of the increasing of the electrochemical peaks of the decay products i.e., Cys and Cd(II). Primarily, temperature stability was investigated within the range from 30 to 90 °C. The Cys-CdSe NPs were heat treated for one hour in the dark and then electrochemically analyzed. The effects of temperature on peaks of Cys and Cd(II) was shown in Fig. 6A. It clearly follows, from the results obtained, that both peaks increased with the increasing temperature, which was associated with the decaying of the Cys-CdSe NPs.

Based on these promising results, it should be following with time stability tests. Cys-CdSe NPs were electrochemically analyzed for three weeks. The height of Cys and Cd(II) peaks were shown in Fig. 6B. It can be concluded that the prepared Cys-CdSe NPs were stable for two weeks without any changes, after which, some degradation occurred.

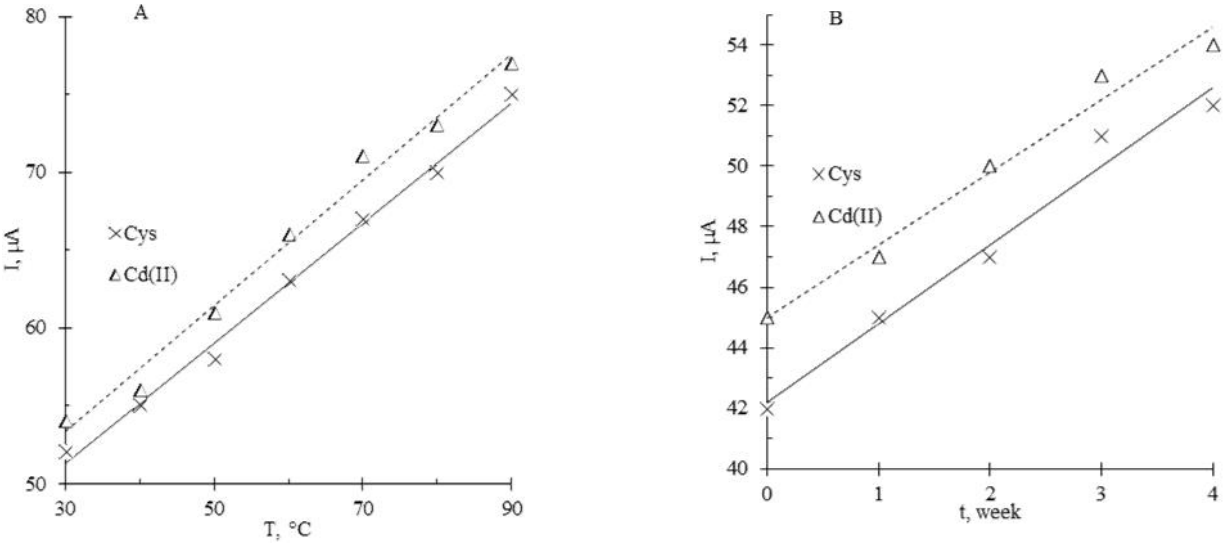


**Fig. 6: Stability of Cys-CdSe NPs, dependences of the height of Cd(II) and Cys oxidation electrochemical peaks (A) on temperature after one hour of heat balance, and (B) on time of store.**

**3.1 Electrochemical behavior of Ova at UTG in in the absence and presence of Cys-CdSe NPs:**

Typical CVgs of Cys-CdSe NPs at UTG, showed few peaks similar to the peaks appear with HMDE but in this test the peaks were vey small, board and have no distinctive shape, so it difficult to determine, but still could use as sensitive electrochemical probe for protein such as Ova. The CVgs of Ova at UTG in the absence and presence of Cys-CdSe NPs was showed in Fig. 7.

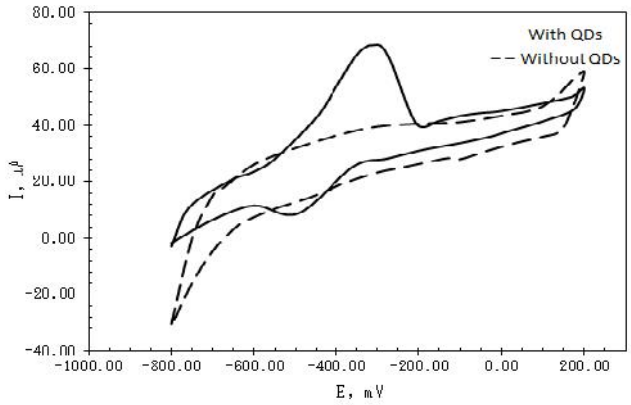


**Fig. 7: CVg of 10 µg.mL$^{-1}$ Ova at UTG in the absence (striped line - - ) and presence (solid line–) of 0.03 mmol.L$^{-1}$ Cys-CdSe NPs. All exper iments carried out at pH 6.8 PBS, 25 °C and 100 mV.s$^{-1}$ scan rates**.

CV of 10 µg.mL$^{-1}$ Ova in the absence of Cys-CdSe NPs showed no oxidation and reduction peaks appeared, when 0.03 mmol.L$^{-1}$ Cys-CdSe NPs was added into the Ova solution, one oxidation and one reduction appearance at -326 mV and -598 mV, successively. The two peaks were sensitive to changes of Ova concentration; all the CVs measured at the same potential scan range and applied the same optimum conditions. The CVgs observations due to the surface interactions with analytes may induce changes in the surface dielectric, resulting in changes in nanoparticle capacitance and voltammetric peak potential spacing. This approach may allow sample detection in the solution phase and in situ detection of proteins [55]. DPV technique was selected for the determination of Ova due to its better accuracy and sensitivity [56]. The higher peak in the CVg was the oxidation one and was chosen in the studies.

**3.2 Method optimization:**

The method optimization was performed by dependence on the oxidation peaks in DPV technique. The study contained determining of the influence of pH, concentration Cys-CdSe NPs and temperature.

3.2.1. Influence of pH:

The effect of pH on the electrochemical response of Cys-CdSe NPs in presence of Ova over the pH range 3.50 to 8.00 were examined, using DPV at a fix scan rate of 100 mV.s$^{-1}$. For this purpose many buffer solution was tested as Britton-Robinson universal buffers for pH 3.50-8.00, sodium citrate /citric acid for pH 3.5-4.2, $CH_3COONa/CH_3COOH$ for pH 4.2-5.5, $NaH_2PO_4/H_3PO_4$ for pH 5.5-7.2, and ammonium tartrate/ tartaric acid for pH 7.20-8.0. The results were shown in Fig. 8.

The electrochemical peak enhanced remarkably with the increase of pH from 3.50 to 6.20, and then remained almost constant. After pH 7.40, the electrochemical peak begins to decrease, so the optimum range of pH was from 6.20 to 7.40, while the electrochemical response decreases out of this range of pH. The reason may be explained as follows: in acid medium, the electrochemical response decreases as a possible result of the deconstruction of the Cd(II)-Cys complexes annulus due to the protonation of the surface-binding thiolates [57]; while when pH increases above 7.4, the electrochemical response decreases may be due to the precipitation product changing into Cd(OH)Y, where Y the anion dominates at the solution. At the pH 6.80 which was the middle of the optimum range of pH adjusted by PBS was chosen for the further studies.

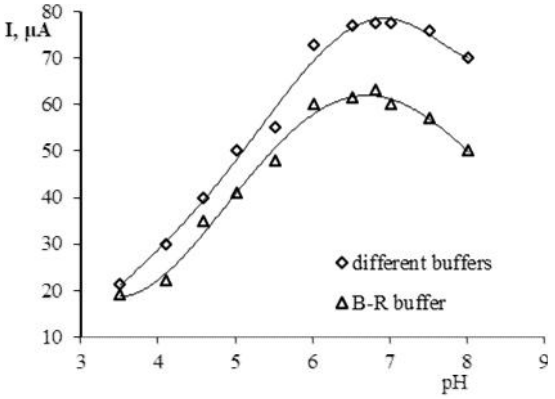


**Fig. 8: Effect of pH on the electrochemical peak**

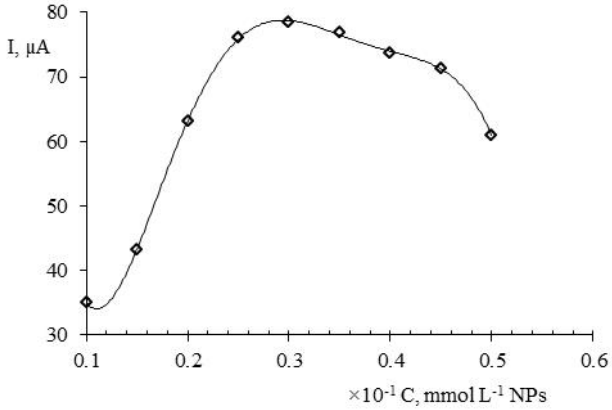


**Fig. 9: Effect of NPs concentration on the electrochemical peak**

3.2.2. Influence of Cys-CdSe NPs concentration:

It was found that the concentration of Cys-CdSe NPs greatly affected the sensitivity of assay as shown in Fig. 9. The low concentration below 0.025 mmol.$L^{-1}$ and high concentration of aqueous Cys-CdSe NPs over 0.04 mmol.$L^{-1}$ decreased the sensitivity significantly, however, between the concentrations of 0.025-0.040 mmol.$L^{-1}$ a little influence on the electrochemical response, so the concentration of 0.03 mmol.$L^{-1}$ Cys-CdSe NPs was chosen for the further studies.

3.2.3. Influence of temperature:

The effect of the electrochemical solution cell temperature on the electrochemical response of Cys-CdSe NPs in presence of Ova was tested at 10, 15, 20, 25, 30, 35 and 40 °C, respectively. The results were shown in Fig. 10. However, there was little influence on the electrochemical response at the study range of temperature with slight increase of the electrochemical peak with increase the temperature from 10 to 20 °C, then remained almost constant to begin little decrease with increase of temperature over 30 °C, so the normal temperature at 25 °C was chosen for the further studies.

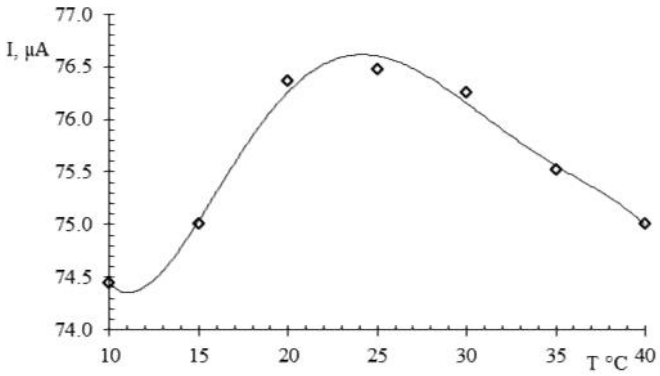


**Fig. 10: Effect of temperature on the electrochemical peak**

3.2.4. Influence of coexisting substances:

The influences of interference substances, as shown at the Table 1, such as metal ions, amino acids and carbohydrates, commonly found in food or blood samples were examined within the concentration range from 0.1-fold until 100- fold, a relative error of ± 5 % on the concentration of Ova was considered tolerable.

**Table 1: Effect of interference substance on the determination of 10 mg.L$^{-1}$ Ova**

| Coexisting substance | Tolerable concentration[a] mg.L$^{-1}$ | Tolerable limit [species]/[Ova] | Coexisting substance | Tolerable concentration[a] mg.L$^{-1}$ | Tolerable limit [species]/[Ova] |
|---|---|---|---|---|---|
| Tryptophan | $1.1\times10^{+2}$ | 11 | $Ca^{2+}$ | $4.8\times10^{+2}$ | 48 |
| Cysteine | $0.3\times10^{+2}$ | 3 | $Fe^{3+}$ | $0.5\times10^{+2}$ | 5 |
| Urea | $6.2\times10^{+2}$ | 62 | $Zn^{2+}$ | $4.0\times10^{+2}$ | 40 |
| Gluocose | $10.4\times10^{+2}$ | 104 | | | |
| Citric acid | $2.5\times10^{+2}$ | 25 | | | |

a Define as ±5% relative error.

## 4. Method validation:

The calibration graph was described by the equation: I = bC + m, (where I: electrochemical peak, m: intercept, b: slope and C: concentration in µg.mL$^{-1}$), obtained by the method of least squares using Excel program. The electrochemical peak increased linearly with the concentration of standard Ova in the range of 0.1-25.0 µg.mL$^{-1}$ ($R^2$ = 0.998), as shown at the Fig. 11.

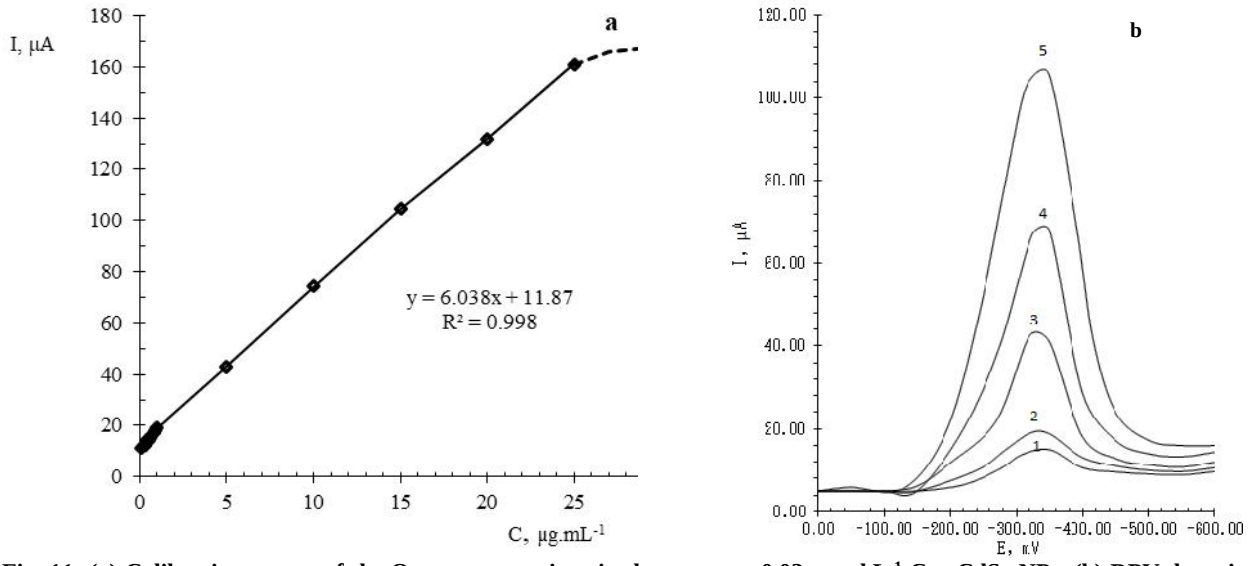


**Fig. 11: (a) Calibration curves of the Ova concentrations in the presence 0.03 mmol.L$^{-1}$ Cys-CdSe NPs, (b) DPV detection of the different concentrations of the Ova in in the presence 0.03 mmol.L$^{-1}$ Cys-CdSe NPs, pH 6.8 PBS, amplitude 0.5 mV, pulse width 0.05 s, sample width 0.05 s, pulse period 0.2 s, quiet time 2 s**.

The LOD (= 0.06 µg.mL$^{-1}$) and LOQ (= 0.20 µg.mL$^{-1}$) were calculated [58]. The precision and accuracy were assessed according to the IUPAC recommendations [59] by analyzing 0.1, 0.5, 1, 5, 10, 15, 20, and 25 µg.mL$^{-1}$ Ova in aqueous solutions, as shown at the Table 2.

The relative accuracy R% was within the range of 97.00-100.02%, with RSD from 0.84% to 3.10%, indicating a good accuracy with high precision of the method. The reproducibility of the method also known as the inter precision was evaluated by performing replicate analyses solution over a period of five days. The inter-day RSD values were less than or equal to 3.50% reflecting the usefulness of the method in routine analysis. Method robustness was tested by making small incremental changes in Cys-CdSe NPs concentration, pH or temperature values were checked at three different concentrations of Ova. The intermediate precision, expressed as RSD percent. The measure of robustness was within the acceptable limits as shown at the

Table 3.

**Table 2 Evaluation of precision and accuracy for Ova determination [a]**

| Taken | Intra-day accuracy and precision (n=6) | | | | | Inter-day accuracy and precision (n=5) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Found ±SD | R | RSD | Smb | CLb | Found ±SD | R% | RSD% | Sm | CLc |
| µg.mL$^{-1}$ | µg.mL$^{-1}$ | % | % | µg.mL$^{-1}$ | µg.mL$^{-1}$ | µg.mL$^{-1}$ | % | % | µg.mL$^{-1}$ | µg.mL$^{-1}$ |
| 00.1 | 00.097 ± 0.003 | 097.00 | 3.09 | 0.001 | 00.097 ± 0.003 | 00.096 ± 0.003 | 96.00 | 3.500 | 0.002 | 00.096 ± 0.004 |
| 00.5 | 00.495 ± 0.012 | 099.00 | 2.50 | 0.005 | 00.495 ± 0.013 | 00.492 ± 0.012 | 98.40 | 2.415 | 0.005 | 00.492 ± 0.015 |
| 01.0 | 01.000 ± 0.020 | 100.00 | 2.01 | 0.008 | 01.000 ± 0.021 | 00.985 ± 0.021 | 98.40 | 2.102 | 0.009 | 00.985 ± 0.026 |
| 05.0 | 05.001 ± 0.090 | 100.02 | 1.80 | 0.037 | 05.001 ± 0.094 | 04.980 ± 0.075 | 99.60 | 1.500 | 0.033 | 04.980 ± 0.093 |
| 10.0 | 09.984 ± 0.152 | 099.84 | 1.52 | 0.062 | 09.984 ± 0.159 | 10.010 ± 0.141 | 100.10 | 1.410 | 0.063 | 10.010 ± 0.175 |
| 15.0 | 14.868 ± 0.159 | 099.12 | 1.07 | 0.065 | 14.868 ± 0.167 | 14.824 ± 0.182 | 98.83 | 1.227 | 0.081 | 14.824 ± 0.226 |
| 20.0 | 19.614 ± 0.186 | 098.07 | 0.95 | 0.076 | 19.614 ± 0.195 | 19.512 ± 0.224 | 97.56 | 1.150 | 0.100 | 19.512 ± 0.278 |
| 25.0 | 24.502 ± 0.205 | 098.01 | 0.84 | 0.084 | 24.502± 0.215 | 24.324 ± 0.271 | 97.30 | 1.114 | 0.121 | 24.324 ± 0.337 |

a The experiments were performed using the optimum condition of 0.03 mmol.L-1, pH 6.8, T 25 °C.

b The value of t for calculating the 9 5% confidence limits was 2.57 for 5 degrees of freedom.

c The value of t for calculating the 95 % confidence limits was 2.78 for 4 degrees of freedom.

**Table 3 Robustness expressed as intermediate precision**

| taken | Parameter altered, RSD% (n=3) | | |
|---|---|---|---|
| | pH (6.2, 6.8, 7.2) | T (20, 25, 30) °C | C NPs (0.25, 0.30, 0.35) mmol.L$^{-1}$ |
| µg.mL$^{-1}$ | | | |
| 0.500 | 4.2 | 2.6 | 0.300 |
| 10.000 | 3.7 | 1.8 | 0.210 |
| 20.000 | 2.5 | 1.2 | 0.140 |

**5.1 Method Applications:**

Study of thermal denaturation of hen egg white protein: The hen egg-white has 9.7-12 % protein, Ova the main protein constitutes 54-61% of it, that has four free sulphydrilic (S-H) groups from cysteine acid amine and a disulphide group (S-S). During storage or terminal treatment, Ova was altered to S-Ova. S-Ova has a slightly lighter molecular weight than Ova and its relative quantity in the egg white can increase during the storage period, from 5% in fresh eggs to 81 % after six months of refrigerated storage. Both pH and temperature also affect the S-Ova formation. The S-Ova formation involves change in sulphydrilic group, and an amino group, or even the hydroxyl ion and it has a less solubility. The conversion of Ova to the S-Ova results from differences in the structure of covalent bonding. As result, thermal treatments applied to the egg white cause undesirable modifications of their physicochemical and functional properties [60]. That change in the free functional groups in the protein effects on the results of the suggested method and causes decrease of electrochemical peak with decrease the Ova concentration during changing it to S-Ova. The goal of this study was to acquire some knowledge on the effect of heat on the change of the concentration of Ova.

The study was done as following: 30 fresh eggs from each source were cleaned and processed immediately or stored at 4 °C upon collection. After washing and cleaning of eggs bought in trade, they were carefully broken and the white and yolk separated, taking the precaution of removing chalazae. The pH of the albumen was measured immediately using a pH meter it was equal to 9.45 ± 0.02. The volume and weight of eggs white were measured, and 1.0 g of egg white was taken and dilution the buffer solution Tris-HCl (20 mM pH 8.2) to 1L in volumetric flask, a gentle stirring for 10 min was done on the samples, followed by filtration. Then control the mixture volume in 1L volumetric flask, identical volumes were transferred into tubes. After heat treatment in a water bath thermo-stated under continuous agitation at temperatures of 56 °C to 89 °C with a step of 3 °C for 3 min, 10 min and 20 min, the tubes were immediately immersed in an ice bath for 1 minute to stop the denaturation process [61], sedimentation the mixture from the turbidity at 3000 cyc/min for 10 min, take 3 mL from the solute to measure immediately used suggested method (section) and reference spectrophotometric method[42] to compared the results.

The reference method depend on destroys the protein which the protein was mixed with the copper and acid solution used in the biuret reaction and then reacting with the Folin-Ciocalteau reagent (a reduced phosphomolybdate-phosphotungstate solution). The Fig. 12 shows the recovery values of remained soluble protein opposite of the temperature with different periods of heating.

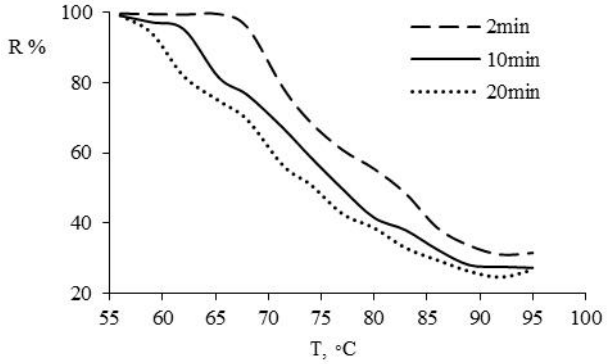


**Fig. 12: effect of temperature on protein solubility at different periods of heating**

The curved shows after heat during 3 min at 89 °C, only 33.62% soluble proteins remained, while after 10 min and 20 min at 89 °C 28.04% and 26.24%, respectively remained soluble. The results show a good agreement between the results obtained by the proposed method and the reference method, which the recoveries produced with the reference method were 32.99, 29.11, and 26.50 % after heat at 89 °C during 3, 10 and 20 min respectively.

## CONCLUSION

The quantification of ovalbumin protein is an example of using our suggested method based on nanomaterial and electrochemical determination has good results. The expanded researches can determined depended on our suggested method for more applications

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