

Biocentri-voltammetric biosensor for acetylcholine and choline

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Abstract We report on the determination of choline and acetylcholine via biocentrivoltammetry. This method combines centrifugation and voltammetry and is based on a carbon paste electrode modified with acetylcholinesterase and choline oxidase. The electrode was placed at the bottom of a biocentrivoltammetric cell. Acetylcholine and choline are accumulated on the enzyme electrode via centrifugative forces, upon which a direct voltammetric scan is applied. Reaction time, pH values, quantities of enzyme and centrifugation parameters were optimized. A linear response is obtained in the 0.07 to 10 μM concentration range of acetylcholine, and a limit of detection as low as 0.5 μM . The linear range is between 0.1 and 500 μM for choline. The method was applied to the determination of acetylcholine and choline in spiked serum samples.

Keywords Biocentri-voltammetry · Biosensor · Acetylcholine · Choline

Introduction

Centri-voltammetry enables to preconcentrate analyte on the electrode surface with proper carrier via centrifugation. Then it provides direct analyte detection without needing

any dissolution or filtration processes. It allows a practical way for voltammetric analysis in application of coprecipitation. As a result, preventing of analyte loss provides the sensitive analysis compared with other preconcentration techniques. Centri-voltammetry is firstly used by us to detect trace amounts of Pb (II) ions. Pb (II) ions were accumulated by means of $\text{Al}(\text{OH})_4$ and XAD-7 resin carrier materials on the Pt planar electrode. The Pt planar working electrode was placed at the bottom of the cell. LOD value of lead (II) ions was calculated as $5.2 \times 10^{-9} \text{M}$ with XAD-7 resin while with $\text{Al}(\text{OH})_4$ this value was calculated as $2.2 \times 10^{-9} \text{M}$ [1, 2]. In another application, mercury (II) ions were preconcentrated on gold film electrode by applying centri-voltammetry [3].

Centri-voltammetry and biosensing system were first used together by our group. We recommended the name “biocentri-voltammetry”. The first biocentri-voltammetric application includes the xanthine detection where xanthine oxidase was immobilized onto planar Pt working electrode. Xanthine accumulation onto this electrode was achieved via centrifugation force without using any carrier precipitate [4]. In another application, acetylcholinesterase (AChE) activity was determined by using biocentri-voltammetry [5]. For this purpose, again carbon paste electrode (CPE) was used as the working electrode. After the optimization of experimental parameters, the developed method was applied for Donepezil-based drug analysis by following inhibition signal of the developed system [5].

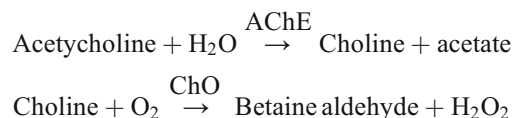
Carbon based electrodes are widely utilized in electrochemical analysis because of wide anodic potential window, short response time, low background current and ease in the modification. They were utilized as transducers in many sensor/biosensor systems like glucose, xanthine, ethanol, aspartame and catechol [6–18]. Especially the composite carbon based electrodes were preferred since they can be easily modified and renew [9–18].

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Acetylcholine (ACh) is the one of the most important neurotransmitters which was firstly discovered in central nervous system. ACh and its metabolite choline (Ch) play vital roles in brain chemistry. They affect learning, memory and attention. An abnormal amount of ACh causes some of neuropsychiatric disorder such as Alzheimer and Parkinson diseases, progressive dementia and schizophrenia. Therefore sensitive and accurate detection of ACh is very important in clinical applications [19–22]. Determination of ACh and Ch is based on the following enzymatic reactions as shown below:



We describe here the first usage of biocentri-voltammetry for ACh and Ch determination. ACh and Ch molecules were accumulated on the composite electrode via centrifugation force. Accumulated analytes were then detected by applying direct voltammetric scan. For this purpose, a specially designed cell compatible to both voltammetry and centrifuge was constructed. This cell contains AChE/ChO modified CPE as a working electrode at the bottom (Fig. 1b, c and d). After optimization of workings conditions, developed method was tested for ACh detection in artificial serum sample.

Experimental

Apparatus

Voltammetric studies were carried out with the AUTOLAB PGSTAT 12 electrochemical measurement system from ECO CHEMIE Instruments B.V. (The Netherlands) (www.ecochemie.nl) driven by GPES software. Sigma3–

16PK centrifuge was used for centrifugation (www.sigma-zentrifugen.de). The cell, made from a delrin tube, was constructed to be compatible to the centrifugation system (Fig. 1A and B). Studies were made with CPE. CPE was placed at the bottom of the cell where reference (Ag/AgCl) and counter (Pt rod) electrodes were immersed in the same cell.

Reagents

AChE from *Electrophorus electricus* (518 Units (U)/mg solid), ChO from *Alcaligenes* (14 Units (U)mg⁻¹ solid), acetylcholine chloride, choline chloride and CaCl₂ were obtained from Sigma (www.sigmaaldrich.com). Graphite powder, glucose, MgCl₂ and Tris–HCl were purchased from Merck and mineral oil was obtained from Aldrich. NaCl was obtained from Pancreac, while KCl was purchased from Riedel-de Haen. Urea was obtained from Horasan Chemistry. Phosphate buffer system was (50 mM pH 7.0) served as supporting electrolyte. All solutions were prepared by dilution of proper amount of the reagents with phosphate buffer.

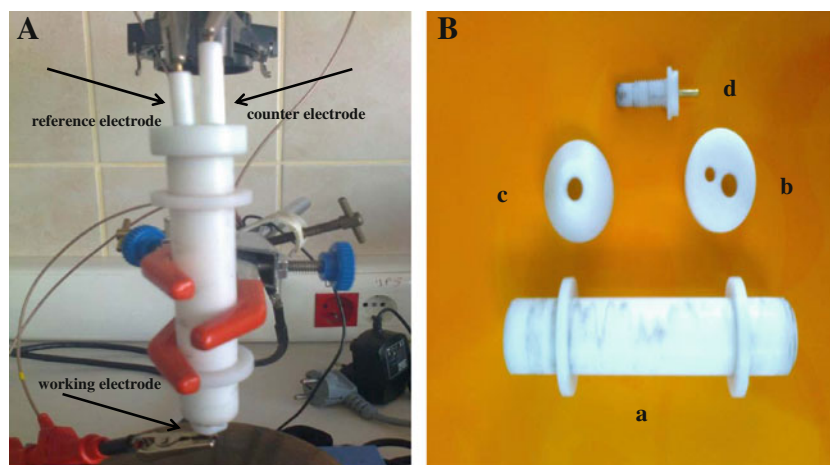
Electrode preparation

AChE/ChO-modified CPE was prepared by hand-mixing of proper amount of graphite powder with mineral oil, AChE and ChO. A portion of the resulting paste was then packed firmly into the electrode cavity. Electrical contact was established via a copper wire. The paste surface was smoothed on a weighing paper and rinsed carefully with double distilled water.

Procedure

Desired amount of ACh or Ch was put into biocentri-voltammetric cell for 5 mins. After the completion of enzymatic reaction, the cell was centrifuged for 4 min at 4000 rpm. Then the cell was carefully placed in the voltammetric stand and reference and counter electrodes

Fig. 1 **A** Biocentri-voltammetric system, **B** Parts of biocentri-voltammetric cell: **a** body, **b** cover for reference and counter electrode, **c** protective for working electrode and **d** carbon paste working electrode



were immersed into the solution. D.P voltammograms were recorded in the range 0 to -0.9 V. The decrease of the reduction current of molecular oxygen through the subsequent enzymatic reaction was monitored for detection of ACh and Ch.

Sample application

Artificial serum solution was prepared by using 140 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl_2 , 0.8 mM MgCl_2 , 2.5 mM urea and 4.7 mM glucose in 10 mM Tris-HCl. The final pH of the solution was adjusted to 7.3 with the addition of concentrated HCl [23]. Differential pulse voltammetric measurements were performed after transferring the corresponding solution to the biocentri-voltammetric cell. The potential range was between 0 and -0.9 V versus Ag/AgCl.

Result and discussions

Effect of reaction time

In order to increase substrate and enzyme interaction, effect of reaction time was investigated. 10 mL of 50 mM phosphate buffer solution including 0.25 mM ACh was put into biocentri-voltammetric cell. Then this solution was allowed to be reacted at room temperature for 0.5, 1, 2, 5, 8 and 10 min. After that, the cell was centrifuged for 5 min. at 3000 rpm. Results were expressed in terms of % biosensor response. The current that was obtained at the optimal working conditions was assumed as 100 % and other measured values were calculated relative to this value [24]. As shown in Fig. 2a, response current of enzyme electrode increases from 0.1 to 5 min. and then a decrease is obtained. Therefore 5 min. was selected as optimum reaction time and used for further studies. From the results it is obvious that the reaction completes at 5 min. and after this period, there is a slight activity loss. At this point, it could be stated that the enzyme could lose the catalytic activity for longer reaction times. The formation of un-reactive species could be the reasons for that. Thiocoline might form a dimer or enzyme can react with cysteine residues and as a result these species can be formed [5]. On the other hand, 5 min. is a very short measuring period demonstrating the practicality of the developed method.

pH influence

The pH of the buffer solution effects the biosensor response because of altering protonation state of enzyme. Therefore, influence of pH on the biosensor response was investigated using phosphate buffer solution in a range from pH 5.5 to

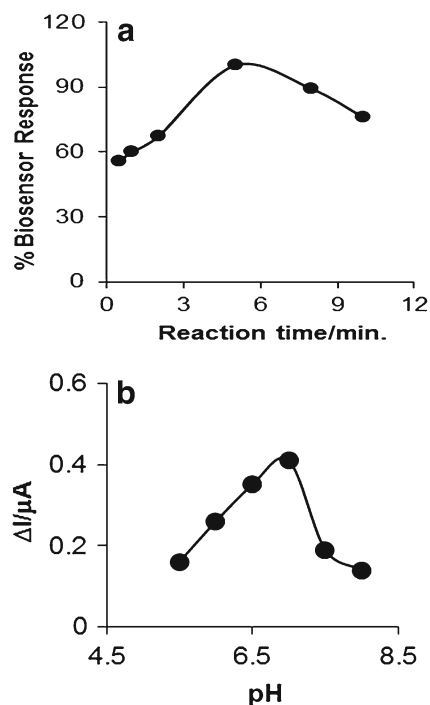


Fig. 2 a The effect of reaction time (0.5, 1, 2, 5, 8 and 10 min.) on the biocentri-voltammetric sensor signal for 0.25 mM ACh, 50 mM phosphate buffer solution (pH 7.0) as supporting electrolyte; V_{cent} ; 5000 rpm, 3 min, 4.2 U ChO, 155 U AChE. b The effect of pH (between 5.5 and 8) on the current values for 0.25 mM ACh. All other conditions as in A

8.0. From Fig. 2b, it is clearly be seen that peak values increase from pH 5.5 to 7.0 and then a sharp decrease is obtained which might be due to the enzyme denaturization. As a result pH 7.0 was chosen as optimum pH for further studies. This value is in accordance with previous study, concerning the detection of ACh [19].

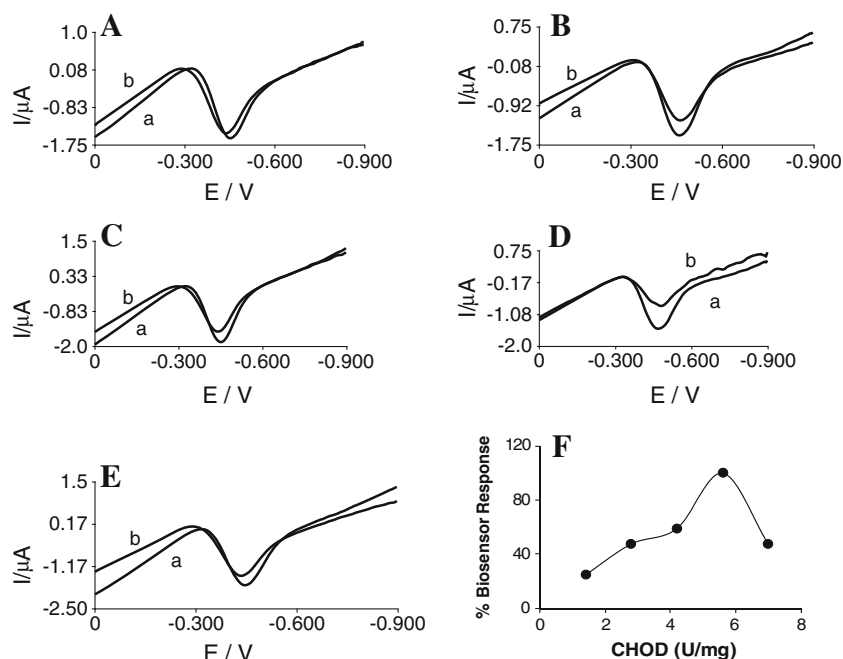
Effect of enzyme amount

Amount of enzymes in the bienzymatic sensor were investigated for examining the effect towards ACh and Ch response. ChO amount was firstly tested (1.4 U to 7.0 U) for 0.25 mM Ch (Fig. 3). As clearly can be seen from Fig. 3F, response current of the enzyme electrode to Ch increases from 1.4 U to 5.6 U, and then a sharp decrease is obtained at 7.0 U. As a result 5.6 U was used as the optimum enzyme amount for this bienzymatic sensor.

For ACh biosensor, AChE amount effect was searched between 51.8 and 259 U and ChO concentration was keep constant at 5.6 U (Fig. 4). As you can see from Fig. 4F, the highest current value was obtained at 207 U. So this AChE amount was used for further experiments.

The decreases after certain enzyme amount for both enzymes might be attributed to the electrode passivation due to the presence of higher protein amount that might

Fig. 3 The effect of ChO amount on D.P voltammograms obtained for **a** without, **b** with 0.25 mM Ch with **A** 1.4 U, **B** 2.8 U, **C** 4.2 U, **D** 5.6 U, **E** 7.0 U ChO. 50 mM phosphate buffer solution (pH 7.0) as supporting electrolyte; V_{cent} : 5000 rpm, 3 min, 155 U AChE. **F** ChO amount effect on biosensor response



also cause diffusion problems [5]. As mentioned above, further experiments were conducted by using these optimum amounts.

Effect of centrifugation parameters

Centrifugation force is a critical key at centri-voltammetric/biocentri-voltammetric systems [1, 2, 4, 5]. Because in these techniques, the analyte was accumulated on the working

electrode with centrifugation force. Hence centrifugation parameters such as centrifugation time and speed are important and needed to be optimized.

Effect of centrifugation time was examined between 0 and 6 min. with an increment of 1 min. for 0.25 mM ACh. Results were presented at Fig. 5. Response current of electrode to ACh, increases from 0 min. to 4 min (Fig. 5). Then a decrease is obtained. The decrease at higher centrifugation time longer than 4 min. is attributed to dispersion of

Fig. 4 The influence of AChE amount on D.P voltammograms obtained for **a** without, **b** with 0.25 mM ACh with **A** 51.8 U, **B** 103.6 U, **C** 155 U, **D** 207.2 U, **E** 259 U AChE. 50 mM phosphate buffer solution (pH 7.0) as supporting electrolyte; V_{cent} : 5000 rpm, 3 min, 5.6 U ChO. **F** AChE amount influence on biosensor response

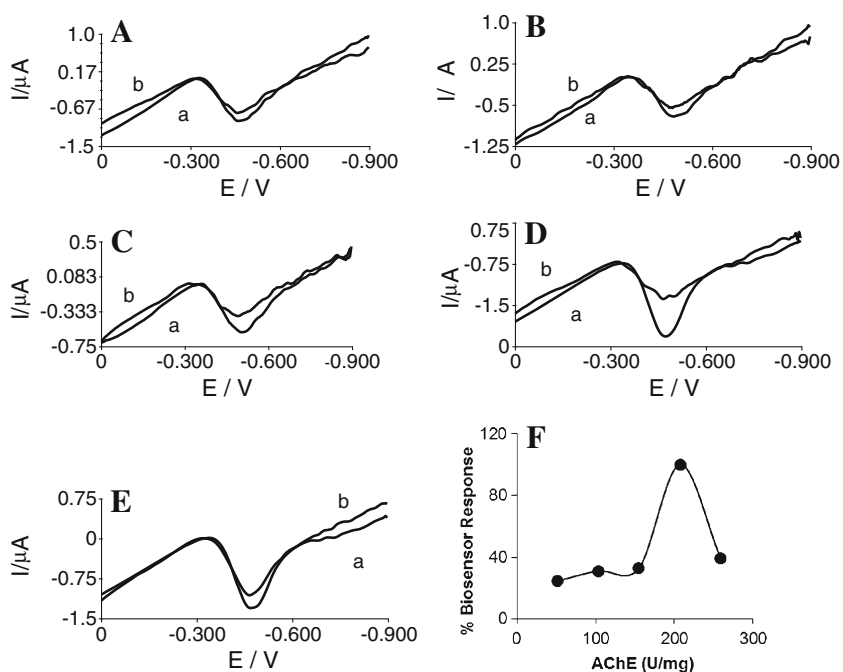
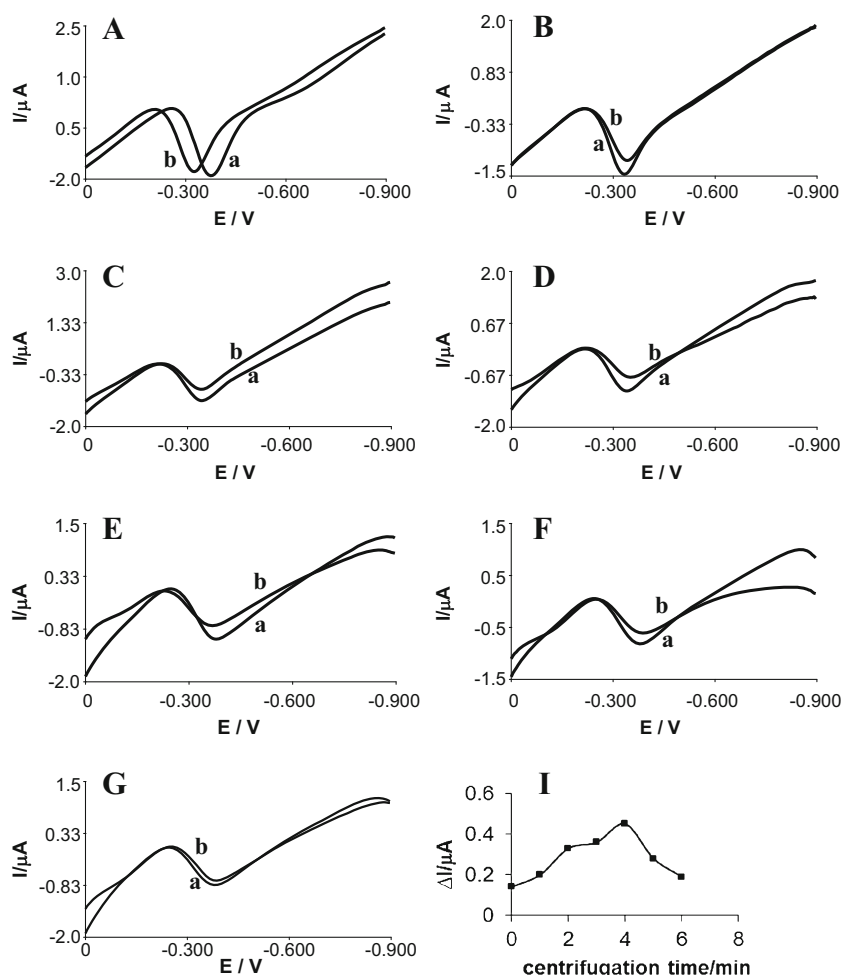


Fig. 5 The effect of centrifugation time on voltammetric signals for **a** without, **b** with 0.25 mM ACh with **A** 0, **B** 1, **C** 2, **D** 3, **E** 4, **F** 5 and **G** 6 min. **I** The curve demonstrating this effect 50 mM phosphate buffer solution (pH 7.0) as supporting electrolyte; Vcent; 3000 rpm, 5.6 U ChO, 207.2 U AChE



accumulated analyte on the electrode surface [4, 5]. So further experiments were conducted by utilizing 4 min. as the optimum centrifugation time.

Centrifugation speed is another centrifugation parameter that is needed to be investigated in biocentri-voltammetry. For this purpose, 0, 1000, 2000, 3000, 4000, 5000 and 6000 rpm were applied to system. As you can see at Fig. 6, best current value was obtained at 4000 rpm. This

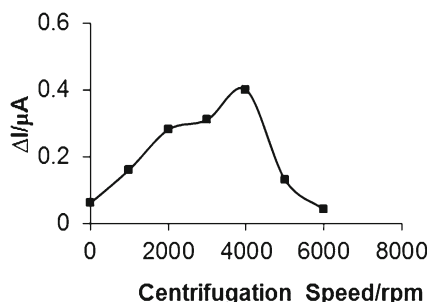


Fig. 6 The influence of different centrifugation speed on the current values (0, 1000, 2000, 3000, 4000, 5000, 6000 rpm), centrifugation time 4 min, All other conditions as in Fig. 5

increment can be attributed to the increase in convection process that causes more analyte accumulation on the electrode surface. On the other hand, higher centrifugation speeds more than 4000 rpm might cause removal of the substrate from the accumulation layer [5]. As a result, 4000 rpm was used for further experiments as centrifugation speed parameter.

Analytical characteristics and sample application

Under the above established experimental results, linear range for ACh (Fig. 7a) was obtained between 0.5 and 10 μM ($R^2=0.9943$) with RSD value of 4.56 ($n=5$). LOD, based on $s/n=3$, was calculated as $5.1 \times 10^{-7}\text{M}$. For comparison, a linear graph was formed for ACh without applying any centrifugation (Fig. 7b). As a result, a linear range between 2 and 25 μM with $R^2=0.7035$ were obtained. As it is clearly be seen biocentri-voltammetry possess wider linear range with better R^2 value. The comparison of biocentri-voltammetric methods with other electrochemical biosensor was also done in terms of linear range and LOD (Table 1). As can be seen from the Table, biocentri-voltammetry offers

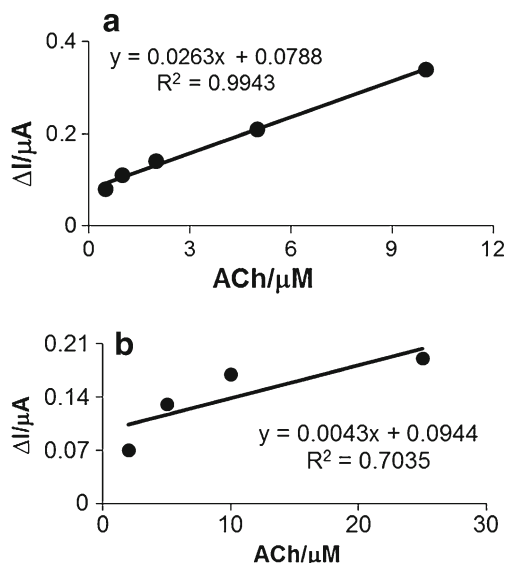


Fig. 7 **a** Calibration graph of ACh with centrifugation (between 0.5 and 10 μM). **b** Calibration graph of ACh without centrifugation (between 2 and 25 μM). Working conditions are same as in Fig. 6

comparable linear range with better sensitivity compared to even nanoparticle based systems. Though some other systems like reference [19] in Table 1 offers wider linear range for ACh detection, when enzyme amounts were considered, biocentri-voltammetry could be accepted as more advantageous technique.

The bienzymatic biosensor was also used for Ch detection. Figure 8 shows the calibration curves of the biocentri-voltammetric biosensor for Ch. A linear range was obtained between 1×10^{-5} and 5.0×10^{-4} M. Wider linear range with better LOD value were obtained for ACh detection. This difference is attributed AChE and ChO enzyme amount in the biosensor. Since 207 U AChE activity is much higher than that of 5.6 U ChO activity, it is expected to get better results for ACh [19].

On the other hand, developed biocentri-voltammetric system was applied for ACh and Ch detection in artificial serum sample. Known amounts of ACh and Ch were added

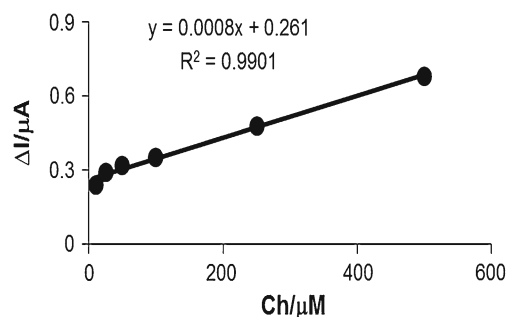


Fig. 8 Calibration curve of Ch in the range of 10 μM to 500 μM . Working conditions are same as in Fig. 6

to the biocentri-voltammetric cell from stock solution that was prepared by artificial serum solution as described in experimental part. Recovery values were calculated as 104.7 ± 0.15 ($n=3$) for ACh and 100.9 ± 0.14 ($n=3$) for Ch.

Conclusion

In the present study, biocentri-voltammetry was used for the first time to detect ACh and Ch. ACh and Ch molecules were deposited via centrifugation force onto the AChE/ChO modified CPE. Concerning analytical characteristic values, it can be concluded that biocentri-voltammetry can be utilized for detection of these two analytes. Moreover when compared with even nanomaterial included systems, this technique provides accessible linear range and higher sensitivity (Table 1). Developed system was also applied for detection of ACh and Ch in artificial serum sample. From the promising recovery values given above section, it is clear that developed system can be applied to complex natured samples and not showed any matrix effect. In our previous studies, biocentri-voltammetry was applied for xanthine detection [4] and also acetylcholinesterase activity detection. [5] These studies demonstrate the practicability of developed method. On going works concerning application of the technique for different analytes continue in our lab.

Table 1 The comparison of linear range and LOD values

| Electrode | Linear Range (M) | LOD (M) | Enzyme Amount | Reference |
|---------------------------------------|---|-----------------------|---|--------------|
| AChE/CHOD/pnAN/Pt | 1.0×10^{-6} – 1.5×10^{-3} | 5.0×10^{-7} | 2148 U ml^{-1} AChE, 262 U ml^{-1} ChO | [19] |
| AChE/CHOD/PEG/Pt | 5.0×10^{-6} – 1.0×10^{-4} | 2.0×10^{-7} | 5 U g^{-1} AChE, 1.5 U g^{-1} ChO | [25] |
| AChE-F127M/CHOD-F127M/Pt | 8.0×10^{-6} – 8.0×10^{-4} | not reported | not reported | [26] |
| MWCNT/AuNP/CHOD (PDDA-AChE)/Pt | 5.0×10^{-6} – 4.0×10^{-4} | 1.0×10^{-6} | 0.2 g/l AChE 5 g/l ChO | [27] |
| AChE/CHOD/CPE (Biocentri-voltammetry) | 5.0×10^{-7} – 1.0×10^{-5} | 0.51×10^{-7} | 207 U AChE 5.6 U ChO | Present work |

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