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β-Cyclodextrin-Based Potentiometric Sensors for Flow-Injection Determination of Acetylcholines

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β-Cyclodextrins (β-CDs) based polyvinylchloride (PVC) and carbon paste (CPEs) electrodes were fabricated and applied for potentiometric determination of different acetylcholine (ACh) drevatives, namely; butrylcholine (BCh), acetylmethylcholine (AmCh) and acetylthiocholine (AtCh). Matrix composition optimization was done referring the effect of type and content of α-, β-, or γ-CDs, anionic sites and plasticizer. Electrodes incorporated with heptakis (2, 3, 6-tri-o-methyl)-β-CD as sensing ionophore, potassium tetrakis (4-florophenyl) borate (KTFPB) as anionic site and *o*-nitrophenyloctyl ether (*o*-NPOE) as electrode plasticizer showed the best electroanalytical performances. The fabricated electrodes worked satisfactorily in the concentration range from 10^{-6} to 10^{-2} mol L^{-1} with detection limit reaching 8.3×10^{-7} mol L^{-1} and fast response time of 2s. The developed sensors possessed improved selectivity, twords various acetylcholine derivatives rather choline. The sensors have been successfully applied for the potentiometric determination of ACh and its derivatives under flow injection analysis (FIA) and potentiometric titration conditions.

Keywords: β-Cyclodextrins (β-CDs); Acetylcholines ; PVC electrode; Carbon paste electrode (CPEs); Flow injection analysis

1. INTRODUCTION

Acetylcholine (ACh) is the first identified neuromodulator, it is one of many neurotransmitters in the autonomic nervous system and the only one in the somatic nervous system of vertebrates and insects. It is hydrolyzed by acetylcholinesterase enzyme into the inactive metabolites choline (Ch) and acetate as the accumulation of Ach, due to AChE inhibition, causes continuous stimulation central nervous system, intense spasms and death. On the other hand, ACh deficiency due to cholineacetyl-transferase enzyme inhibition causes disturbance in the transmission of nerve impulses, paralysis (symptoms analogous to Alzheimer's disease) and death [1]. Choline serves both as the precursor and metabolite of ACh, it is a natural amine classified as a water soluble essential nutrient and usually grouped within vitamin B complex [2]. Butrylcholine (BCh), acetylmethylcholine (AmCh), and acetylthiocholine (AtCh) are synthetic ACh derivatives (ACh mimetic molecules) and do not occur in the body naturally.

The development of methods for rapid and sensitive determination of neurotransmitters and their precursors and/or metabolites had been a challenging goal for many years since they provide valuable information for disposition dynamics rather than the static levels of neurotransmitters. Spectrophotometric [3, 4] and chromatographic [5] techniques have been suggested for various AChs determination. However, the aforementioned methods possess several disadvantages including high cost, long analysis time and specialized personnel with laboratory facilities. On the other hand, the electroanalytical techniques provide simple instrumentation and short analysis time. Uni-, bi-, and tri-enzyme/mediators biosensors including chemiluminometric [6], amperometric [7-9], conductometric [10] and voltammetric [11] methods have been applied for AChs determination. Ion selective electrodes (ISEs) appeared to have a considerable analytical interest as a reliable alternative to the classical methods for the rapid and simple quantification of AChs. Potentiometric electrodes based on dipicrylamine [12], K-tetraflorophenylborate [13, 14] or macrocycle carriers [15] have been developed and applied for potentiometric ACh determination.

The beauty of electrochemical techniques is to utilize a chemically modified electrode (CME) tailor fabricated by incorporation of a suitable modifier for sensitive and selective analytical applications. Cyclodextrins (CDs) are naturally occurring macrocyclic oligosaccharides formed of 1,4-glucosidic bond linked D(+) glucopyranose oligomers of 6, 7, and 8 glucose units yielding α-, β-, and γ-CD, respectively. The chiral recognition (host-guest interaction) leads to encapsulation of the guest molecule into the CDs toroidal cavity without the formation of chemical bonds or changing their structure [16, 17]. Such unique properties introduced β-CD for chiral separation of drugs based on chromatographic capillary zone electrophoretic and mass spectroscopic methods [18, 19] as well as a sensing material in potentiometric sensors for many pharmaceutically important drugs [20-23].

In the present work, simple potentiometric PVC and CPE electrodes applying β-CDs as a sensing material, have been characterized and optimized for rapid, accurate and low cost quantification of ACh derivatives.

2. EXPERIMENTAL PART

2.1. Reagents

All reagents were of the analytical grade and bidistilled water was used throughout the experiments. Acetylcholine bromide (ACh, Acros Organics) and its derivatives; choline chloride (Ch, Fluka) butrylcholine iodide (BCh, Fluka), acetylthiocholine iodide (AtCh, Fluka) and acetylmethylcholine chloride (AmCh, Aldrich) were used without further purification.

Different CD derivatives tested in the present study were: α-CD (Bio Basic Inc.), γ-CD (Bio Basic Inc.), β-CD (**I**, Sigma), heptakis (2,6-di-o-methyl)-β-CD (**II**, Aldrich), heptakis (2,3,6-tri-o-methyl)-β-CD (**III**, Aldrich) and 2-hydroxypropyl-β-CD (**IV**, Aldrich). Different lipophilic anionic additives namely; sodium tetraphenylborate (NaTPB, Fluka), sodium tetrakis (4-fluorophenyl)borate (NaTFPB, Fluka), potassium tetrakis (4-chlorophenyl)borate (KTClPB, Fluka), phosphotungstic acid (PTA, Sigma), phosphomolybdic acid (PMA, Sigma), sodium dodecyl sulfate (SDS, Fluka) and reineckate ammonium salt (RAS, Sigma) were used.

o-Nitrophenyloctylether (*o*-NPOE, Sigma), dibutylphthalate (DBP, Sigma), dioctylphthalate (DOP, BDH), dioctylsebacate (DOS, Avocado), 2-fluorophenyl 2-nitrophenyl ether (FPNPE, Fluka) and tricresylphosphate (TCP, Fluka), paraffin oil (Aldrich) or Silicon oil (Aldrich) were used as electrode plasticizers. Polyvinylchloride (PVC, relative high molecular weight) and graphite powder (synthetic 1-2 μm) were purchased from Aldrich.

2.2. Apparatus

All the potential measurements were carried out using a 692-pH meter (Metrohm) with Ag/AgCl double-junction reference electrode (Metrohm 6.0726.10) and combined pH glass electrode (Metrohm 6.0202.100). A single line flow injection system composed of a four channel peristaltic pump (MCP Ismatec, Switzerland), sample injection valve (ECOM, Ventil C, Czech Republic) with injection sample loop (200 μL) and a homemade Perspex wall-jet cell, was used [24]. The response time of the electrode was measured using 46-Range Digital Multimeter with PC interface.

2.3. Procedure

2.3.1. Sensor construction

For PVC electrodes, a matrix cocktail composed of 2.5 mg β-CD (**III**), 2 mg KTFPB, 240 mg *o*-NPOE, 240 mg PVC and 6 mL tetrahydrofuran was prepared and poured in a Petri dish (5cm diameter). After slow solvent evaporation, a master PVC sheet was cut and mounted to plastic electrode body filled with an internal reference solution of the corresponding ACh derivative (10^{-3} mol L^{-1}) and 10^{-2} mol L^{-1} KCl using Ag/AgCl as internal reference electrode. The fabricated electrodes were conditioned for 2h in 10^{-3} mol L^{-1} of the target AChs before use.

Carbon pastes electrodes were prepared by hand mixing of 2 mg β-CD (**III**), 5 mg KTFPB, 250 mg carbon powder and 100 μL of *o*-NPOE. The result paste was packed into a piston driven Teflon electrode holder [25] and conditioned in 10^{-3} mol L^{-1} ACh derivative solution for 2h.

2.3.2. Sensors calibration

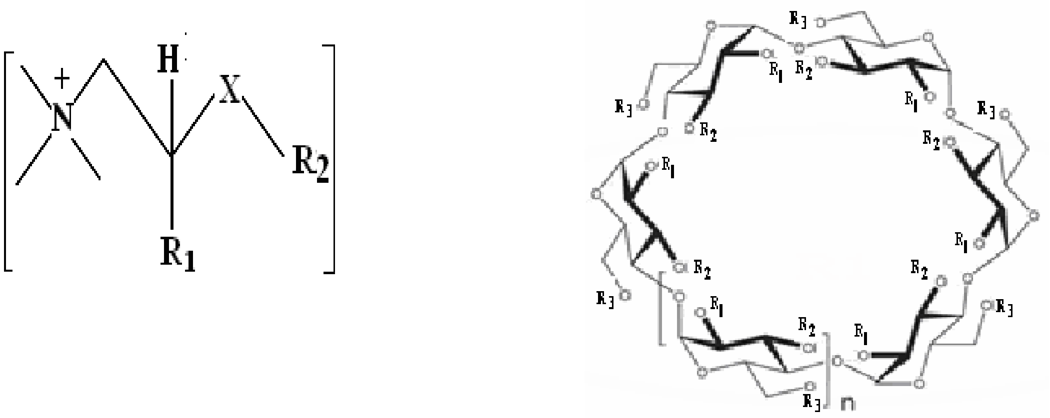
The fabricated sensors were calibrated by transferring 25 mL aliquots of different ACh derivatives (10^{-7}-10^{-2} mol L^{-1}) into a 50 mL double jacket thermostated glass cell at 25°C followed by immersing the developed sensor in conjugation with reference electrode in the solution. The potential readings were recorded after stabilization and plotted against AChs concentration (log [ACh]).

3. RESULTS AND DISCUSSION

3.1. Chiral essence of CDs

The most important property of CDs is their ability to form supramolecular (inclusion) complexes with many appropriately sized organic ions and molecules in aqueous, non-aqueous and mixed media. The driving forces for the complexation are non-covalent, including van der Waals forces and directed hydrogen bonding. Water molecules in CD cavity are displaced by more hydrophobic guest molecules present in the solution to attain a non-polar/non-polar association and decrease of CD ring strain resulting in a more stable lower energy state. Mechanisms of chiral recognition by CDs have been elucidated and thoroughly reviewed, they could be mainly explained by “three points binding” and “lock and key” rules [16, 26, 27].

In the present work, PVC and CPE potentiometric sensors incorporated with different CDs in combination with lipophilic anionic sites were fabricated and applied for determination of various ACh derivatives (Scheme 1).



Ch: X= O, R_1= H, R_2= H
ACh: X= O, R_1= H, R_2= $COCH_3$
AmCh: X= O, R_1= CH_3, R_2= $COCH_3$
BCh:X= O, R_1= H, R_2= $COCH_2CH_2CH_3$
AtCh: X= S, R_1= H, R_2= $COCH_3$

Ionophore I: R_1, R_2, R_3= H
Ionophore II: R_1, R_3= CH_3, R_2= H
Ionophore III: R_1, R_2, R_3= CH_3
Ionophore IV: R_1, R_3= H, R_2= $CH_2CHOHCH_3$

Scheme 1. Structural formula for the tested ACh and CD derivatives.

3.2. Optimal electrode matrices compositions

Due to the critical role of the electrode matrix composition on its performance, parallel studies were carried out on PVC and CPEs. The influence of the nature and amount of ionophore, ionic additives and plasticizer, were tested to elect the optimal electrode possessing the best sensitivity and selectivity towards ACh derivatives.

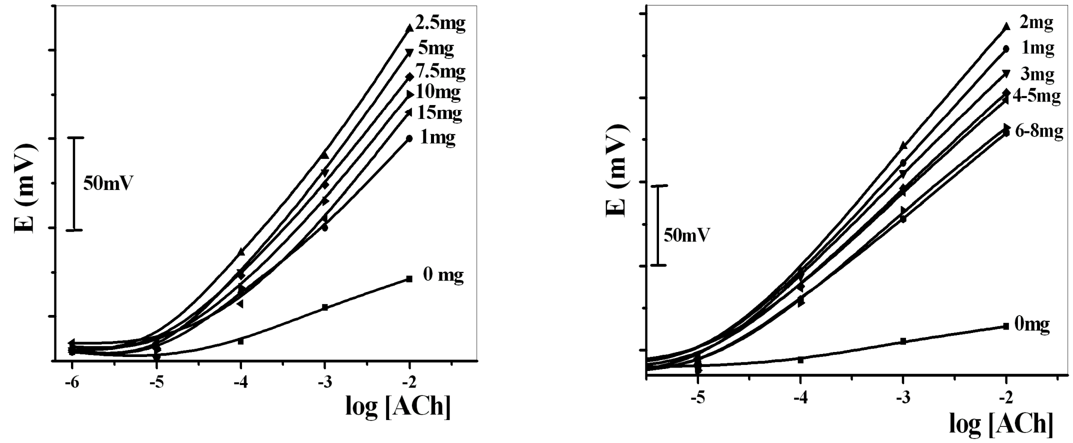
3.2.1. Effect of the ionophore type

In preliminary experiments, α, γ-CDs and four derivatives of β-CDs were incorporated in the electrode matrices and the response of the fabricated electrodes towards ACh derivatives was tested. Electrodes modified with both α- and γ-CDs showed non-significant response towards ACh derivatives which may be related to the incompatible cavity size for inclusion complex formation.

Sensors incorporating β-CD derivatives (**I, II, III**, and **IV**) showed calibration graphs with cationic slope values towards ACh depending upon the type of the ionophore, demonstrating the crucial rule of the ionophore on the electrode response. The election of the sensing ionophore is not restricted to higher response but extends to the stability of the fabricated electrodes. Sensors modified with ionophores (**I, II** or **III**) showed more stable Nernstian response during an operation period of 3 weeks (50.7±2.1, 52.7±1.4, and 55.0±1.1 mV/decade, respectively). The slope values of senors modified with ionophore **IV** decreased significantly after 5 days of operation (45.9±3.0 mV/decade). The potentiometric response of the fabricated sensors prepared using ionophores **III** was the best among the tested ionophores with slope values of 54.4±1.6 and 51.3±3.6 mV/decade for PVC and CPE, respectively.

3.2.2. Effect of the ionophore content

On constructing an ISE, the amount of the sensing in the electrode matrix should be sufficient to obtain reasonable complexation at the electrode surface that is responsible for the electrode potential. If such ionophore is present in excess, over-saturation occurs in the network hindering the complexation process and leading to unsatisfactory measurements. Thus, the influence of the β-CD (ionophore **III**) concentration in the PVC and CPEs matrices was investigated by varying the ionophore content from 0 to 25 mg. It was found that incorporation of 2.5 and 2.0 mg of the aforementioned ionophore was sufficient to the proper performance of both PVC (Fig. 1a) and CPE electrode (56.1±1.0 and 55.0±0.9 mV/decade, respectively).



(a)

(b)

Figure 1. Effect of (a) β-CD (III) and (b) anionic sites (KTFPB) content on PVC electrode performance.

3.2.3. Effect of anionic sites

Lipophilic ionic additives promote the interfacial ion exchange kinetics and decrease the electrode resistance through enhancing the ionic mobility in the electrode matrix. The response of ISEs containing ionic sites can be distinguished whether the incorporated ionophore acts as an electrically charged or uncharged carrier. ISEs based on a neutral carrier function only if the ionic sites with opposite charge to that of the target analyte are added to the electrode matrix [28- 31].

Results revealed that, all electrodes based on ionophores (**III**), without ionic additives, showed non Nernstian response towards ACh (slope values ranged from 15 to 18 mV/decade) Since the ionophore behaved as a neutral carrier, the incorporation of such ionic sites in the electrode matrix was necessary in order to obtain proper performances. Tetraphenylborate derivatives such as NaTPB, KTClPB and NaTFPB are usually the anionic sites of choice in ionophore based sensors. It was found that the incorporation of ionic sites into various electrodes matrices improved their sensitivity values depending upon the ionic site lipophilicity according to the order KTFPB ≥ NaTClPB > NaTPB > PTA > PMA > RAS > SDS (Fig. 2).

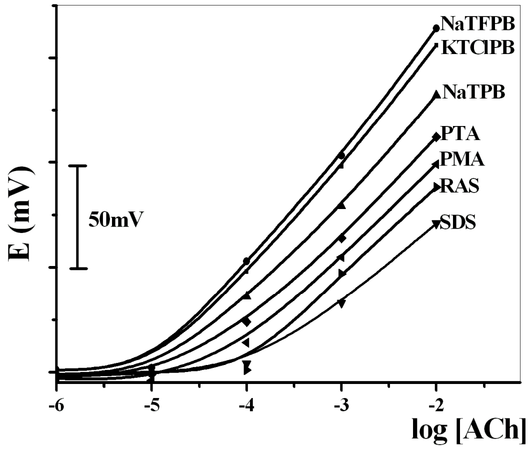


Figure 2. Effect of anionic sites type on PVC electrode.

The influence of the amount of KTFPB in the electrodes matrices on their responses was also studied by addition of 0-8 mg to the PVC and CPE electrode matrices. Addition of 3 mg of KTFPB in the PVC matrix containing the ionophore **III** exhibited the highest sensitivity to ACh (55.9±0.9 mV/decade) (Fig. 1b), while the corresponding content for CPEs was 5 mg (53.2±1.5 mV/decade). A noticeable decrease in the electrode sensitivity was observed when the amount of KTClPB exceeds the optimal content, which may be due to competitive interactions between the ionophore and the anionic site to combine with the cationic analyte.

3.2.4. Effect of the plasticizer

The sensitivity and selectivity of ionophore based ISEs is greatly influenced by the polarity of the electrode mediator, which is defined by the plasticizer dielectric constant (ε). It should be noted that the nature of the plasticizer affects not only the polarity of the electrode phase but also the mobility of ionophore molecules and the state of the formed complex [32-34]. The plasticizers effect on ACh electrode performance was investigated by using six plasticizers having different dielectric constant, namely; FPNPE, o-NPOE, TCP, DOS, DBP or DOP (ε = 50, 24, 17.6, 5.2, 4.7 and 3.8, respectively) as well as non-polar binders; paraffin oil and silicon oil for CPEs.

For CPEs, application of less polar plasticizers reduced the Nernstian slope, which may be related to lower solvation of the ionophore and the formed complex in the electrode plasticizer (50.8±0.9, 47.3±1.5, 44.3±2.1, 44.8±1.1, 17.5±2.9 and 25.5±2.2 mV/decade for TCP, DOP, DBS, DOS, paraffin oil and Silicon oil plasticized CPEs). High sensitivity was observed for electrodes containing aromatic plasticizers; *o*-NPOE and FPNPE (56.3±1.2 and 57.4±0.8 mV/decade) where the presence of aromatic rings in the plasticizer structure can enhance the solubility of the ionophore in the electrode matrix [34]. For PVC electrodes, the corresponding slope values were 55.6±1.9, 53.1±1.7, 51.2±1.2, 50.2±2.5, 48.3±1.5 and 46.3±1.3 mV/decade for *o*-NPOE, FPNPE, TCP, DOP, DBS and DOS, respectively.

The choice of the plasticizer should consider not only its polarity (value of dielectric constant) but also the possibility of hydrogen bonding or Lewis acid–base interactions with ionophore molecules. Such interactions can be favorable (enhance solvation of ionophore) which improves the ionophore solubility, or unfavorable (blocking of complexing site of ionophore) which decreases the ability of ion recognition by the ionophore [34]. The application of the most polar plasticizer FPNPE did not significantly improve the electrodes response in comparable to *o*-NPOE plasticized electrodes.

3.3. Electrodes performances

Besides application of the developed electrodes to potentiometric determination of ACh, the response of such electrodes towards different ACh derivatives (with different side chain substitutions, scheme 1) was also investigated. The proposed electrodes showed potentiometric response characteristics depending on the nature of the electrode and the derivative tested. Generally, PVC electrodes showed better performances than CPEs, regarding the linear range and detection limit (Table 1). The developed electrodes were more sensitive towards ACh derivatives than the native choline itself and this sensitivity increased by the aliphatic substitution of the side chain as well as replacing of the oxygen with sulfur which may be attributed to the more favorable host-guest interactions due to presence of such side chain.

The prepared sensors displayed Nernstian cationic responses depending on the type of the electrode. For PVC electrodes, the slope values were 55.6±1.9, 52.3±2.2, 54.2±1.5, 55.2±1.1 and 42.5±2.5 mV/decade for ACh, AtCh, AmCh, BCh, and Ch, respectively. The corresponding slope values for CPEs were 54.3±1.3, 56.8±1.0, 62.5±2.2, 55.3±1.4 and 35.3±1.1 mV/decade for the aforementioned derivatives in the same order.

Table 1. Performance characteristics of PVC and CPEs modified with β-CD (III) towards various ACh derivatives

Electrode Performance	PVC					CPE				
	ACh	AtCh	BCh	AmCh	Ch	ACh	AtCh	BCh	AmCh	Ch
Concentration range (mol L^{-1})	10^{-5}-10^{-2}	10^{-6}-10^{-2}	10^{-6}-10^{-2}	10^{-6}-10^{-2}	10^{-4}-10^{-2}	10^{-5}-10^{-2}	10^{-5}-10^{-2}	10^{-5}-10^{-2}	10^{-5}-10^{-2}	10^{-4}-10^{-2}
Slope (mV $decade^{-1}$)	55.6±1.9	52.7±1.3	55.2±1.3	54.2±2.0	42.5±1.2	54.3±0.5	56.8±0.3	62.5±1.5	55.3±2.7	35.3±1.1
r	0.9988	0.9991	0.9992	0.9990	0.9993	0.9999	0.9999	0.9995	0.9988	0.9955
LOD (mol L^{-1})	2.7×10^{-6}	8.3×10^{-7}			10^{-4}	10^{-5}				10^{-4}
Response time (s)	4					2				
Lifetime (day)	21					25				
pH range	3-10					4-9				

On a parallel study, the performances of electrodes incorporated with AChs-TPB ion pairs were also tested. Such electrode showed linear Nernstian response (60.0±2.5 mV/decade) in linear range 10^{-4}-10^{-2} mol L^{-1} which confirmed the superiority of ionophore modified sensors.

Day to day calibration was performed to evaluate the useful lifetime throughout a period of 14-21 days, PVC electrodes showed Nernstian slopes which did not change significantly (within ±2.0 mV/decade). This short lifetime may be related to the leaching out of the sensing material in both the internal filling and external bathing solutions. CPEs showed a relatively longer working lifetime of 30 days on storing them in bidistilled water when not used. This improvement of the electrode lifetime may be related to electrode nature without internal filling solution.

For analytical applications of a novel sensor, the fast electrode response with stable potential measurement is of critical importance especially when monitoring kinetic or enzymatic reactions or incorporating in FIA systems. CPEs showed fast response of 2s while PVC electrodes showed a longer dynamic response time of 4s.

The influence of the pH on the response of different ACh sensors was investigated in the pH range from 2 to 12 (Fig. 3). The fabricated PVC electrodes showed stable Nernstian responses in the working pH range 3-10, while CPE showed stable potential values in the pH range 4-9.

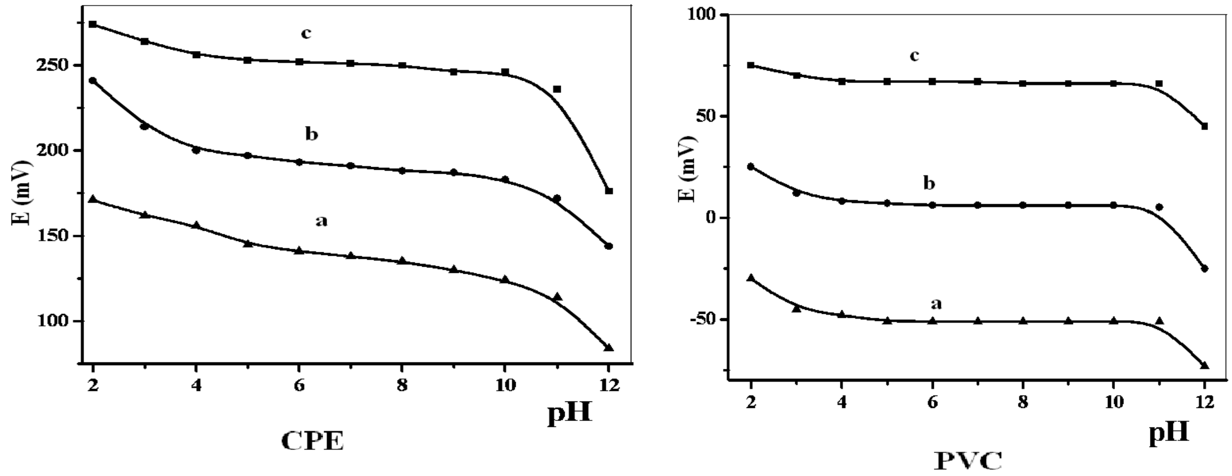


Figure 3. Effect of the pH on the potential readings of both PVC and CPE electrodes (a) 1 $\times10^{-4}$, (b) 1 $\times10^{-3}$, and (c) 1 $\times10^{-2}$ mol L^{-1} ACh.

3.4. Selectivity coefficient measurement

Interference evaluation was performed using Matched Potential Method (MPM) in order to assess the effect of interferents in the optimized analytical procedure. The selectivity coefficient is defined as the activity (concentration) ratio of the primary ion and the interfering ion which gives the same potential change in a reference solution [35, 36]. An improvement in selectivity for most of the cations studied was observed (Table 2), which may be related to the buffered activity of the free main cation (ACh) in the electrode due to the host-guest complex equilibrium, excluding other ions of the same charge. The presence of β-CD as ionophore besides anionic site increased the target extraction into the organic mediator phase (plasticizer).

Table 2. Selectivity coefficients of ACh sensors.

Interferent	$-\log K_{A,B}$				
	PVC	CPE		PVC	CPE
K^+	3.89	3.63	Glycine	2.45	2.69
Li^+	3.76	3.55	Citrate	2.53	2.62
NH_4^+	3.80	3.98	Maltose	2.41	2.68
Choline	2.50	2.39	Starch	2.53	2.79
Citrate	2.53	2.62	Sucrose	2.01	2.64
Caffeine	2.30	2.82			

3.5. Analytical application

FIA becomes a wide spread methodology characterized by versatility, ease of automation and high sampling frequency. It is viewed as a well efficient mean of improving the performance characteristics of ISEs [37, 38]. As FIA techniques are based on non-equilibrium measurements, they require high sensitivity, short response time, and operational stability of the applied electrodes. The developed electrodes were incorporated successfully in flow injection systems previously optimized [24] where 200 µL of freshly prepared Ach solutions covering the range 10^{-6}-10^{-2} mol L^{-1} were injected into the flowing stream (flow rate of 45 mL min^{-1}) and the corresponding peak heights were recorded and used to draw the calibration graphs (Fig. 5).

The calibration graph was linear in the concentration range from 10^{-5} to 10^{-2} mol L^{-1} with Nernstian slopes of 57±2.2 mV/decade and sampling throughput of 120 sample h^{-1}. The peak heights were highly reproducible as the average peak height was 145.0±3.2 mV for 12 replicates injection of 200 200 µL 10^{-3}mol L^{-1} ACh solution.

In addition, the proposed electrodes were successfully used as an indicator electrode in the potentiometric titration of ACh derivatives against NaTPB, where the titration curves were of sigmoid shape showing 1:1 stoichiometric ratios.

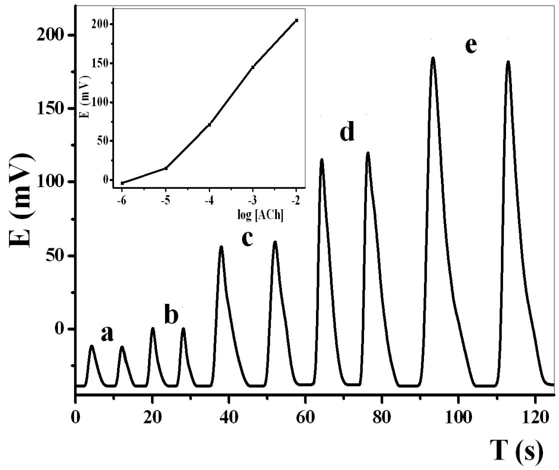


Figure 4. Flow injection potentiometric determination of ACh using CPE: (a) 1 ×10^{-6}, (b) 1 ×10^{-5}, (c) 1 ×10^{-4} (d) 1 ×10^{-3}, and (e) 1 ×10^{-2} mol L^{-1} ACh.

4. CONCLUSIONS

The present work has demonstrated the fabrication of PVC and CPEs electrodes for ACh derivatives quantification using heptakis (2,3,6-tri-o-methyl)-β-CD as ionophore, KTFPB as anionic site and *o*-NPOE as electrode plasticizer. The fabricated potentiometric electrodes showed fast dynamic response time (2s) and long operational lifetime (30 days) with LOD reaching 8.3x10^{-7} mol L^{-1}. The developed electrodes were successfully applied for the potentiometric determination of ACh and its mimetic molecules using FIA and potentiometric titration techniques with high accuracy and precision. The present study enabled the choice of β-CD derivative possessing the highest affinity towards inclusion complex formation with its most favorable ACh derivative and with minimal interference from Ch. Moreover, the current work form the basis for further studies on applying the developed sensors for potentiometric assay of different acetylcholine esterase (AChE) activity by selecting the most suitable enzyme substrate as well as pesticides monitoring.

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