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Acetylcholinesterase Based Colorimetric Dipsticks for Military Performance: Principles and Construction

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Abstract:

Colorimetric dipsticks based on recognition capability of acetylcholinesterase enzyme (AChE) are a suitable tool for a fast and sensitive detection of nerve agents and some pesticides. In this review, the detectors are digestedly presented and basic biochemical methods appropriate for the colorimetric estimation of AChE enzymatic activity are included as well. Moreover, the available matrices suitable for construction of dipsticks and chemical, as well as physical protocols for AChE immobilization on detectors surface are introduced.

Keywords:

Colorimetry, nerve agents, acetylcholinesterase, dipstick, detector

1. Introduction

Acetylcholinesterase (AChE EC 3.1.1.7.) and butyrylcholinesterase (BChE EC 3.1.1.8) enzymes are suitable biorecognition elements for assay of organophosphorus neurotoxic compounds. Nerve agents used as chemical warfare and some pesticides can be mentioned as examples. In addition, some natural toxins such as aflatoxins can be also measured using the mentioned cholinesterases [1]. The AChE enzyme is participating in cholinergic neurotransmission in both peripheral and central nervous system. It is engaged in hydrolysis of neurotransmitter acetylcholine into choline and acetic acid in neurosynaptic cleft or neuromuscular junction. The hydrolysis causes termination of cholinergic receptors stimulation and it is an irretrievable physiological mechanism [2]. Quite extensive amount of AChE molecules can be found on blood cells' surface where it participates in neuroendocrinologic regulation. Compared to AChE, BChE role in human body is not well understood. Some detoxification

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mechanisms, such as protection from cocaine, are proposed for BChE [3]. In the past, BChE was also called plasmatic cholinesterase by some investigators. The mentioned name points out to the fact that only BChE remains in plasma after blood cells separation. Though the AChE is called according to the physiological substrate; natural substrate of BChE was not found. Butyrylcholine is an artificial substrate to be converted by BChE with a high turnover rate. It is not present in living organisms. For analytical purposes, BChE was used in the past in a large scale due to simple isolation from blood. The turnover number of BChE for acetyl(thio)choline is lower when compared to the AChE. For this reason, AChE is considered as more suitable enzyme for analytical purposes. Both AChE and BChE are serine hydrolases. Active serine is localized in esteratic part of the active site. The serine moiety is irreversibly modified by organophosphorus neurotoxins. Carbamates cause pseudo-irreversible inhibition due to the fact that the carbamate moiety slowly leaves the enzyme by spontaneous hydrolysis. After modification, neither AChE nor BChE can participate in hydrolysis of their substrates [4]. The present review is aimed at the introduction of some important facts of AChE biochemistry and the performance of cholinesterases for colorimetric disposable dipsticks construction.

2. Nerve Agents and Pesticides

From the toxicological point of view, organophosphorus inhibitors of AChE may be divided into two groups: pesticides and nerve agents. Beside the organophosphorus toxins, reversible and pseudo-irreversible inhibitors of AChE, such as Alzheimer's disease drugs rivastigmine, donepezil and galantamine are well known and used in the current medicine [2]. Because of the demand of high agricultural production, large quantities of pesticides are used around the World [5]. From the pesticides family, organophosphorus compounds and carbamates are the most widely used insecticides. Although the named pesticides are highly toxic, their performance still expands, especially in the less developed countries [6]. On the other hand, neurotoxic pesticides are often misused in suicide attempts, because of free access to these toxic compounds [7]. Pirimicarb, fenoxycarb, carbofuran, paraoxon, parathion, chlorpyrifos, and dichlorvos belong to a wide range of pesticides. In the Czech Republic, desmedipham, fenoxycarb, methiocarb, pirimicarb, phenmedipham, propamocarb, dimethoat and fosetyl Al are commercially available. Sarin (abbreviation in compliance with NATO: GB), soman (GD), tabun (GA), cyclosarin (GF) and VX can be mentioned as typical examples of nerve agents. Structures of selected nerve agents are depicted in Fig. 1 and those of pesticides in Fig. 2.

It is not a surprise that nerve agents were originally developed as potent pesticides. They were synthesized by Dr. Shrader's group in Germany shortly before World War II. Some of the prepared candidates were extremely toxic to mammals. The political situation changed their research priority to the development of a new class of chemical warfare agents, so nerve agents were prepared [8]. The first members of nerve agent family, sarin and tabun, were synthesized in time interval from 1936 to 1938. Soman, the most potent AChE inhibitor from nerve agent synthesized in Germany, was prepared in 1944.

After the World War II, the political situation was changed again. The time period called the Cold War commenced; many other derivatives of nerve agent were synthesized and manufactured in large quantities. Among them, V type compounds such as VX have been introduced as novel chemical warfare agents [9]. Nowadays, the

novichok, probably the newest nerve agent prepared in the former Soviet Union, is of a great importance among scientists around the World. Unfortunately, the novichok agents remain mysterious as many controversial facts appeared in the past.

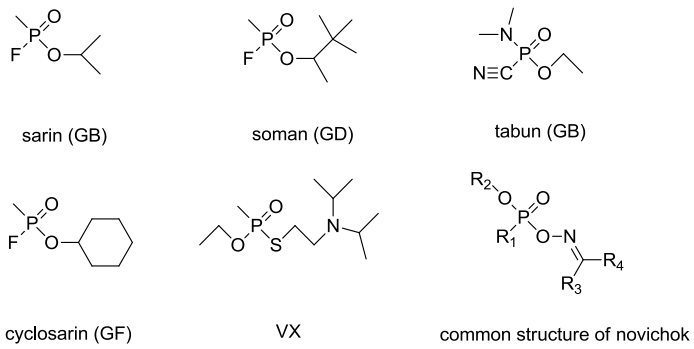

Fig. 1 Structure of selected nerve agents.

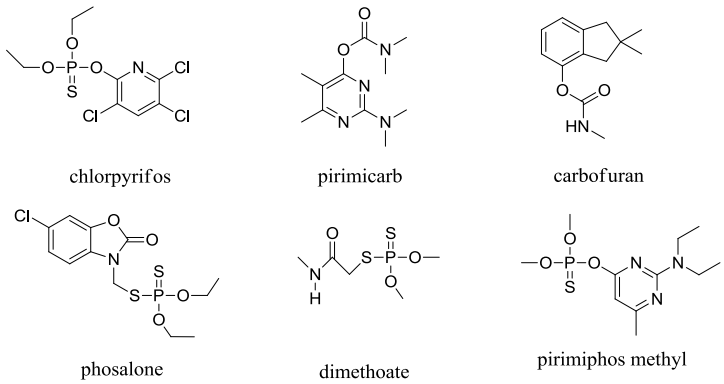

Fig. 2 Structure of selected pesticides inhibiting cholinesterases.

3. Biochemistry of AChE Based Colorimetric Assay

The principle of detectors ChP 71, 05, Detehit sensors and clinical examination of cholinesterases is based on modified Ellman's method [10]. The chemical principle is depicted in Fig. 3. The reaction is suitable for clinical examination of AChE activity in blood as a marker suitable for diagnosis of intoxication by nerve agents or neurotoxic pesticides. Mechanism of the reaction is following: substrate acetylthiocholine is hydrolyzed by AChE into acetic acid and thiocholine. In the next step, thiocholine reacts with 5,5'- dithiobis-(2-nitrobenzoic) acid (known as Ellman reagent) providing 2-nitro-5-thiobenzoic acid. The reaction is clearly visible and the absorbance can be measured at 412 nm. BChE can be examined in a similar way as AChE. However, acetylthiocholine is replaced by butyrylthiocholine. The first step of both the reported reactions is blocked by organophosphorus and carbamate neurotoxic compounds.

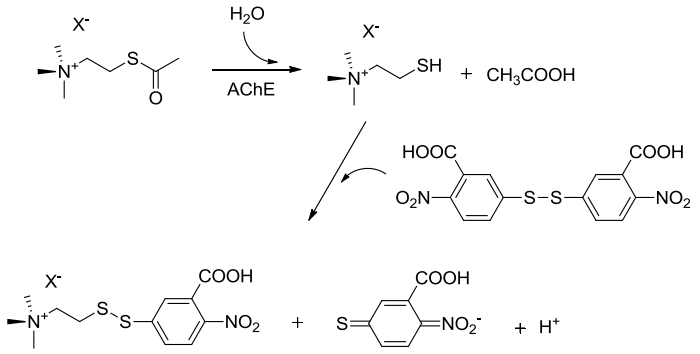

Fig. 3 AChE assay using Ellman's method.

Performance of indoxyl derivates is another way how to assay AChE activity. Indophenyl acetate, 2.6-dichloroindophenyl acetate, β-naphthyl acetate and N-methylindoxyl acetate are the available compounds [11]. Indoxyl acetate is probably used in the largest scale. In comparison with the Ellman's reagent, the above-mentioned compounds produce (display) blue colour with strong fluorescence. On the other hand, the enzymatic conversion is significantly slower comparing to the acetylthiocholine. In case of indoxyl acetate, the reaction mechanism is based on hydrolysis catalyzed by AChE providing hydroxylindole and acetic acid. The reaction is stopped by AChE inhibitors. Hydroxylindole spontaneously changes into blue coloured indigo in the presence of oxygen. The reaction mechanism is depicted in Fig. 4. Performance of acetylcholine and pH indicative reagent can be introduced as the last type of colorimetric method. Acetic acid liberated from acetylcholine causes solvent acidification. The acidification can be clearly visible when pH indicative reagent is added. The main disadvantage of this type of assay is a wide interference from acidic solvents and vapours. Moreover, extensive amount of nerve agents and pesticides provide false positive indication of active AChE activity due to non-catalyzed medium acidification.

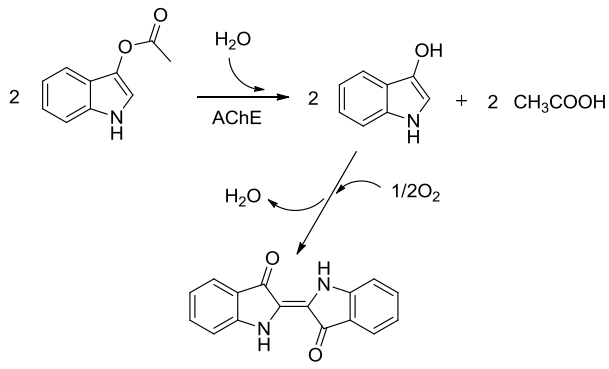

Fig. 4 AChE activity assay using indoxylacetate.

Both the above reported methods have some advantages and disadvantages. The main advantage of Ellman's method is a fast conversion of acetylthiocholine comparing to other artificial substrates such as indoxylacetate. The same amount of AChE causes probably ten times higher conversion of acetylthiocholine in comparison with indoxylacetate. On the other hand, Ellman's method has some disadvantages, too. It is a large interference of some compounds. False positive reaction can be provided by samples containing a lot of thiol bearing molecules. E.g. glutathione is another compound being assayed by modified Ellman's method [12]. Hemoglobin is also strongly absorbing at 412 nm and thus interfering in the assay. In available literature, there is clearly described reaction of Ellman reagent and some drugs containing oxime moiety [13]. The compounds provide oximolysis appearing as a false positive enzyme activity leading to over-expected efficacy of oxime drugs. Similarity between native substrate acetylcholine and acetylthiocholine is an undisputable advantage of Ellman's method. For this reason, Ellman's method is preferred in clinical laboratories. Advantage of indoxyl acetate is colorimetric change better observable by a naked eye and allowing spectrophotometric as well as fluorimetric assay. In the past, Ellman's method was used instead of indoxylacetate based assay in order to save expensive AChE.Currently, the price of recombinant AChE is not much higher comparing to the other laboratory costs and priorities for substrate selection can alter.

4. Available Colorimetric Detectors Based on Cholinesterases

Several methods were proposed as suitable for estimation of cholinesterase activity. Two colorimetric methods seem to be the most suitable and they are widely used for analytical purposes. The first group of detectors is based on Ellman's method (see the previous chapter). Two available instruments can be introduced as practical examples. The first is a ChP 71 respectively 05 (Oritest, Czech Republic) semiquantitave chemical detectors currently used in some NATO countries including the Czech Republic. Boththe versions are able to detect the most important chemical warfare agents. The assay of nerve agents is based on colorimetric tube with bound AChE. The device ChP 71 was constructed in former Czechoslovakia in the 1960s and 1970s and it can be loaded with disposable detectors for assay of nerve agents, mustard gas, phosgene, cyanide etc. Both the ChP 71 and 05 include air sampling parts, filtration of outputting air and source of light. Detectors are glass tubes filled with sensitive matrix. In case of nerve agents, AChE and Ellman's reagents are included within the tube. Assessment of chemical warfare agents is based on evaluation of coloring using a naked eye. Comparing with the ChP devices, GSP and GSA devices were the first automatic devices available during the Cold War era. The devices were produced in the former Soviet Union. Disposable detector dipsticks, such as e.g. Detehit (Oritest, Czech Republic), are based on Ellman's method, too.

Indoxylacetate represents another option for the construction of colorimetric dipsticks. The advantage of the indoxylacetate based assay is a better color change, as it provides contrast blue coloration. The disadvantage of indoxyl acetate usage is a lower turnover rate comparing to the Ellman's method. Simple dipstick provided by Neogen Corporation (Lansing, Michigan, USA) can be introduced as an example of commercialized device based on indoxylacetate.

It can be stated that the contemporary universal detectors for chemical warfare agents are mobile mass spectrometers RAID-1, RAID-M, RAID-S a RAID-E (Bruker Daltonics). This devices process the measured spectra with standard spectra in device

memory. The advantage of mobile spectrometers is a better reproducibility of measurement with lower sensitivity to poor storage if compared to the detector with biologically active macromolecules, such as AChE. The major disadvantage of mass spectrometers is quite a high price and inability to recognize compounds that have not saved spectra in the memory.

5. Carrier Matrix Used in Detectors Based on Acetylcholinesterase

For the design of colorimetric detector, a wide range of carrier matrix is used and this corresponds to the number of different immobilization technologies (see the next chapter). The general requirements for the carrier matrix are following: compatibility with the sample, i.e. the sample matrix may not dissolve, extract the folder etc. Other requirements are: a sufficiently large contact area with sample analyte, quick access to AChE with sufficient permeability, hydrophilic character, chemical and thermal stability, mechanical resilience, resistance to microbial degradation, low cost, etc.

The carrier matrix can be divided into three major groups:

- Natural polymers (polysacharides, proteins)
- Synthetic polymers (polystyrene, polyacrylates, hydroxylalkyl methacrylate, etc.)
- In-organic carriers (minerals, activated carbon, glass fibres, porous metal oxides).

Natural polymers are derived from a relatively wide range of natural resources, such as wood fiber waste from slaughterhouses or microorganisms. Typical representatives of this group are cellulose or paper, dextran and gelatin [14]. Certain disadvantage of these substances is a possibility of loss of AChE by elution. Among synthetic polymers, respectively inorganic carriers, PVC [15], polycarbonates [16], polyester [17], polyurethane [18], ceramics [19], glass fiber or cellulose fabric are often used for AChE immobilization purposes. From the less known synthetic materials, we can mention polymer AQ 55 (see reference [20]) which dissolves in water and whose mixture with an enzyme after dropping and drying on the surface of the carrier matrix can be used in the presence of organic solvents. Biocers (biologically modified ceramics) [21] or nanofibers matrix based on carbon or other nano-structured carbon [22] are novel and available matrices. Biocers are a class of ceramic nanocomposites which combines biocomponents as a matrix. It can be prepared as a powdery material or as a film to prevent critical conditions (high temperature, organic solvents), which would lead to denaturation of proteins. Detectors based on these carrier matrices often exhibit a remarkably low level limit of detection. The disadvantage of the matrix is a high cost of reagents.

In regards to the low production prices, the last group of carrier matrix based on simple physical sorption or mechanical recording under a thin layer is undoubtedly very interesting. We can mention enzyme solution saturated porous medium, generally inorganic, such as glass fiber, sol-gel and glass membrane. Owing to the projected one-shot detector based on AChE, there is an optimum price / performance ratio.

6. AChE Immobilization

AChE can be immobilized in similar way as the other molecules of biological origin. The immobilization procedure should be corresponding to the purpose of molecule capturing and expected conditions of performance. AChE immobilized for purposes of

neurotoxic cholinesterase inhibitors assay does not need to be bound with huge affinity equilibrium constant. Colorimetric dipsticks with AChE are typically disposable and they are removed after the assay. The one-step immobilizations are preferred due to saving production costs. Sorption on matrix surface and immobilization into membranes can be mentioned as relevant one-step procedures. From the available literature, AChE binding into gelatin [23] or cellulose [24] membranes seems to be suitable. Some specific techniques such as sol-gel, binding on nanoparticles and magnetic microparticles are known as well [25]; the procedures are reasonably effective; on the other hand, it increases costs and heftiness of the dipsticks production.

There are well described procedures based on cross-linking of AChE and some co-polymering molecules by glutaraldehyde. Amino groups of AChE structure play a crucial role during the immobilization. Glutaraldehyde is a homo-bifunctional reagent covalently linking two molecules of AChE by amino groups or AChE with another biomolecule such as albumin. There can also be connection of AChE and another enzyme, such as cholineoxidase [26]. Thus the connected biomolecules are moderately resistant to washing out from matrix. AChE could be immobilized in a similar way on cellulose. It is a slight adoption of referred protocol [27]. Partial oxidation of cellulose by proper reagents, such as periodate, is necessary before immobilization. The second step is similar to precipitation by glutaraldehyde. This type of immobilization procedures is fairly common in construction of many analytical devices including biosensors [28, 29].

7. Conclusions

Colorimetric dipsticks based on AChE are frequently used tools for a fast, cheap and highly precise assay of neurotoxic compounds. Some natural toxins can be simply assayed, too. Methods for disposable dipsticks construction currently available are able to provide firmly bound AChE complex – matrix without any difficult manipulation. There are also known biochemical methods for evaluation of AChE activity using chromogenic substrates that are converted with sufficient turnover rate.

References

[1] POHANKA, M., KUCA, K. and JUN, D. Aflatoxin assay using an amperometric sensor strip and acetylcholinesterase as recognition element. *Sensor Letters*, 2008, vol. 6, no. 3, p. 450-453.

[2] POHANKA, M. Cholinesterases, a target of pharmacology and toxicology. *Biomedical Papers*, 2011, vol. 155, no. 3, p. 219-230.

[3] DUYSEN, E.G., LI, B., CARLSON, M., LI, Y.F., WIESLER, S., HINDRICHS, S.H. and LOCKRIDGE, O. Increased hepatotoxicity and cardiac fibrosis in cocaine-treated butyrylcholinesterase knockout mice. *Basic and Clinical Pharmacology and Toxicology*, 2008, vol. 103, no. 6, p. 514-521.

[4] CASIDA, J.E. and QUISTAD, G.B. Serine hydrolase targets of organophosphorus toxicants. *Chemico-Biological Interactions*, 2005, vol. 157, no. 158, p. 277-283.

[5] BAJGAR, J. Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Advances in Clinical Chemistry,* 2004, vol. 38, no. 1, p. 151-216.

[6] EDDLESTON, M., BUCKLEY, N.A., EYER, P. and DAWSON, A.H. Management of acute organophosphorus pesticide poisoning. *The Lances,* 2008, vol. 371, no. 9612, p. 11, 4-9.

[7] GUVEN, M., UNLUHIZARCI, K., GOKTAS, Z. and KURTOGLU, S. Intravenous organophosphate injection: an unusual way of intoxication. *Human and Experimental Toxicology,* 1997, vol. 16, no. 5, p. 279-280.

[8] DELFINO RT., RIBERIO TS. and FIGUEROA-VILLAR JD. Organophosphorus compounds as chemical warfare agents: a review. *Journal of the Brazilian Chemical Society,* 2009, vol. 20, no. 3, p. 1-22.

[9] KARASOVA, J.Z., BAJGAR, J., JUN, D., PAVLIKOVA, R. and KUCA, K. Time-course changes of acetylcholinesterase activity in blood and some tissues in rats after intoxication by Russian VX. *Neurotoxicity Research*, 2009, vol. 16, no. 4, p. 356-360.

[10] ELLMAN, G.L., COURTNEY, D.K., ANDRES, V. and FEARSTONE, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 1961, vol. 7, no.1, p. 88-95.

[11] NO, H.Y., KIM, Y.A., LEE, Y.T. and LEE, H.S. Cholinesterase-based dipstick assai for the detection of organophosphate and carbamate pesticides. *Analytica Chimica Acta,* 2007, vol. 594, no. 1, p. 37-43.

[12] DAVIES, M.H., BIRT, D.F. and SCHNELL, R.C. Direct enzymatic assay for reduced and oxidized glutathione. *Journal of Pharmacological Methods,* 1984, vol. 12, no. 3, p. 191-194.

[13] SINKO, G., CALIC, M., BOSAK, A. and KOVARIK, Z. Limitation of the Ellman method: cholinesterase activity measurement in the presence of oximes. *Analytical Biochemistry*, 2007, vol. 370, no. 2, p. 223-227.

[14] CUI, G., KIM, S.J., CHOI, S.H., NAM, H., CHA, G.S. and PAENG, K.J. A disposable amperometric sensor screen printed on a nitrocellulose strip: a glucose biosensor employing lead oxide as an interference-removing agent. *Analytical Chemistry*, 2000, vol. 72, no. 8, p. 1925-1929.

[15] ANDREESCU, S., BARTHELMEBS, L. and MARTY, J.L. Immobilization of acetylcholinesterase on screen-printed electrodes: comparative study between three immobilization methods and applications to the detection of organophosphorus insecticides. *Analytica Chimica Acta,* 2002, vol. 464, no. 2, p. 171-180.

[16] SANCHEZ, S., PUMERA, M., CABRUJA, E. and FABREGAS, E. Carbon nanotube / polysulfone composite screen-printed electrochemical enzyme biosensors. *Analyst*, 2007, vol. 132, no. 2, p. 142-147.

[17] ARDUINI, F., RICCI, F., TUTA, C.S., MOSCONE, D., AMINE, A. and PALLESCHI G. Detection of carbamic and organophosphorous pesticides in water samples using a cholinesterase biosensor based on Prussian Blue-modified screen-printed electrode. *Analytica Chimica Acta*, 2006, vol. 580, no. 2, p. 155-162.

[18] CHO, Y.A., LEE, H.S., CHA, G.S. and LEE, Y.T. Fabrication of butyrylcholinesterase sensor using polyuretane-based ion-selective membranes. *Biosensors and Bioelectronics*, 1999, vol. 14, no. 4, p. 435-438.

[19] SANTHI, H., DOBAY, R. and HARSANYI, G. Amperometric uric acid biosensors fabricated of various types of uricase enzymes. *IEEE Sensors Journal*, 2003, vol. 3, no. 3, p. 282-287.

[20] KONASH, A. and MAGNER, E. Characterization of an organic phase peroxide biosensor based on horseradish peroxidase immobilized in Eastman AQ. *Biosensors and Bioelectronics,* 2006, vol. 22, no. 1, p. 116-123.

[21] BOTTCHER, H., SOLTMANN, U., MERTIG, M. and POMPE, W. Biocers: ceramics with incorporated microorganisms for biocatalytic, biosorptive and functional materials development. *Journal of Materials Chemistry*, 2004, vol. 14, no. 14, p. 2176-2188.

[22] SOTIROPOULOU, S. and CHANIOTAKIS N.A. Lowering the detection limit of the acetylcholinesterase biosensor using a nanoporous carbon matrix *Analytica Chimica Acta,* 2005, vol. 530, no. 2, p. 199-204.

[23] TIMUR, S. and TELEFONCU, A. Acetylcholinesterase (AChE) electrodes based on gelatin and chitosan matrices for the pesticide detection. *Artifical Cells, Blood Substitutes, and Immobilization Biotechnology,* 2004, vol. 32, no. 3, p. 427-442.

[24] ZHANG, J., LUO, A., LIU, P., WEI, S., WANG, G. and WEI, S. Detection of organophosphorus pesticides using potentiometric enzymatic membrane biosensor based on methylcellulose immobilization. *Analytical Science*, 2009, vol. 25, no. 4, p. 511-515.

[25] POHANKA, M., MUSILEK, K. and KUCA, K. Progress of biosensors based on cholinesterase inhibition. *Current Medicinal Chemistry,* 2009, vol. 16, no. 14, p. 1790-1798.

[26] GUERRIERI, A., MONACI, L., QUINTO, M. and PALMISANO, F. A disposable amperometric biosensor for rapid screening of anticholinesterase activity in soil extracts. *Analyst*, 2002, vol. 127, no. 1, p. 5-7.

[27] WANG, S., LI, S. and YU, Y. Immobilization of cholesterol oxidase on celulose acetate membrane for free cholesterol biosensor development. *Artifical Cells, Blood Substitutes, and Immobilazition Biotechnology*, 2004, vol. 32, no. 3, p. 413-425.

[28] POHANKA, M., SKLADAL, P. and KROCA, M. Biosensors for biological warfare agent detection. *Defence Science Journal*, 2007, vol. 57, no. 3, p. 185-193.

[29] POHANKA, M., BINDER, J. and KUCA, K. Nerve agent assay using different origin acetylcholinesterases and an electrochemical sensor strip. *Defence Science Journal,* 2009, vol. 59, no. 3, p. 300-304.

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