

Acetylcholinesterase inhibitors assay using colorimetric pH sensitive strips and image analysis by a smartphone

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Abstract

Smartphones are widely spread and their usage does not require any trained personal. Recently, smartphones were successfully used in analytical chemistry as a simple detection tool in some applications. This paper focuses on immobilization of acetylcholinesterase (AChE) onto commercially available pH strips with stabilization in the gelatin membrane. AChE degrades acetylcholine into choline and acetic acid which causes color change of acid-base indicator. Smartphone served as a tool for measurement of indicator color change from red to orange while inhibitors blocked this process. AChE inhibitors were measured with limits of detection 149 nM and 22.3 nM for galanthamine and donepezil respectively. Organic solvents were measured for method interferences. Measurement procedure was performed on 3D printed holder and digital photography was evaluated using red-green-blue (RGB) channels. The invented assay was validated to the standard Ellman's test and verified on murine plasma samples spiked with inhibitors. We consider the assay is fully suitable for practical performance.

Keywords

Acetylcholinesterase; smartphone assay; pH strips; biosensor; colorimetry; 3D print; image analysis

1. Introduction

AChE is an enzyme splitting the neurotransmitter acetylcholine in cholinergic synapsis into choline and acetic acid [1]. Sensitivity of AChE to neurotoxic compounds (anti-Alzheimer's drugs, pesticides, nerve agents) can be use in their measurement [2]. Commonly used method is based on reaction of thiocholine, formed from acetylthiocholine during enzymatic hydrolysis, with Ellman's reagent producing yellow 5-thio-2-nitrobenzoate measurable by spectrophotometry in 412 nm [3]. Despite broad use, this method has some drawbacks like instability of Ellman's reagent and hemoglobin interference [2, 4, 5]. Furthermore there is possibility to measure enzyme activity electrochemically [6, 7]. Colorimetric detection can be also based on pH measurement. Many acid-base indicators are known for this purpose, [when phenol red was used in our work previously](#) [8, 9]. Different techniques for enzyme

immobilization and matrix like gelatin were described as well [10-13]. Simple physical immobilization of enzyme onto cellulose and stabilization into gelatin matrix was successfully used for preparation of biosensor in an application where AChE was embedded into gelatin membrane on a paper matrix and performed for the assay of neurotoxic compounds [14, 15]. In the cited papers, there was not, however, made evaluation of enzyme activity by a camera because a naked eye assay and another type of substrate were preferred. Gelatin provides good properties for enzyme immobilization combine with biocompatibility and zero toxicity [16]. Modern mobile phones dispose high resolution cameras which gives them ability to serve as a tool for diagnostics [17, 18]. Previously mobile phones have been used for some applications in analytical chemistry [19, 20]. This paper deals with preparation of biosensor based on commercial pH strips with immobilized AChE and stabilized in gelatin membrane and his performance in measurement of neurotoxic compounds. This approach offers easy way for AChE inhibitors determination, especially if we considered well established spectrophotometric assay, for here presented method no special equipment or trained personal are required. There is also innovation in evaluation of color based reaction compare to previously described methods. The major advantage of our assay is based on the opportunity to link it to a smartphone which is considered as the detector device providing wide availability to less equipped laboratories and for field tests without any expensive gear.

2. Material and methods

2.1. Materials and devices

Acetylcholinesterase from electric eel (≥ 1000 units/mg protein), acetylcholine chloride (AChCl), acetylthiocholine chloride (ATChCl), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), donepezil hydrochloride monohydrate, galanthamine hydrobromide, tetraisopropyl pyrophosphoramidate (iso-OMPA), phosphate buffer saline (PBS) pH 7.4, dimethyl sulfoxide (DMSO) and isopropyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, USA), denatured ethanol, gelatin were supplied by PENTA (Prague, Czech Republic). Indicator strips pH-Fix 6.0 – 7.7 were obtained from Macherey-Nagel (Düren, Germany). Color change was detected by Sony Xperia MT27i with 5 Mpx camera and LED light using operation system Android 2.3.7., device version number 6.0.B.3.184 (Tokyo, Japan). For 3D print, 3D printer Prusa i3 from Prusa Research (Prague, Czech Republic) was used. Murine plasma samples were obtained from 20 female BALB/c mice which were purchased from Velaz (Unetice, Czech Republic). The mice were kept under standard ambient temperature and humidity 50 ± 10 %. Light and dark periods lasted each equally 12 hours. The mice were sacrificed in the age 8 weeks by cutting of carotid under carbon dioxide narcosis and the blood was taken into tubes pre-treated with lithium heparin (Dialab, Prague, Czech Republic) and centrifuged at $1,000 \times g$ for 5 minutes. Fresh plasma was kept at -80 °C until use in the assay. The whole experiment was the both permitted and supervised by ethical committee Faculty of Military Health Sciences (Hradec Kralove, Czech Republic).

2.2. Solutions preparation

AChCl solutions were prepared in concentration range from 0.31 to 10 mM and placed microtubes. Galanthamine solutions were prepared in concentration range from 1.6 to 25.00

μM. Donepezil solutions were prepared in concentration range from 0.31 to 5.00 to μM. Each solution was prepared in PBS 7.4 and final concentration in micro tube was 10 times lower. Gelatin was prepared in 1 % concentration by stirring of 10 mg of gelatin in 1 ml of water for 20 min. All solutions for Ellman's assay were prepared in PBS 7.4. DTNB solution was prepared in concentration 1 mM and ATChCl in 10 mM. Concentration range of galanthamine was from 62.5 to 100 μM and 13 to 20 μM in case of donepezil. Final concentrations in cuvette were 10 fold less concentration of ATChCl and 40 fold less in case of galanthamine or donepezil. Iso-OMPA was prepared in PBS 7.4 in concentration 1 mM, final concentration in plasma samples were 0.1 mM.

2.3. Preparation of pH strips with AChE

To pH strips 10 μl of AChE (activity for acetylthiocholine 1.73×10^{-8} mol/s/μl) were added and let it dry in laboratory temperature. Then pH strips were covered by 10 μl of 1 % gelatin. After dry in laboratory temperature pH strips were stored in 4 °C until used in the assay.

2.4. Preparation of 3D printed holder

Holder was created in Autodesk 123D Design (open source software). 3D printer setup was following: acrylonitrile butadiene styrene shaped in 3 mm filaments was used as material, nozzle temperature 285 °C, bottom temperature 100 °C and individual deposited layers 0.1 mm thick. Size of holder was 80 mm height, 105 mm length and inner diameter of tube was set to 40 mm (Figure 1).

2.5. Smartphone assay

The smartphone assay was made in following way: 450 μl of PBS pH 7.4 and 50 μl of 10 mM AChCl solution were added into microtubes and strip was put into it. After incubation lasting 15 min, excess of reaction medium was drain and picture of the still wet pH strip was taken. During photographing, the camera was placed on the 3D printed holder and the strip inside hence no outer light influenced the photography and integrated LED light was the only one source. Distance between the strip and camera was also constant just due to the holder.



Figure 1. Tube shaped holder printed by 3D print technology (upper picture) and the holder with a smartphone adjusted on the hole to provide photographs by an integrated camera

2.6. Ellman's assay

To standard cuvette 400 μl of DNTB solution, 25 μl AChE, 475 μl PBS and 100 μl of ATChCl were added. Absorbance was measured in 412 nm immediately and after incubation lasting 2 min.

2.7. Data processing

RGB color values were obtained by processing of photography in GIMP 2.8.16 (open source software) using Color Picker function. Δ Color intensity was obtained as follow: Intensity of strip before reaction – Intensity of strip after the reaction. This difference corresponds to AChE activity in different concentration of used substrate or inhibitor. K_M value for AChE and AChCl substrate was calculated using non-linear curve fitting by Hill function with

coefficient of cooperativity $n = 1$. Limit of detection was calculated as signal to noise ratio equal to three. For these purposes, Origin software 8 PRO (OriginLab, Northampton, MA, USA) was used.

3. Results and discussion

AChE splitting acetylcholine into choline and acetic acid resulted in decreasing pH of medium. Our method is based on color change of indicator in pH sensitive zone of pH strip from red to orange (Figure 2). During photography processing color change was observed in green channel while red and blue ones were without change.

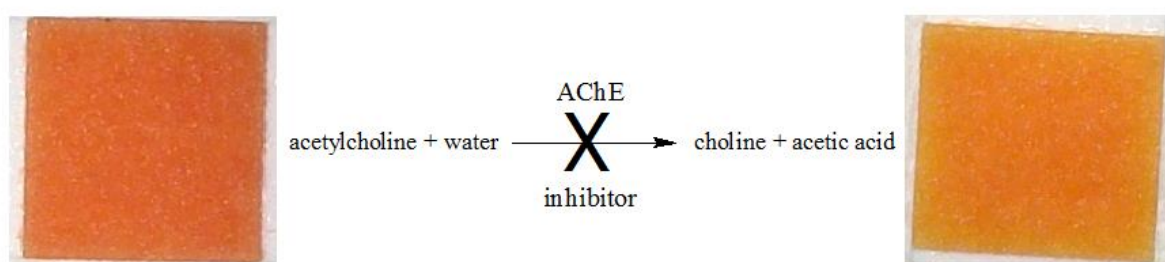


Figure 2. pH sensitive method using AChE principle.

3.1. Gelatin optimization

Amount of gelatin was tested in 0 %, 0.001 %, 0.01 %, 0.1 %, 1 % and 10 % concentration. The strips were covered with 10 μ l of gelatin in tested concentration and dipped into PBS 7.4 and color change of strip into red was observed after 5 min. Gelatin in 10 % concentration did not allow to enter buffer to pH sensitive zone and color did not change into red one while stayed orange which was represented by decreasing of color intensity (Figure 3). Color change into red was held while using gelatin in 1 % and less concentration, but for further measurement 1 % gelatin was chosen just for the better stabilization effect for AChE.

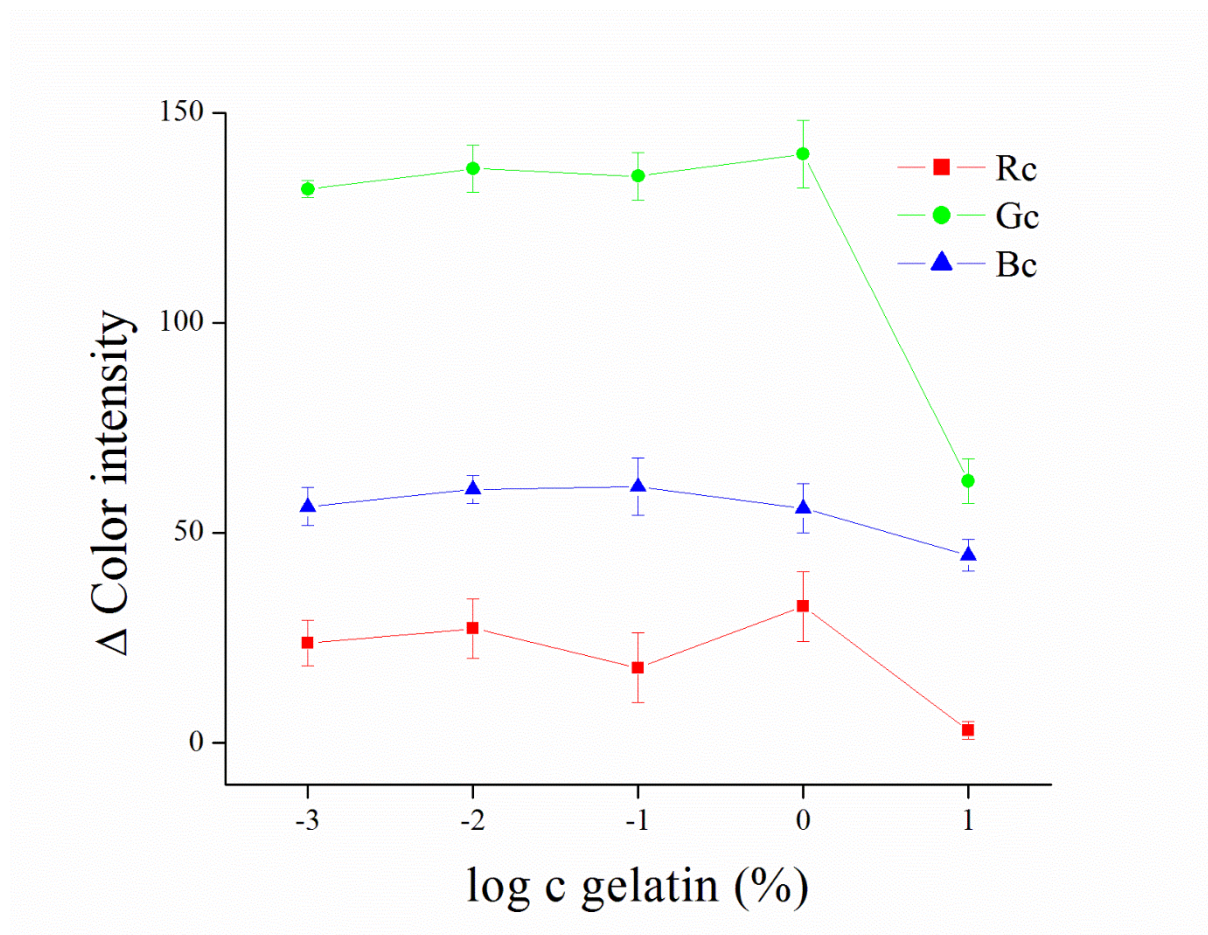


Figure 3. Gelatin optimization. Drop in Δ Color intensity indicates that pH strip did not change into red color. Error bars represent standard error of the mean for $n = 3$. Rc = red channel, Gc = green channel, Bc = blue channel.

3.2. Time optimization

Strip with AChE and 1 % gelatin was incubated with 1 mM AChCl. Incubation time with AChCl was observed in 5 min intervals from 0 to 30 min. The biggest color change was held up to 15 min. Over this time color change was not significant for longer incubation time (Figure 4).

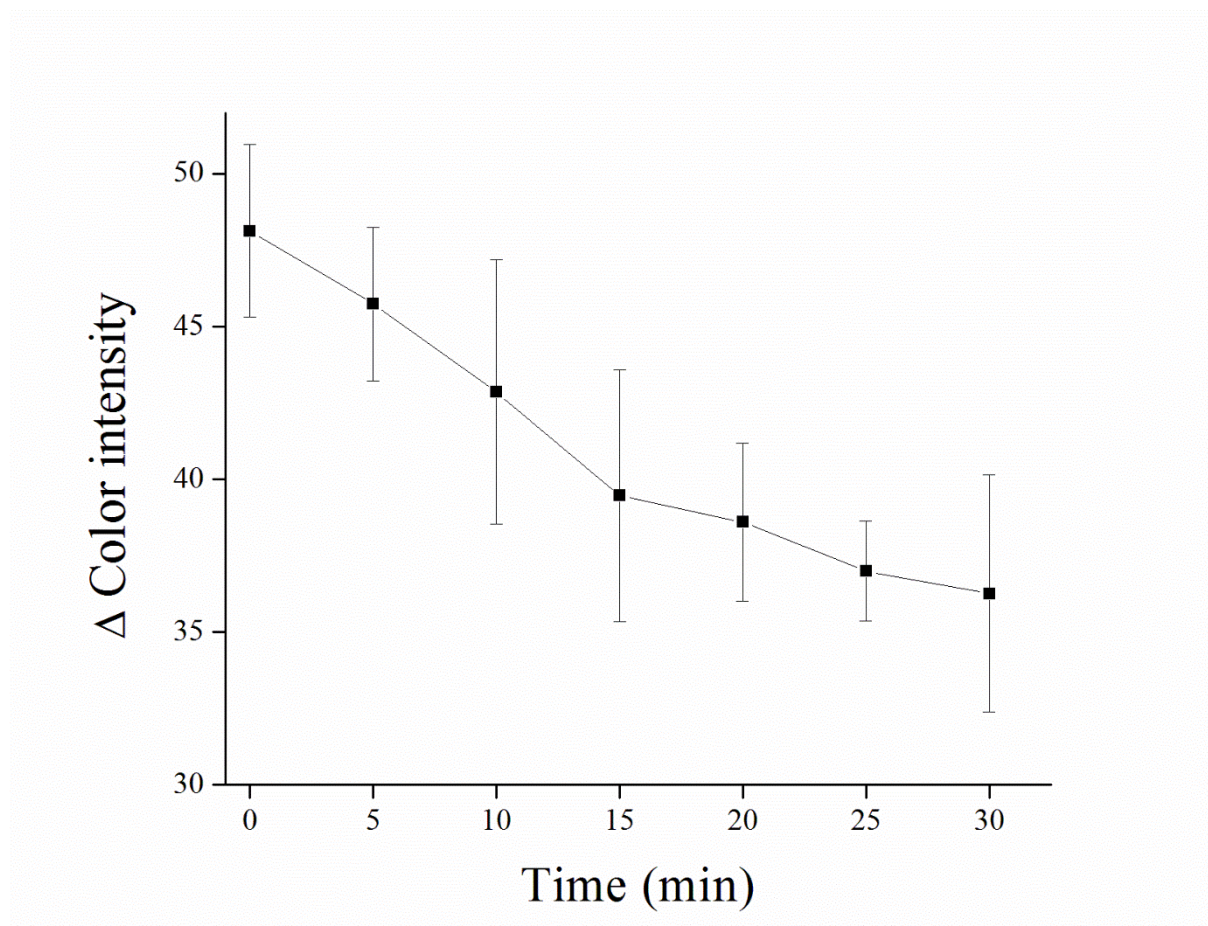


Figure 4. Optimization of pH strip incubation time with ATChCl as a substrate. Error bars represent standard error of the mean for $n = 3$.

3.3. Substrate measurement

Saturation curve for AChE and AChCl as a substrate was measured in concentration range from 0.031 to 1.0 mM and K_M value was calculated as described above to 54.26 μM (Figure 5) while 73.9 μM was reported by Xu and coworkers [21]. K_M value however depends on type of used buffer as proved by Wille et al. using human AChE isolated from erythrocytes and achieved K_M equal to 71.4 μM in MOPS buffer, 98.2 μM in PBS buffer, 0.1 mM in Tyrode buffer and 0.122 mM in Tris buffer when pH was set to 7.4 [22]. There are also big differences between organisms as shown by Shaonan et al. who worked with AChE isolated from fish species and found out values above 0.1 mM [23] and by Jiang et al. who reported K_M to be 63.85 μM when measured with mosquito AChE [24]. Recombinant enzymes exhibit slightly higher K_M values compare to wild types as showed experiments with mice AChE carried out by Boyd et al. who found out K_M to 46 μM in wild type and 58 μM for recombinant AChE [25].

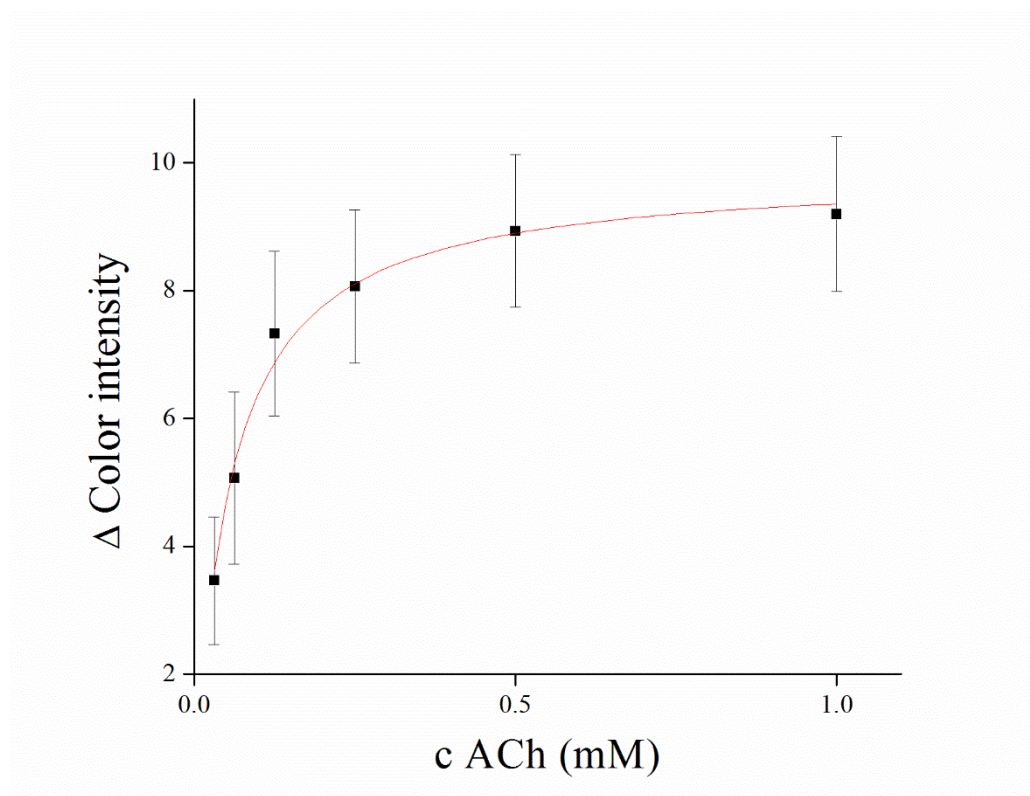


Figure 5. Saturation curve for AChE and AChCl as a substrate. Error bars represent standard error of the mean for $n = 3$.

3.4. Inhibitors measurement

AChE is sensitive to neurotoxic compounds like drugs, nerve agents or pesticides. Some of these compounds are widely used in treatment of Alzheimer disease [26], galanthamine and donepezil, currently using drugs, can be mentioned as example [27]. We performed calibration curve of galanthamine in concentration range from 0.156 to 2.5 μM with limit of detection calculated to 149 nM and quantification limit 0.5 μM was achieved (Figure 6). Linearity of the assay is limited to 2.5 μM when higher concentrations appeared to be indistinguishable. Comparing to the standard Ellman's assay, it is only 10 times higher detection limit, when 18.3 nM of galanthamine was achieved in our experiment. From previously published methods for galanthamine measurement based on AChE inhibition we can conclude that our method is competitive [28, 29]. Although detection limits are similar, there is advantage in fabrication time of pH strip with immobilized AChE which is not time consuming.

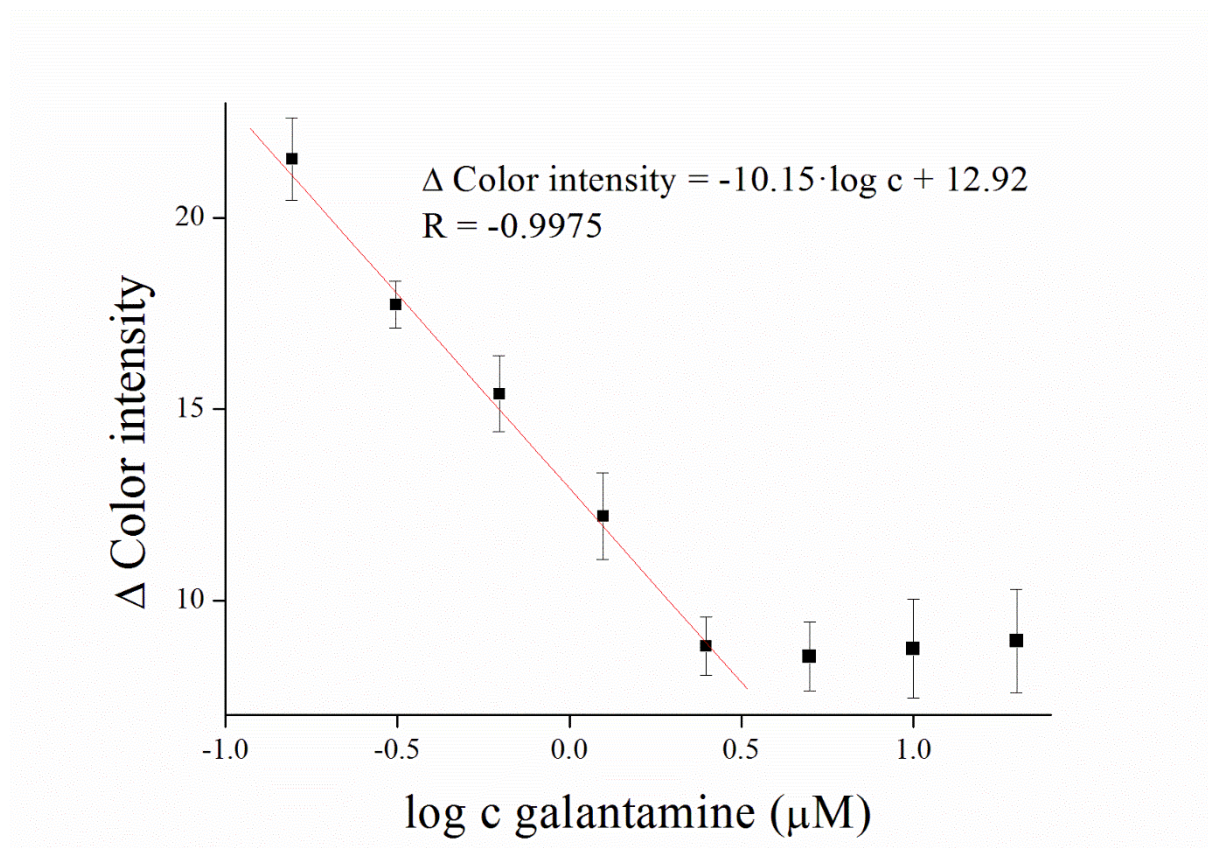


Figure 6. Calibration curve of galanthamine, concentration is given in logarithm. Error bars represent standard error of the mean for $n = 3$.

Validation of method for galanthamine measurement was done using standard Ellman's assay with correlation coefficient 0.9922 (Figure 7).

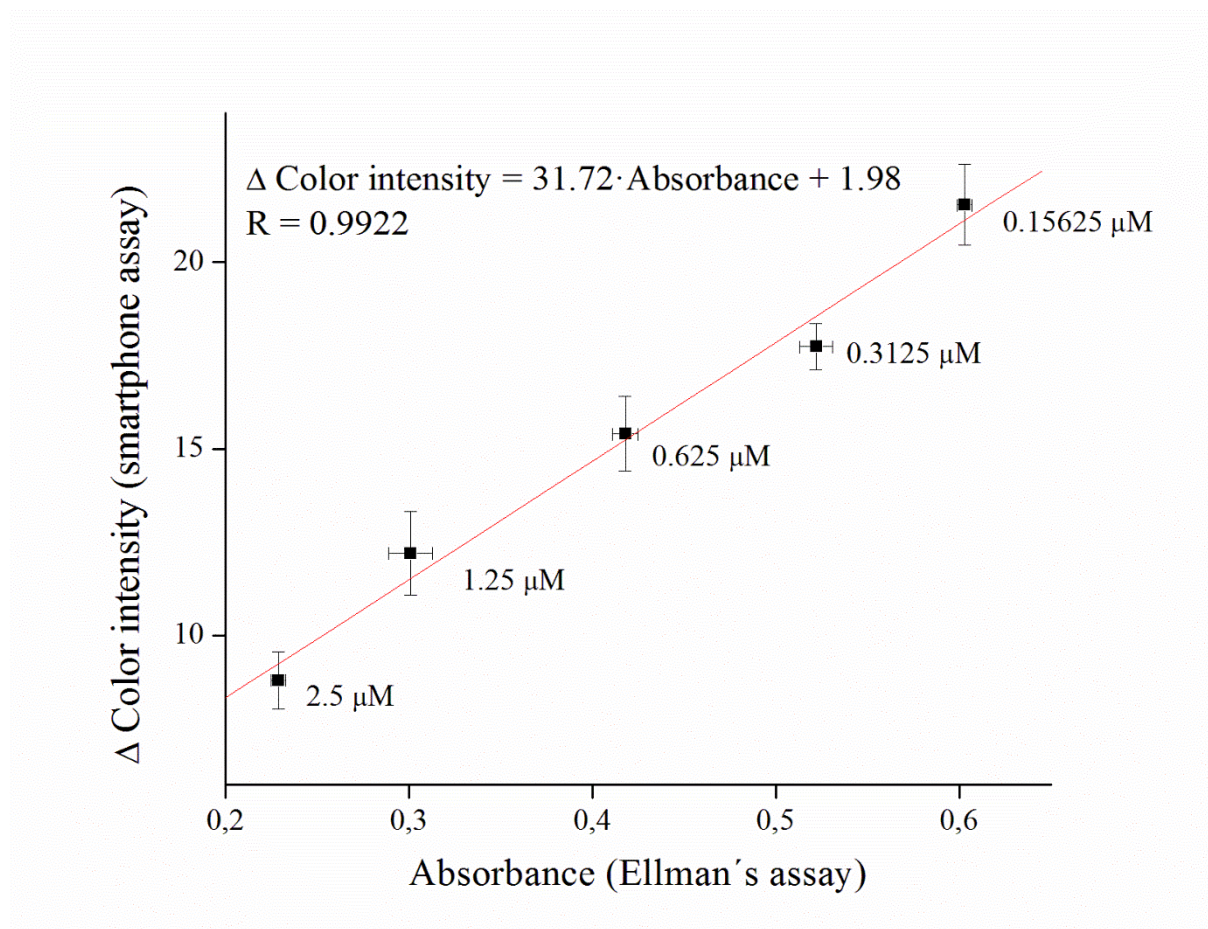


Figure 7. Validation of galanthamine assay compared to standard Ellman's assay. Error bars for smartphone assay represent standard error of the mean and for Ellman's assay standard deviation for n = 3.

Method was verified using murine plasma. Plasma samples were pre-treated by iso-OMPA, selective inhibitor of butyrylcholinesterase [30], which naturally occurs in plasma and has ability to splits acetylcholine. Then galanthamine was spiked into plasma samples in appropriate concentrations and smartphone assay has been performed (Figure 8). For measurement with plasma PBS buffer had to be replaced by water, because plasma strongly buffered itself then PBS buffer was not required.

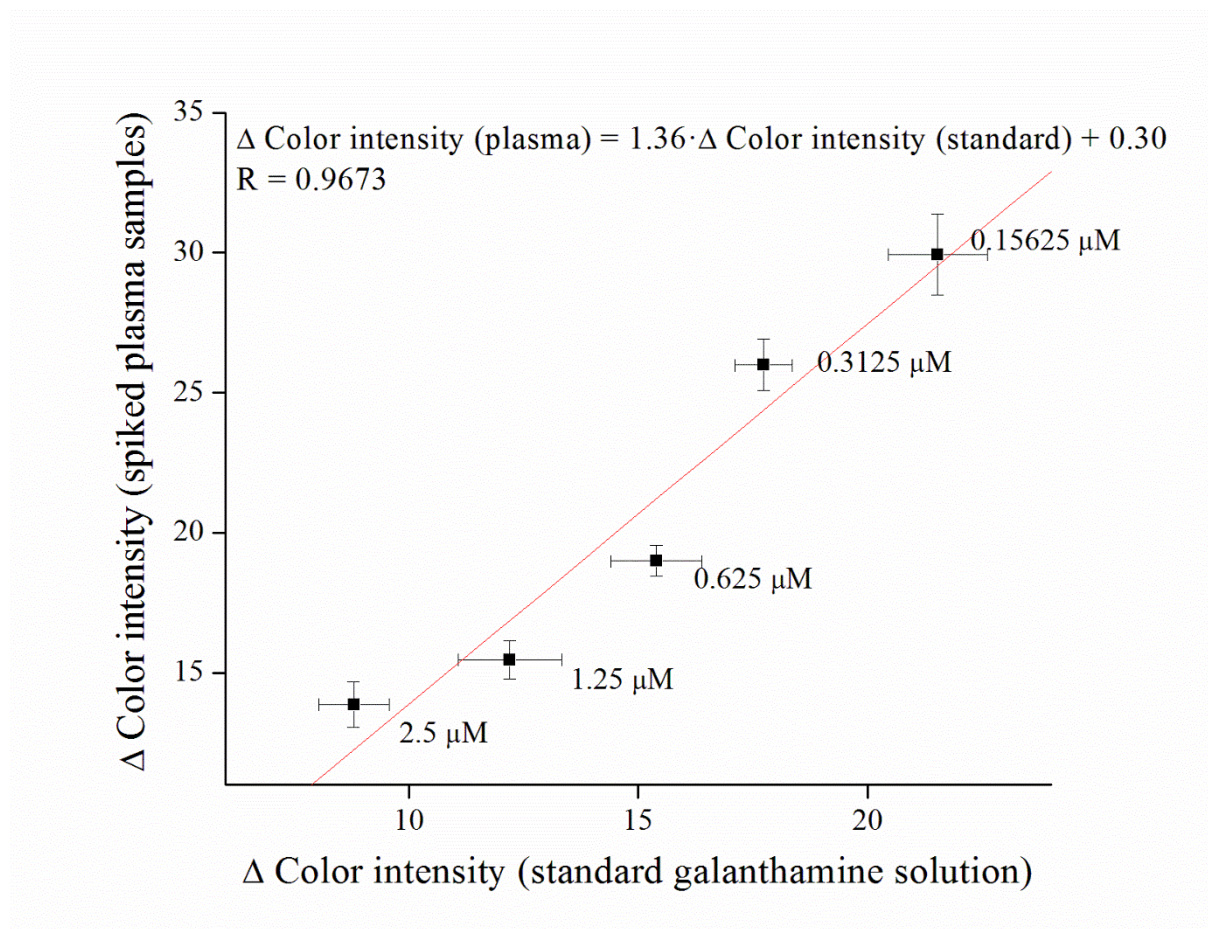


Figure 8. Verifying of galanthamine assay in plasma samples compare to standard galanthamine solution. Error bars represent standard error of the mean for n = 3.

In literature, there are plenty of references about determination of donepezil by chromatography techniques [31, 32]. Likewise, there are electrochemical methods for donepezil measurement with similar detection limits as our method [33, 34] and some spectrophotometric methods for quantification of donepezil in pharmaceuticals or human plasma [35, 36]. However, no evidence about determination of donepezil via inhibition of AChE was found. Donepezil calibration curve was done in concentration range from 0.031 to 0.50 μM and limit of detection equal 22.3 nM was achieved while limit of quantification was found out 0.2 μM (Figure 9). Also, above calibration range there is limited linearity. For comparison, detection limit achieved by Ellman's assay in this experiment was equal to 3.82 nM.

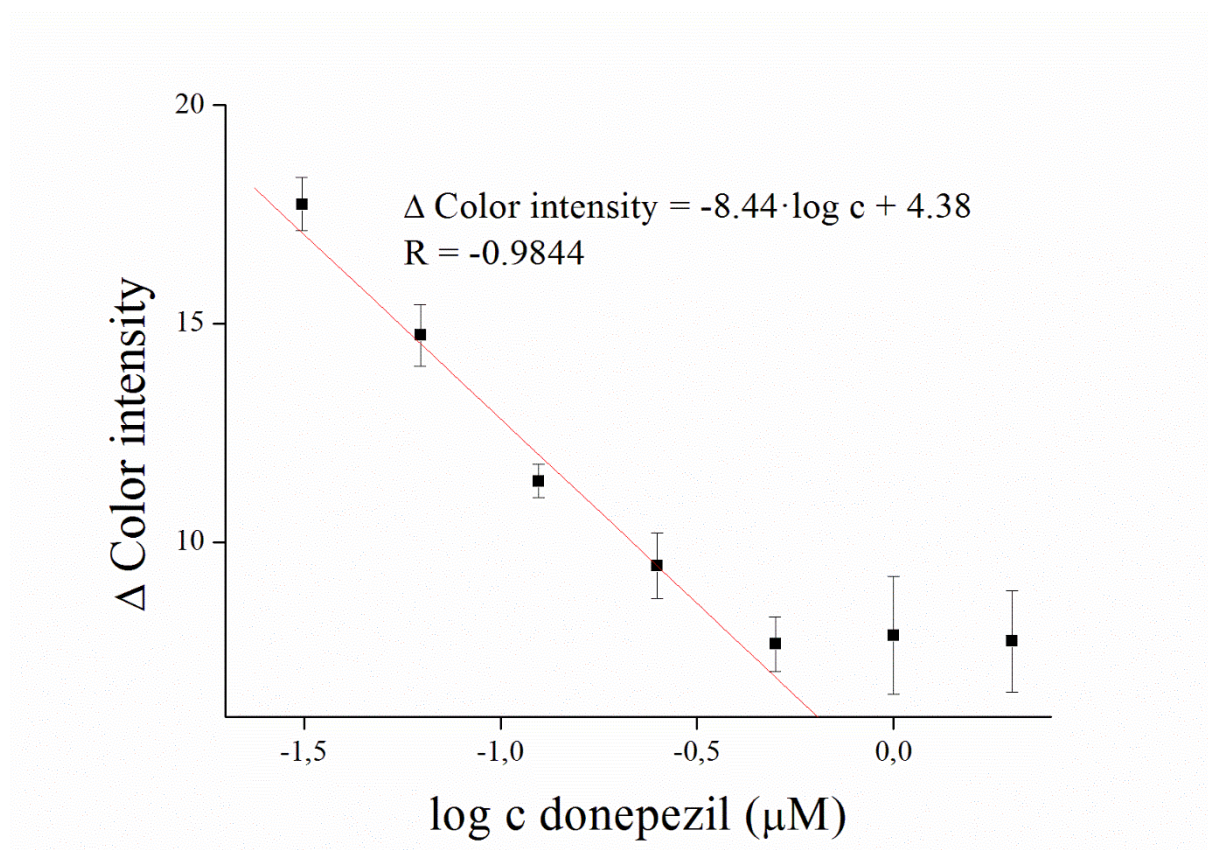


Figure 9. Calibration curve of donepezil, concentration is given in logarithm. Error bars represent standard error of the mean for n = 3.

Validation of method for donepezil measurement was performed using standard Ellman's assay with correlation coefficient equal 0.9895 (Figure 10).

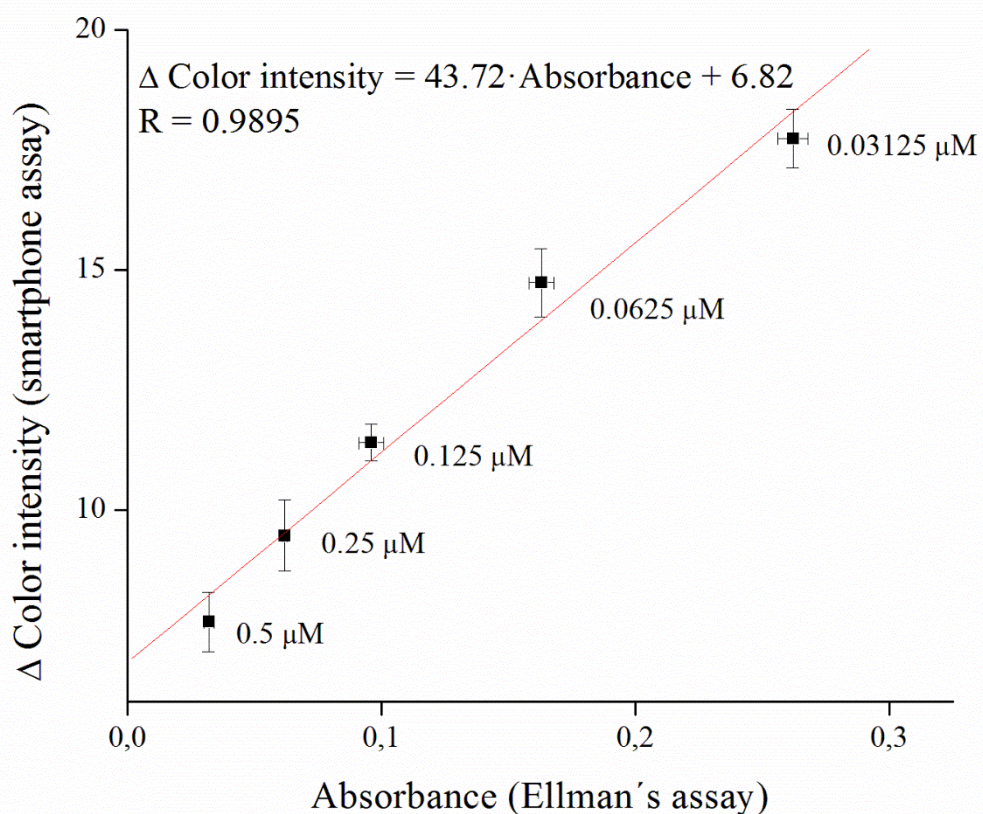


Figure 10. Validation of donepezil assay compared to standard Ellman's assay. Error bars for smartphone assay represent standard error of the mean and for Ellman's assay standard deviation for n = 3.

Verifying of donepezil in plasma samples were done as well. Donepezil was spiked in appropriate concentrations into pre-treated plasma samples and smartphone assay was performed (Figure 11). PBS buffer was also replaced by water for reason mentioned above. It appears that the both measurements are in a good correlation.

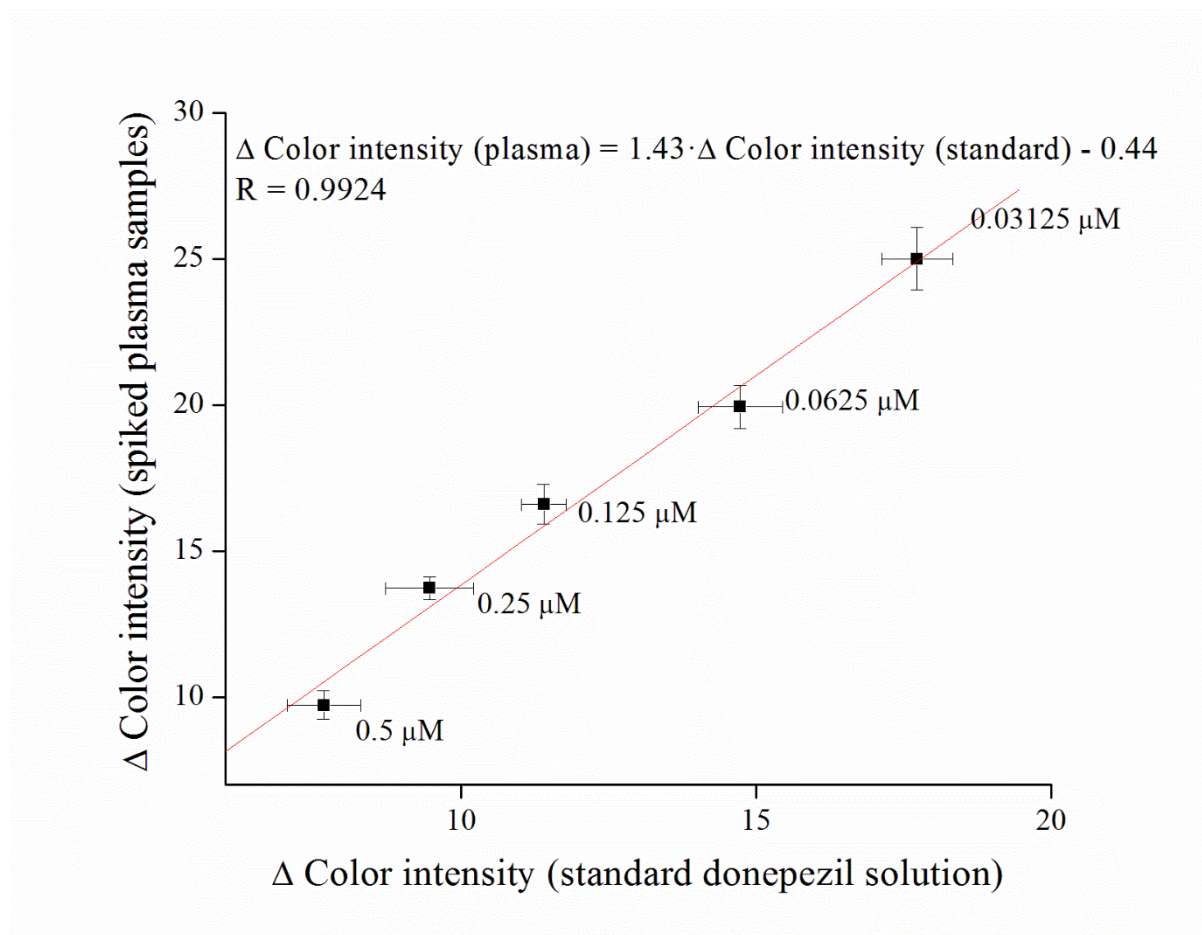


Figure 11. Verifying of donepezil assay in plasma samples compare to standard donepezil solution. Error bars represent standard error of the mean for $n = 3$.

3.5. Organic solvents

Because AChE activity can be reduced by organic solvents in small concentration [37], we decided to consider them as possible interferents in the AChE based assay. For this purposes ethanol, isopropyl alcohol and dimethyl sulfoxide were tested, all in 5 % concentration. Influence of DMSO to AChE activity has been investigated before and results showed that DMSO slightly decreases enzyme activity which was confirmed in our work [38]. Ethanol has been reported to decreases of AChE activity [37, 39], however there are evidence at low concentration it can enhance enzyme activity [38, 40]. No activity change against uninhibited enzyme was observed in our work. Isopropyl alcohol as well as ethanol, can drop AChE activity [41] which is in agreement with our results. To compare, used inhibitors donepezil and galanthamine are included in graph (Figure 12).

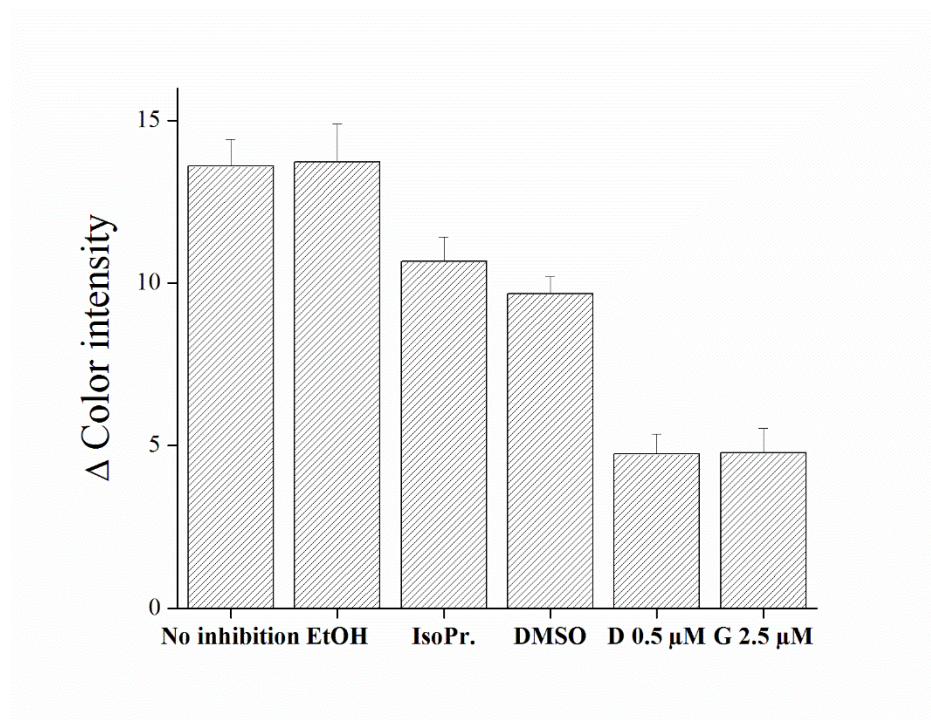


Figure 12. Method interferences by organic solvents. EtOH = ethanol, IsoPr. = isopropyl alcohol, DMSO = dimethyl sulfoxide, D 0.5 μ M = donepezil in 0.5 μ M concentration, G 2.5 μ M = galanthamine in 2.5 μ M concentration. Error bars represent standard error of the mean for $n = 3$.

4. Conclusion

Here presented method proved its ability for determination of neurotoxic compounds with promising limits of detection for galanthamine and donepezil, 149 nM and 22.3 nM respectively. Assay was successfully validated to the standard Ellman's spectrophotometric test and showed feasibility of measurement in plasma samples. Additionally, no specialized equipment and trained personnel are required, combine with low cost, portability, easy preparation and miniaturization are considered as big advantages of the here invented method. Comparison of presented method with standard Ellman's assay and literature is given in Table 1.

Table 1. Comparison of presented assay with Ellman's assay and literature

	Detection limit	Fabrication time	Assay time	Necessary equipment
Presented smartphone assay	Galanthamine: 149.1 nM Donepezil: 22.3 nM	Aprox. 1 hour	15 min	Smartphone
Standard Ellman's assay	Galanthamine: 18.3 nM Donepezil: 3.82 nM	NA	10 min	Spectrophotometer
Spectrophotometric assay [29]	Galanthamine: 0.05 nM	Aprox. 3.5 hours	Aprox. 20 mn	Spectrophotometer
Potentiometric	Galanthamine:	Aprox. 10	Aprox.	Electrodes,

assay [28]	5.4 nM	hours	10 min	potentiometr
Square-wave voltammetry [34]	Donepezil: 151 nM	NA	Aprox. 20 min	Electrodes, electrochemical analyser

Conflict of interest

The authors declare that there is no conflict of interest with regards to the publication of this paper.

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5. References

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