


A NOVEL COLORIMETRIC BIOSENSOR FOR DETERMINATION OF CATIONIC SURFACTANTS

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Authors have offered a novel colorimetric biosensor for determination of acetylcholinesterase (AChE) activity and its inhibitors on the example of cationic surfactant Benzalkonium chloride (BAC) in aqueous solutions by taking utilization of H_2O_2 – 4-ethoxyaniline (p-Ph) detection system. In the presence of AChE, acetylcholine (ACh) was hydrolyzed to choline and acetic acid. H_2O_2 could interact with unreacted ACh, and in situ formed CH_3CO_3H can oxidize p-Ph to azoxyphenetole (ox p-Ph), resulting in the developing light brown colour and improvement of absorbance at 350 nm. A colorimetric method, developed for estimating the acetylcholinesterase activity using ACh as the substrate, measures the rate of ox- p-Ph formation and assay of the cationic surfactant BAC, being highly sensitive with a lower detection limit at $6 \cdot 10^{-7}$ mol/L. The obtained assay is fairly simple, inexpensive, which may be used for screening the trace amount of cationic surfactants.

Keywords: cationic surfactant, colorimetric biosensor, Benzalkonium chloride.

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Introduction

Currently the monitoring of environment, in particular, permanent control of the presence of toxicants, has become a priority. Surfactants, in particular, quaternary cationic nitrogen atoms (QAC), are one of the widespread environmental pollutants.

Cationic surfactants are one of the most widespread environmental pollutants. They can be found in personal hygiene products, numerous washing and cleaning agents, etc. After being used, surfactants are discharged into the environment in huge volumes that result in contamination of water ecosystems [1, 2]. Moreover, due to their ability to increase solubility of other pollutants, they are found in water in higher concentrations [3].

The main analytical methods of cationic surfactants determination include different types of LC [4-8], spectrophotometry with extraction of liquid-liquids [9-13], spectrophotometry with solid-phase extraction [14], spectrofluorimetry (SFI) [15], colorimetry [16-18], and potentiometric determination with ISE [19, 20]. Nuclear Magnetic Resonance spectroscopy without the requirement for solvent extraction, dilution or filtration was proposed as well [12].

The complex and expensive equipment, highly skilled personnel to work on it, as well as time- and cost-consuming sample pretreatment are the limitations of the discussed

methods. Consequently, creation of more functional accurate, selective, fast and low-cost method is actually necessary.

It should be noted, that in recent years, biosensors become an accepted part of analytical chemistry [21]. The literature review presented several biosensors used to determine cationic surfactants. At this point, the development of biosensors seems to be a promising approach.

Cholinesterase is an enzyme vital for human organism. It is needed for proper functioning of the nervous system. Simultaneously, the use of Cholinesterase-based biosensors allows determining the trace amounts of surfactants in the environment. Due to its properties, Cholinesterase-based biosensors are worth paying attention to.

The detection schemes include amperometric [22, 23], potentiometric [24, 25], conductometric [26, 17], optical [16], fluorometric [27], and piezoelectric [28] transduction models.

Here we present an alternative method used to determine cationic surfactants, namely Benzalkonium chloride (BAC).

BAC belongs to a series of quaternary ammonium chloride homologues with the structure shown on Figure 1.

The pervasive use of BAC in consumer products results from its antiseptic and antifungal properties. It is widely applied: from cleaning products and disinfectants to sanitizing wipes and ophthalmic solutions.

Because of its extensive use BAC

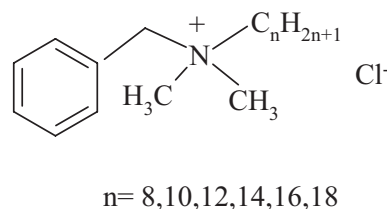


Fig. 1. Structure of Benzalkonium chloride. C_{12} , C_{14} and C_{16} homologues are the most common homologues found in consumer products

has been the subject of numerous studies, including the evaluation of its reactivity with the ocular tissue, and the wider study of municipal wastewater, which found BAC to be the most prevalent quaternary ammonium compound in wastewater, with concentrations ranging between 200 and 300 mg/L [20].

2. Materials and methods

For light absorbance of solutions "photoelectric concentration colorimeter («CPC-2»)" was used (Zagorsky Optical & Mechanical Plant, Russia). The filter No. 2 ($\lambda_{max} = 354$ nm) and quartz cell of 1.0 cm were used.

pH value was measured at Ionomer I - 160M laboratory (Belarus) by using the EGL 43-07 pH glass laboratory electrode together with auxiliary chloride silver electrode of EAL-1M3.1 type, saturated with potassium chloride.

For the research the following reagents were used: *p*-Phenetidine

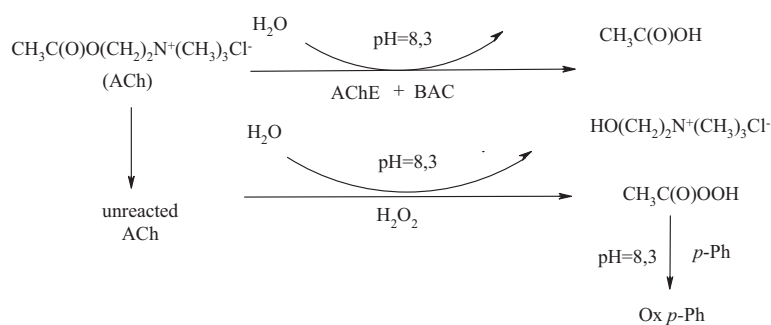


Fig. 2. Scheme of the reactions underlying the determination of Benzalkonium chloride by the enzyme-kinetic photometric method

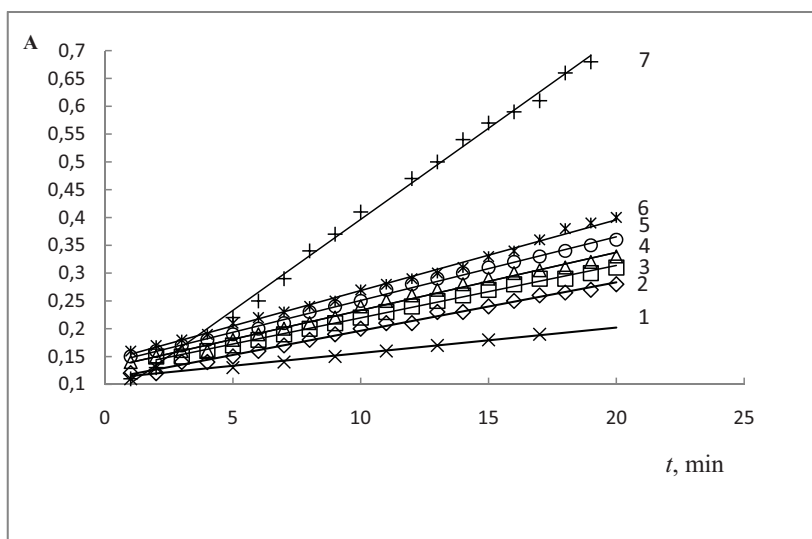


Fig. 3. Kinetic curves of couple oxidation of p-phenetidine by hydrogen peroxide in presence of the system: 1 - ACh+ChE, 2- 6 - ACh+(ChE+BAC), 7 - ACh. $w(\text{ACh}) = 0.1\%$; $\text{ChE} = 0.25 \text{ U}$; $c(\text{BAC}, 10^{-6} \text{ mol/L})$: 2-1.4, 3- 2.8, 4-3.4, 5 -5.6, 6 - 7.0.

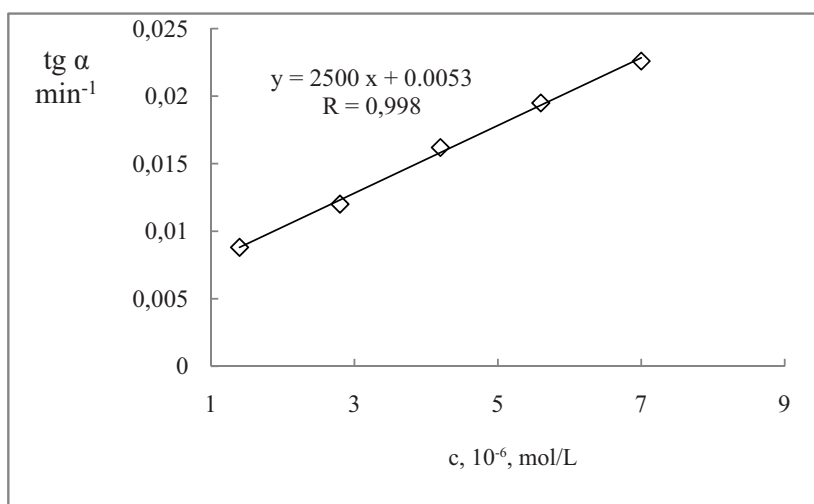


Fig. 4. Curve of the graduated dependence of the indicator reaction rate of ACh hydrolysis in presence of (ChE+BAC) vs. BAC concentration.

(4 - ethoxyaniline - 98%) (SIGMA hydrochloride (p-Ph), extracted from the - ALDRICH); A0281408 series, base by hydrogen chloride precipitation New Jersey, USA; p-Phenetidine in the chloroform solution.

Pharmacopoeial acetylcholine chloride drug - 0.2 g per amp/5 ml (manufactured by "VECTOR" - State Science Center of virology and biotechnology in Russian Federation) (Russia).

Dry protein drug of cholinesterase from horse serum was taken - 80 mg / fL (VI class), 22 AE / mg (manufactured by SMU "Biomed", Russia).

"Stabilized Hydrogen Peroxide 30-40%" (LLC "Inter - Synthes", Boryslav, Ukraine); The content of hydrogen peroxide was determined by SPU.

Benzalkonium chloride (Arquad MCB-50 alkylbenzyltrimethylammonium chloride, MW=352.5 g/mol), produced by «Akro Nobel Surface Chemistry AB», Stenungsund, Sweden.

Preparation of the solution of enzyme substrate of acetylcholine chloride (ACh)

The ampoule's content of pharmacopoeia drug acetylcholine chloride 0.2 g is dissolved in 200 ml of double-distilled water. For that end, open an ampoule, add 4.0 ml of water with pipette, and shake until acetylcholine is completely dissolved. Then pour the acetylcholine solution quantitatively transferred into the 200 ml capacity measuring bottle and dilute the double-distilled water to the required volume.

Cholinesterase solution preparation (ChE).

Add 10.0 ml of double-distilled water in a flask containing 80 mg of dry cholinesterase drug, shake and thermostate for 10 minutes at 38 °C.

Phosphate buffer solution preparation (pH 8.35).

Pour 35.75 g of disodium hydrogen phosphate into the 500 ml flask, add 300 ml of double-distilled water, dissolve it, add 19 ml of 0.1 mol/L solution of hydrochloric acid, stir and dilute double-distilled water up to 500.0 ml. The prepared solution of pH is potentiometrically controlled.

10% hydrogen peroxide solution.

It is prepared by the appropriate high-test hydrogen peroxide dilution with double-distilled water. The exact hydrogen peroxide content in the prepared 10% solution is determined permanganometrically.

1% p-Phenetidine hydrochloride solution preparation.

Dissolve 1.00 g of p-phenetidine hydrochloride in 80 ml of double-distilled water in the 100 ml measuring bottle and dilute it to volume.

Preparation of the working standard solution (WSS) $2.8 \cdot 10^{-5}$ mol/L

WSS is prepared in double-distilled water. Sweep down 0.09910 g (precisely weighed quantity) of the standard solution of Benzalkonium chloride in the 500 ml capacity measuring flask and dilute up to the mark. 1.00 ml of the prepared solution is transferred with pipette into the 100 ml measuring flask. After this, dilute with double-distilled water to volume at 20°C, cork the flask and mix thoroughly.

WSS was prepared containing $2.8 \cdot 10^{-5}$ mol/L of BAC.

Preparation of the Calibration Curve

In graduated test tubes with the ground plug gradually add phosphate buffer (pH = 8,4) - 10.0 ml of 0.2 mol / L in each one, respectively, from 1.00 ml to 5.00 ml of Benzalkonium chloride solution (WSS) and add 2.0 ml of cholinesterase while stirring; switch a timer, shake up each solution thoroughly and thermostate for 20 min, then quickly add 1.0 mL of 1% acetylcholine solution, switch on timer, shake thoroughly and thermostate for 10 min again, then add 2.0 ml of 10% hydrogen peroxide solution, keep for 10 min in the thermostat and add 1.0 ml of 1% p-phenetidine solution (p-Ph). Dilute distilled water to volume at 20 ml. Switch on timer and every other minute scan each solution photometrically for 20 min on the photoelectric colorimeter CPC-2, use colour filter No. 2 and 1.0 cm cuvette. Every time before shaking the test tube contents, plug it thoroughly. Buffered solution with double-distilled water is used as reference solution.

According to the optical-time relations the kinetic curves are plotted and the slope of the first 10 minutes is found. According to data received a slope-finite analyte concentration calibrated relation is obtained, $c, \mu\text{mol/L}$. A calibration curve equation is solved by the least squares method (Linear regression): $tg\alpha = b \cdot c + a$, where a, b are Y-axis intercept and slope, (tg α , min⁻¹) respectively.

Standard technique for determining Benzalkonium chloride in a sample of model solution.

In graduated test tubes with ground plug gradually add phosphate buffer (pH = 8.4) - 10.0 ml of 0.2 mol / L, than a certain amount (volume) (1.0 - 5.0 ml) of test inhibitor and carry out determination like in

an experiment of the "Calibration Curve". All experiments were repeated 5 times. Concentration of BAC in standardized test solution is calculated using the formula:

$$C_x = \frac{tg\alpha - a}{b}$$

tg α - is a slope, available from operational experiment, min⁻¹;

Tab. 1.

Metrological characteristics of results of Benzalkonium chloride kinetic determination in model solutions

No.	BAC taken, mol /L	BAC found, n, 10 ⁻⁶ mol /L	Metrological characteristics (P=0.95, n=5))
1.	$1.41 \cdot 10^{-6}$	1.33 1.37 1.41 1.42 1.45	$\bar{X} = 1.39 \cdot 10^{-6}$ $S = 0.05 \cdot 10^{-6}$ $S\bar{x} = 0.02 \cdot 10^{-6}$ $\Delta \bar{x} = 0.06 \cdot 10^{-6}$ $RSD = 3.34\%$ $\delta = 1.42\%$
2.	$2.815 \cdot 10^{-6}$	2.87 2.73 2.85 2.83 2.83	$\bar{X} = 2.82 \cdot 10^{-6}$ $S = 0.05 \cdot 10^{-6}$ $S\bar{x} = 0.02 \cdot 10^{-6}$ $\Delta \bar{x} = 0.75 \cdot 10^{-6}$ $RSD = 1.91\%$ $\delta = 0.18\%$
3.	$4.22 \cdot 10^{-6}$	4.32 4.19 4.22 4.22 4.23	$\bar{X} = 4.23 \cdot 10^{-6}$ $S = 0.05 \cdot 10^{-6}$ $S\bar{x} = 0.02 \cdot 10^{-6}$ $\Delta \bar{x} = 0.06 \cdot 10^{-6}$ $RSD = 1.2\%$ $\delta = -0.24\%$
4.	$5.62 \cdot 10^{-6}$	5.60 5.69 5.55 5.69 5.62	$\bar{X} = 5.63 \cdot 10^{-6}$ $S = 0.06 \cdot 10^{-6}$ $S\bar{x} = 0.03 \cdot 10^{-6}$ $\Delta \bar{x} = 0.075 \cdot 10^{-6}$ $RSD = 1.00\%$ $\delta = 0.18\%$
5.	$7.00 \cdot 10^{-6}$	7.00 7.05 7.09 7.01 7.00	$\bar{X} = 7.03 \cdot 10^{-6}$ $S = 0.039 \cdot 10^{-6}$ $S\bar{x} = 0.01 \cdot 10^{-6}$ $\Delta \bar{x} = 0.05 \cdot 10^{-6}$ $RSD = 0.56\%$ $\delta^* = -0.47\%$

$$\delta^* = (\bar{X} - \mu) \cdot 100\% / \mu$$

a, b - Y-axis intercept and slope of calibration curve equation ($tg\alpha = bc + a$), respectively.

Results and their consideration:

We proposed a new sufficiently sensitive original biosensor for determining small amounts of surfactants – acetylcholinesterase inhibitors.

The functioning of a biosensor is based on the ability of surfactants to inhibit the catalytic activity of the enzyme acetylcholinesterase (AChE) in the hydrolytic reaction of decomposition of acetylcholine-substrate (ACh). At present the mechanism of competitive inhibition effect on enzymatic hydrolysis of acetylcholine by surfactants was established: anionic active site on the surface of AChE interacts with positively charged nitrogen atom surfactants (BAC), which prevents sorption of positively charged substrate of acetylcholine and thus leads to slower reaction of its hydrolysis [30].

Biosensor work is based on the conjugated system of two consecutive reactions - perhydrolysis of acetylcholine and the caused reaction of peroxyacetic acid oxidation of p-Phenetidine. As a result of the last indication the reaction of azoxyphenetole (ox p-Ph) was produced, which is capable to provide intense light absorption. Measuring the rate of change of the absorption of light in time (conditional reaction rate) can determine the content of a surfactant - AChE inhibitor (Fig. 2)

It was found that in the range of pH 8.2-8.5 the rate of formation of 4-azoxyphenetoll (as the result of oxidation of p-Phenetidine by peracetic acid, which was formed in the previous reaction of perhydrolysis of residue acetylcholine) is strictly proportional to the concentration of inhibitor [31].

Figure 2 shows the kinetic curves of couple oxidation of p-phenetidine by hydrogen peroxide in presence of different concentrations of BAC with a linear character at the initial stage. This enables the use (in order to assess the reaction rate) of slope angle tangent (angular coefficient of slope) of the derived kinetic lines, built in the coordinates optical density (A) - time (t, min) min^{-1} as the value of the analytical signal, corresponding to a certain content of an inhibitor in a sample.

The resulting dependence of the calibration indicator reaction ($tg\alpha$, min^{-1}) on concentrations of BAC allowing to determine it in the model solutions, was shown in Fig. 3. The equation of calibration curve looks like: $tg\alpha = 2500c + 0.0053$ ($r = 0.998$).

Table 1 shows the results of quantitative determination BAC in model solutions by the calibration curve.

Limit of quantitation (LOQ) was calculated of the reaction (which was $0.6 \cdot 10^{-6}$ mol/L) was calculated as:

$$LOD = 10 \frac{S\alpha}{b}$$

a – free term in the equation calibration curve $tg\alpha = 2500 \cdot c + 0,0053$ ($r = 0,998$);

b – angular coefficient in the equation calibration curve.

When determining the BAC in the concentration range of $1.4 \cdot 10^{-6}$... $7 \cdot 10^{-6}$ mol/L, the $RSD \leq 3.34\%$ ($\delta = -0.47... +1.42$). Since $\delta < RSD$ the results of the analysis are accepted as correct.


Conclusion

A new sensitive and specific enzyme-kinetic method for determination of cationic surfactant BAC in water solutions was presented. LOQ of the reaction was calculated, which was $6 \cdot 10^{-7}$ mol/L. The method has satisfactory reproducibility and accuracy. When determining the concentration of BAC within $1.4 \cdot 10^{-6}$... $7 \cdot 10^{-6}$ mol/L in model solutions the RSD were $\leq 3.34\%$ ($\delta = +1.42\% \dots -0.47\%$).


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