ENZYMATIC SENSOR FOR ALCOHOLS DETERMINATION

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Abstract
A simple, rapid and sensitive sensor for ethanol, *n*- and *iso*propanols determination based on these alcohols oxidation in the presence of alcoholdehydrogenase from baker's yeast (YADH) was proposed. The optimum conditions (nature, pH and ionic strength of buffer solutions; concentrations of the reagents) of the indicator reaction for methanol, ethanol, propanol and *iso*propanol determination were established. The kinetic parameters (K_M and V_m) of the indicator reaction in the presence of different alcohols were calculated. The high specificity of YADH towards ethanol was used for its selective determination (c_{min} = 0,006 *v/v*%) in the presence of other alcohols. The possibility of the individual determination of propanol (0,007 *v/v*%) and *iso*propyl alcohol (0,08 *v/v*%) after their preliminary separation was shown. Flow-injection system for ethanol determination was developed.

Introduction

Alcohols as metabolites of different biochemical processes are widespread in environment; besides ethanol and methanol are used as a vehicle fuel in some countries (for instance in Brazil). Therefore monitoring of aliphatic alcohols is of great importantce.
The widely used methods for their determination such as chromatography (1), refractometry and steam distillation (2) require expensive equipment and long time for analysis. However several simple, rapid and sensitive alcohols sensors based on enzymatic or microorganisms activity were reported (3-4).
Alcohol dehydrogenase from baker's yeast was chosen for a new sensor producing. YADH belongs to the class of oxidoreductases and catalyses primary aliphatic alcohols oxidation to corresponding aldehydes by β-nicotinamide adenine dinucleotide (NAD^+):

$$R\text{-}CH_2OH + NAD^+ \xleftrightarrow{ADH} R\text{-}CHO + H^+ + NADH.$$

Determination of aliphatic alcohols using YADH is the goal of this work.

Methods

Reagents
Solid preparation of YADH (E.C. 1.1.1.1, "Sigma", USA) was used. Solutions with the enzyme concentration of 3 μM were prepared by dissolving accurately weighed amounts of solid YADH in phosphate buffer solution (pH 7,6). Solutions with a lower enzyme concentration were prepared daily by dilution of an initial solution with phosphate buffer (pH 7,6). Solid simple and solutions of the enzyme were stored at +4°C.
β-Nicotinamide adenine dinucleotide (NAD^+) from baker's yeast ("Sigma", USA) was used. Solutions of NAD^+ were prepared daily by dissolution of accurately weighed amounts of the solid coenzyme in water.

Solutions of alcohols were prepared by dilution of their concentrated solutions in water.
0,05-0,2 M TRIS (pH 9,0), 0,05-0,2 M sodium pyrophosphate (pH 9,0) and 0,1 M phosphate (pH 7,6) buffers were used.
Water purified by "Simplicity" system ("Millipore", France) was used throughout.

Instrumentation
The absorbance of solutions was measured with photoelectrocolorimeter KFK-2 (Russia) (λ_{eff} = 315 nm).
Plunger pump ("Akvilon", Russia) was used to pump carrier at flow-rate of 0,3 – 1 mL/min. A Rheodyne Model 7725i sample injection loop was used to inject a 20-μL sample aliquot into the career. Absorption of flow solution was measured by spectrophotometric detector UVV 104 ("Akvilon", Russia) at λ=315 nm.
The pH of the buffer solutions was measured by pH-millivoltmeter "Econix-expert" (Russia).

Measurement of the reaction rate
The rate of the indicator reaction of alcohols oxidation was monitored by spectrophotometric method measuring an increase in absorbance of solutions due to NADH formation. The reaction rate was characterized by a slope (tanα) of the initial portion of kinetic curves on the absorbance-time coordinates.

Results and discussion
Determination of individual alcohols
The optimum conditions for the oxidation of methanol, ethanol, *n*- and *iso*propanols were studied. The enzyme and cofactor concentrations, which are optimum for spectrophotometric control of the rate of the different alcohols oxidation are presented in Table 1.

Table 1: Optimum concentrations of the components of the reactions of enzymatic oxidation of aliphatic alcohols (pH 9,0)

Reagent	Ethanol	*n*-Propanol	*iso*-Propanol
NAD^+	0,45 mM		
YADH	0,01 μM	0,03 μM	0,2 μM
TRIS	0,05 – 0,1 M	0,05 – 0,1 M	0,2 M
Pyrophosphate	0,05 – 0,2 M	0,05 – 0,2 M	0,2 M

The dependences of the rate of enzymatic oxidation of above-mentioned alcohols on pH and nature of different buffer solutions were studied. Maximum conversion of all alcohols in the presence of YADH is achieved at pH 9,0.
The character of the dependence of the rate of ethanol oxidation on pH in different buffer solutions is presented in Figure 1 by an example.
It was stated that the oxidation rate of all studied alcohols was higher in pyrophosphate buffer solution.

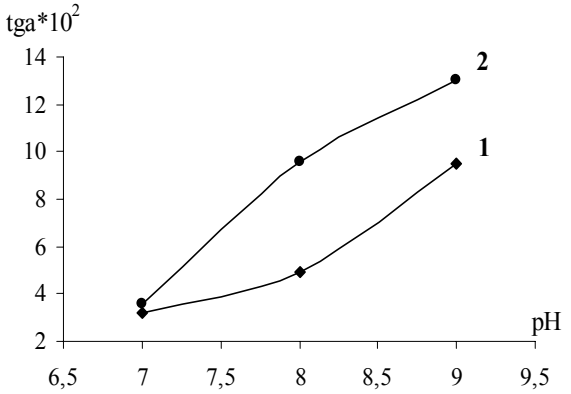


Fig.1 pH-dependence of the rate of ethanol oxidation in the presence of YADH (1 – TRIS, 2 – pyrophosphate buffer solution)

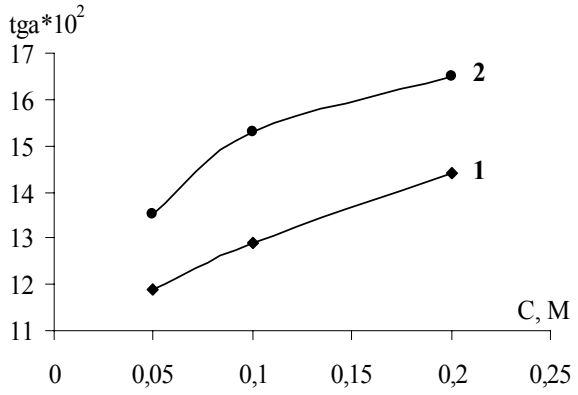


Fig.2 Influence of the buffer solution concentration on the rate of *iso*propanol oxidation in the presence of YADH (1 – TRIS, 2 – pyrophosphate buffer solution)

It has been shown that the rate of *iso*propanol oxidation depends on the concentration of the buffer solution (Figure 2). This alcohol is the least polar among all studied alcohols and its mobility and reactivity become higher with increasing the buffer solution concentration.

Optimum concentrations of buffer solutions for enzymatic YADH oxidation of individual ethanol, *n*- and *iso*propanols are presented in Table 1.

It should be noted that methanol with concentration of 0,03 – 0,6 μM is not oxidized by NAD^+ in the presence of YADH solution.

The values of Michaelis constant (K_M) and maximum rate of different alcohols oxidation (V_m) were calculated (Table 2).

The same V_m values for different alcohols mean that the selected conditions are actually optimum. The studied alcohols may be placed in the following sequence of YADH's subsrate specificity according to K_M values:

Ethanol > *n*-Proranol > *iso*-Proranol.

Table 2: Kinetic parameters of alcohols oxidation in the presence of YADH

	Kinetic parameters	Ethanol	*n*-Propanol	*iso*-Propanol
0,1 M TRIS buffer solution (pH 9,0)				
Lineweaver-Burk plot	$K_M \times 10^2$, M	1,0	2,7	9,7
	$V_m \times 10^4$	2,0	1,7	2,0
Eadie-Hofstee plot	$K_M \times 10^2$, M	1,0	2,8	10,4
	$V_m \times 10^4$	2,0	2,0	2,0
0,1 M pyrophosphate buffer solution (pH 9,0)				
Lineweaver-Burk plot	$K_M \times 10^2$, M	1,1	5,5	9,5
	$V_m \times 10^4$	1,7	1,4	2,0
Eadie-Hofstee plot	$K_M \times 10^2$, M	1,3	5,4	10,4
	$V_m \times 10^4$	2,0	1,5	2,0

The comparison of K_M-values in TRIS- and pyrophosfate buffer solutions for the reaction of different alcohols oxidation demonstrates the absence of any effect of buffer nature on YADH specificity. It should be mentioned that the indicator reaction rate and therefore sensitivity of the determination of all alcohols in pyrophosphate buffer solution are higher (Table 3).

Table 3: Analytical characteristics of the procedure for alcohols determination in the presence of YADH in TRIS (I) and pyrophosphate (II) buffer solutions

	Alcohol	Applicable concentration range, mM	Calibration equation	RSD, %
I	Ethanol	1 – 50	y = 89,6x + 1,4	10
II		1 – 50	y = 102,2x + 3,6	9
I	*n*-Propanol	5 – 20	y = 238,3x + 0,7	6
II		1 – 20	y = 4,5x + 0,2	3
I	*iso*-Propanol	10 – 100	y = 56,6x + 0,8	10
II		10 – 100	y= 76,8x + 0,8	10

Interference of different alcohols with ethanol determination

The highest YADH specificity to ethanol can be used for its individual determination in the presence of methanol, *n*- and *iso*propanols. It was stated that 0,005 M ethanol might be determined in the presence of 10-fold excess of *iso*propanol.

The optimum conditions of ethanol and *n*-propanol oxidation in the presence of YADH are almost similar. Therefore even 0,005 M *n*-propanol influences the determination of 0,01 M ethanol (Figure 3). This effect can be overcome by decreasing the enzyme concentration. As the result, the rate of *n*-propanol oxidation decreases and becomes sufficiently low to be ignored. It was stated that 0,01 M ethanol can be determined in the presence of equimolar amount of *n*-propanol.

30-fold excess of methanol prevents the determination of 0,005 M ethanol although the individual methanol isn't oxidized by NAD^+.

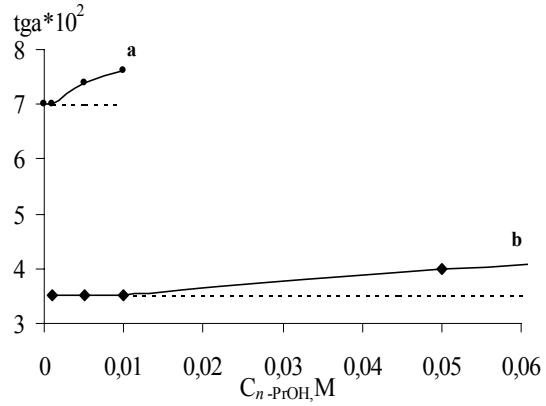


Fig.3 Influence of *n*-propanol concentration on the rate of 0,01 M ethanol oxidation in the presence of 10 nM (a) and 5 nM (b) of YADH

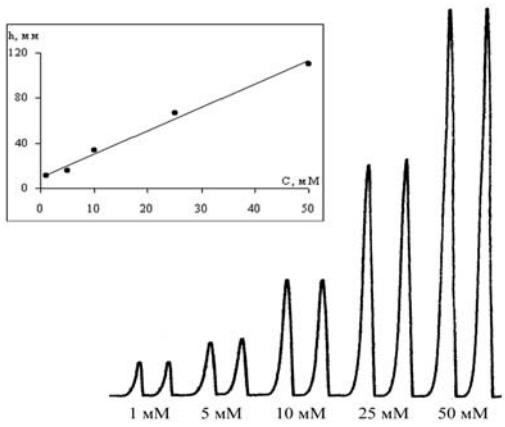
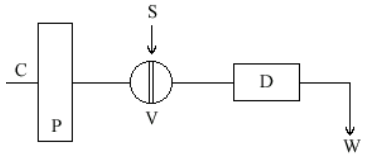


Fig.4 FIA-gramm and the dependence of the signal value of flow-injection system on the concentration of ethanol

Enzymatic flow-injection determination of ethanol
Flow-injection system for individual ethanol determination under selected optimum conditions of the indicator reaction was developed. Scheme of this system is shown in the figure:



The solution (C) containing YADH and NAD^+ in pyrophosphate buffer passed through the pump (P) and injection valve (V). The solution containing ethanol was injected to the stream through a valve (V). NADH absorption was used as analytical signal for conversion control.
Under the flow rate of 0,5 ml/min the detecting signal dispersion is minimal, but the signal value is appropriate for the detection.
It was shown that a signal value was directly proportional to ethanol concentration within the range of 1-50 mM (Figure 4). The detection limit of ethanol is 1 mM (0,006 *v/v*%). Selectivity of flow-injection determination of ethanol in the presence of other alcohols and its determination in stationary regime is the same. A sample frequency of 30 h^{-1} was achieved.
The developed procedure was used for ethanol determination in beer and different wines. The results are presented in Table 4.

Table 4: The results of ethanol determination in beer and wines

Sample	Ethanol concentration, *v/v*%	
	Announced	Found
Beer “Zolotaya Bochka” (Russia)	> 4	4,4 ± 0,2
White dry wine “Sieur de Trinquelage” (France)	12,5	12,6 ± 0,4
White sweet wine “Muskat” (Moldova)	11 – 13	11,4 ± 0,3
Red sweet wine “Zori Moskovskie” (Russia)	9 – 11	10,5 ± 0,5

Conclusion
The enzymatic procedures for the determination of ethanol (C_{min}=1 mM), *n*- (C_{min}=1 mM) and *iso*propanols (C_{min} = 10 mM) were developed.
The procedure of flow-injection ethanol determination (C_{min}=1 mM, RSD=3%) was developed and used for analysis of different beverages.

References
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