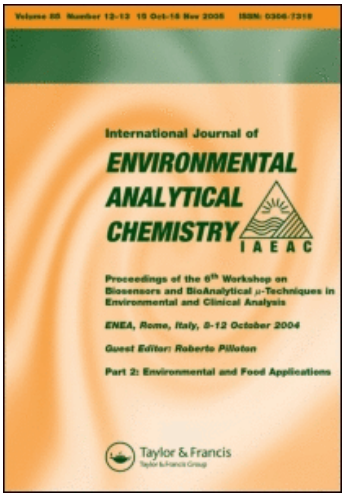

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Photometric assay of methanol and formaldehyde in industrial waste-waters using alcohol oxidase and 3-methyl-2-benzothiazolinone hydrazone

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Photometric assay of methanol and formaldehyde in industrial waste-waters using alcohol oxidase and 3-methyl-2-benzothiazolinone hydrazone

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An enzymo-chemical method using alcohol oxidase (AO) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) for the simultaneous analysis of methanol and formaldehyde in mixtures, including industrial waste-waters, is described. The enzyme oxidizes methanol to formaldehyde, while MBTH plays a double role: (1) in the first step of the reaction, it forms a colourless azine adduct with pre-existing and enzymatically formed formaldehyde and prevents its further oxidation by AO; (2) in the second step, non-enzymatic oxidation of azine product to cyanine dye occurs in the presence of ferric ions in acid medium. Pre-existing formaldehyde content is evaluated by a colorimetric reaction with MBTH before sample treatment with AO, and methanol content is determined by the increase in a coloured product after enzymatic oxidation of methanol to formaldehyde. The possibility of a differential assay of methanol and formaldehyde by the proposed method has been proved for model solutions, for real samples of technical formalin and real industrial waste-waters from the Pustkow plant for phenol-formaldehyde resins (Poland). The sensitivity of the assay for both analytes approaches 30 ng of analyte per millilitre of reaction mixture, that is 3.2-fold higher than with the chemical method with the use of permanganate and chromotropic acid. The linearity of the calibration curve is significant ($p < 0.0001$), and the standard deviation does not exceed 7%.

Keywords: Methanol; Formaldehyde; Photometric assay; Enzymo-chemical method; Alcohol oxidase; 3-Methyl-2-benzothiazolinone hydrazone

1. Introduction

Simple, fast, sensitive, and reliable methods for monitoring toxic pollutants in environment, commercial products and food are becoming increasingly important.

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Formaldehyde and methanol are considered as the most important commercial chemicals due to their broad application in industrial synthesis of a large number of organic compounds. Methanol is used for the synthesis of formaldehyde and other compounds, and is also used as an organic solvent. Formaldehyde is employed as a chemical intermediate for production of plastics and different consumer goods (detergents, soaps, and shampoos), and as a preservative in pharmacology and medicine [1]. Recently, a new risk factor associated with formaldehyde has been revealed. Some advanced technologies of potable water pre-treatment include the ozonation process during which formaldehyde is generated as a result of ozone interaction with humus traces [2]. At the same time, formaldehyde is a natural metabolite of living organisms. It has been found in fruits, vegetables, flesh, and biological fluids of human origin [1]. Some frozen fish, especially of gadoid species, can accumulate up to 200 mg of formaldehyde per kilogram of moist weight due to enzymatic degradation of a natural fish component, trimethylamine oxide [3, 4]. Formaldehyde is classified as a mutagen and possible human carcinogen [5], one of the chemical mediators of apoptosis, i.e. programmed cell death. These considerations are sufficient to demonstrate a need for monitoring formaldehyde content in the environment, industrial and consumer goods, as well as in food and biological liquids. Such control requires the development of novel methods for detection of this extremely toxic agent. Among the novel analytical approaches suitable for this aim, enzymatic [6, 7] and biosensor methods [8–17] could be of a great importance if stable and cheap enzymes of the required selectivity are available.

In spite of a high demand, no suitable enzymatic methods and corresponding enzymatic kits for formaldehyde assay are available on the world market. This could be explained by the fact that among formaldehyde-selective enzymes of bioanalytical importance, only two representatives are produced commercially, namely bacterial NAD-dependent formaldehyde dehydrogenase from *Pseudomonas putida* [18, 19], and NAD- and glutathione-dependent formaldehyde dehydrogenase from the yeast *Candida boidinii* [20, 21]. These enzymes are expensive; they require the addition of one or two co-factors into the analysed mixture, and their stability is insufficient. Obviously, analytical application of the above-mentioned enzymes is more rational in biosensor technologies when the corresponding bioselective element is immobilized and thus could be used many times [9, 10, 12, 13].

Quite often, methanol is present in formaldehyde solutions, e.g. in formalin or real waste-waters of chemical plants producing and utilizing formaldehyde, e.g. at plants producing phenol-formaldehyde or phenol-urea polymers [1]. Methanol normally is added to formaldehyde solutions to stabilize the last compound and prevent its spontaneous polymerization. If the analysis of formaldehyde is a rather complicated task, one can imagine how complicated it would be to analyse both formaldehyde and methanol in a mixture of these compounds.

Alcohol oxidase (AO) from the cells of the thermotolerant methylotrophic yeast *Hansenula polymorpha* [22] or from *Pichia pastoris* [23, 24] can be considered as an alternative to formaldehyde dehydrogenases, regarding use for analytical purposes. This enzyme is fairly stable, contains tightly bound FAD, and does not need any exogenous co-enzyme for catalytic activity. AO is able to oxidize primary alcohols to

aldehydes, and formaldehyde, which in the hydrated form is structurally similar to alcohols, is oxidized to formic acid (see reactions 1–3):

$$RCH_2OH + O_2 \xrightarrow{AO} RCHO + H_2O_2 \quad (1)$$

$$CH_2O + H_2O \leftrightarrow HOCH_2OH \quad (2)$$

$$HOCH_2OH + O_2 \xrightarrow{AO} HCOOH + H_2O_2. \quad (3)$$

Previously, we have proposed an oxidase–peroxidase-based method to evaluate formaldehyde content in hake and similar fish food [4]; it is based on the use of an enzymatic kit, 'Alcotest', that we have developed [25]. However, direct application of this method for sample analysis simultaneously containing formaldehyde and methanol is not possible, because AO oxidizes both substrates.

The aim of this article is to develop a new enzymo-chemical method for simultaneous assay of methanol and formaldehyde in their mixtures using alcohol oxidase (AO) and an aldehyde-selective reagent, 3-methyl-2-benzothiazolinone hydrazone (MBTH) [26, 27]. The enzyme is used for methanol oxidation to formaldehyde, while MBTH plays a double role: (1) in the first step of reaction, it forms a colourless azine adduct with pre-existing and enzymatically formed formaldehyde and prevents its oxidation by AO; (2) in the second step, non-enzymatic oxidation of azine product to cyanine dye occurs in the presence of ferric ions in acid medium. Besides, 3-methyl-2-benzothiazolinone hydrazone (MBTH) does not inhibit the AO reaction. Pre-existing formaldehyde content is evaluated by a colorimetric reaction with MBTH without treating samples by AO, and methanol content is determined by an increase in coloured product content after the enzymatic methanol oxidation to formaldehyde is complete.

The proposed method has been compared with other analytical approaches for assaying target analytes, namely: for formaldehyde, a standard method using chromotropic acid; and for methanol, gas chromatography and a method recommended by the Polish Standard [28], which is based on methanol oxidation by permanganate under strongly acidic conditions followed by determination of the product formed by the chromotropic method. The proposed method has been tested for a differential assay of methanol and formaldehyde in model mixtures as well as in real samples of technical formalin and waste-waters.

2. Experimental

2.1 *Materials*

MBTH and 3-(*N*-morpholino)propanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO); chromotropic acid was purchased from Chemapol (Prague); $FeCl_3$ was purchased from Aldrich (Milwaukee, WI); and paraformaldehyde was purchased from Serva (Heidelberg, Germany). All other chemicals were of the highest quality available in Ukraine and Poland.

2.2 *Preparation of samples*

The following samples were used to test the analytical method: model mixtures of formaldehyde and methanol with different molar ratios of both analytes, waste-water from a Polish factory in Pustkow from a final phase of treatment procedure (a so-called 'waste-water pool') taken at different times from different places in the pool, and a commercial preparation of formalin.

The waste samples chosen were frozen immediately and stored at −25°C. An assay of formaldehyde and methanol in their distillates were obtained according to the recommendations of the corresponding Polish Standard PN-71C/04568 [28]. After distillation, samples were diluted (two- to 100-fold), immediately analysed or frozen, and stored at −25°C before analysis.

A chemical assay of formaldehyde and methanol was carried out according to Polish Standard PN-71C/04568 [28] using chromotropic acid and chemical oxidation of methanol to formaldehyde by permanganate. For this, 0.2 mL of diluted samples was mixed with 1.4 mL of water and 2.4 mL of 4% solution of chromotropic acid ('Chemapol') in concentrated sulfuric acid. Samples were incubated for 10 min in a boiling water bath, cooled, and their optical density at 570 nm measured against a blank containing water instead of the test sample. Formaldehyde content in the samples was calculated using a treatment of formaldehyde calibration solution (initial concentration: 20 mg L^{-1}; addition of 0.2 mL into the reaction mixture) with regard to the initial dilution of the samples.

A formaldehyde calibration solution was prepared by hydrolysis of paraformaldehyde ('Serva') in water (1 M concentration) in a sealed ampoule at 105°C for 12 h and by dilution of hydrolysate to the necessary concentration.

A formaldehyde assay using MBTH [26, 27] was carried out by mixing 0.2 mL of diluted samples and 1.8 mL 0, 05% MBTH-hydrochloride ('Sigma') solution in 25 mM 3-(*N*-morpholino)propanesulfonic acid/KOH buffer (MOPS–K^+ buffer), pH 7.0. Samples were incubated for 15 min at 30°C, and 2 mL of 0.1% $FeCl_3$ solution in 30 mM HCl was added. Mixtures were incubated for 20 min at 30°C, and their optical densities were measured at 670 nm against a blank containing all components except a sample. The formaldehyde content in the test samples was calculated using a calibration curve.

An enzymo-chemical assay of methanol was carried out using AO as a methanol oxidizing agent and MBTH as a reagent for colorimetric determination of generated formaldehyde. The preparation of AO (EC 1.4.3.6) used was isolated from the cells of AO-overproducing catalase-minus mutant strain of the methylotrophic yeast *Hansenula polymorpha* C-105 (*gcr1 catX*) according to Gonchar *et al.* [25]. The specific activity of the enzyme preparation was 4 μmol min^{-1} mg^{-1} of protein (4 U mg^{-1}).

The analysis was performed as follows. A test sample (0.2 mL) was added to 1.8 mL of reaction mixture containing AO (final activity 0.5 U mL^{-1}), 0.05% MBTH-hydrochloride in 25 mM MOPS–K^+ buffer (3-morpholinopropanesulfonic acid-KOH), pH 7.0. After incubation for 15 min at 30°C, 2 mL of 0.1% solution of $FeCl_3$ in 30 mM HCl was added. After a 20-min colouring reaction at 30°C, the optical density of samples at 670 nm was measured. For calibration, methanol solution (5 mg L^{-1}) was used. Methanol and formaldehyde contents were calculated using corresponding calibration solutions.

A methanol assay using gas chromatography [29] was carried out on a 'Crystall-2000' chromatograph using a flame-ionization detector according to a standard procedure with some modifications to allow analysis of methanol content in the presence of high levels of formaldehyde. For a better resolution of the indicated compounds, 15% Apiezon-L on a column Chromaton N-AW-HMDS (0.2–0.25 mm) was used as a carrier. The methanol and formaldehyde were separated under the following conditions: air flow rate 300 $cm^3 min^{-1}$; hydrogen flow rate 30 $cm^3 min^{-1}$; carrier gas flow rate 40 $cm^3 min^{-1}$. The column, evaporator, and detector temperatures were 170, 200, and 250°C, respectively.

3. Results and discussion

In this work, we describe a novel enzymo-chemical method for a simultaneous assay of methanol and formaldehyde in a mixture of both compounds, based on the use of AO-mediated oxidation of methanol to formaldehyde with subsequent determination of the last compound with an aldehyde-specific reagent, MBTH. Pre-existing formaldehyde content is detected without treating samples by AO (CD_0 in reaction 4; and methanol content is determined by the increase in coloured product amount due to the methanol-oxidizing reaction (CD_Δ in reaction 5).

$$[CH_2O]_0 + \mathrm{MBTH} \rightarrow \underset{\text{azine}}{[\mathrm{MBTH} - CH_2O]_0} \xrightarrow{FeCl_3} \underset{(\lambda_{max} = 670\,\mathrm{nm})}{\text{Cyanine dye } (CD_0)} \tag{4}$$

$$CH_3OH + O_2 \xrightarrow[-H_2O_2]{\mathrm{AO}} [CH_2O]_\Delta \xrightarrow{\mathrm{MBTH}} [\mathrm{MBTH} - CH_2O]_\Delta \xrightarrow{FeCl_3} \text{Cyanine dye } (CD_\Delta). \tag{5}$$

The rationale of the proposed method is as follows. Methanol is oxidized by AO to formaldehyde. In the presence of MBTH and AO, formaldehyde reacts with MBTH, forming an azine adduct that prevents any further enzymatic oxidation of formaldehyde by AO. Thus, MBTH in the proposed method plays a dual role: it serves as a chemical 'trap' (masking agent) for formaldehyde, thus avoiding its oxidation by AO to formate as well as a reagent for formaldehyde in the next reaction with Fe(III) in which the azine adduct is converted into a cyanine dye of tetraazopentametine nature (see below).

To check the reliability of the proposed enzymo-chemical method for simultaneous detection of methanol and formaldehyde in mixtures, model solutions containing these analytes separately or in mixtures were prepared. The formation of coloured product with MBTH in the presence of Fe^{3+} ions with and without AO in the first step of the reaction was studied. The dependence of the optical density of the reaction mixture on the analyte concentration without AO is illustrated in figure 1. It was found that only formaldehyde (line A), not methanol and formic acid (line B and C, respectively), forms a coloured product with MBTH. AO addition at the first step of the reaction in the presence of MBTH resulted in oxidation of methanol to formaldehyde, to form a coloured product (figure 2). Linear regression coefficients for line A in figure 1 and the line in figure 2, which correspond to micromolar extinctions of reaction products, are nearly equal: 0.0435 ± 0.001 and 0.0433 ± 0.001, respectively. The very high similarity of

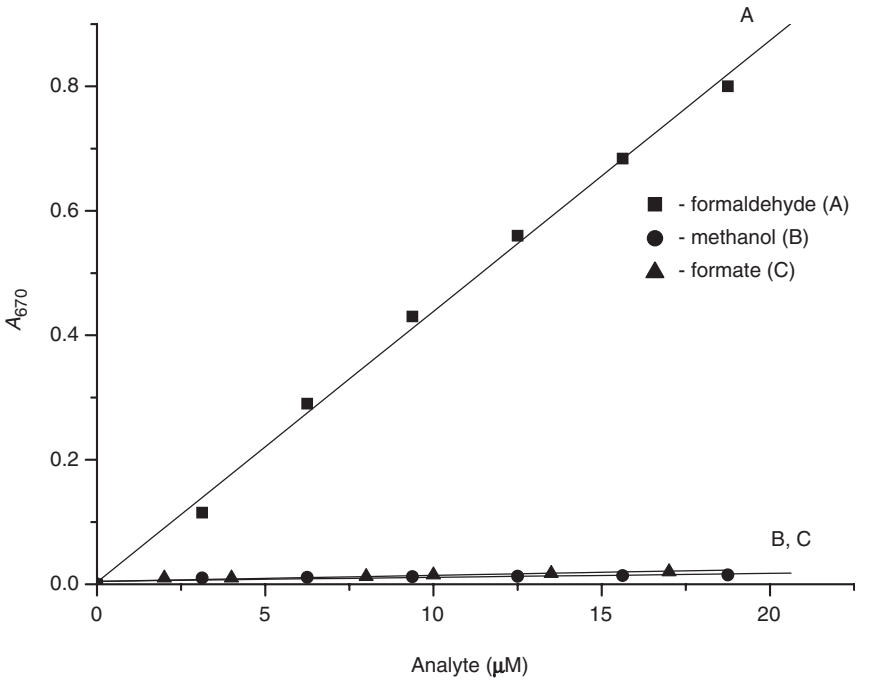

Figure 1. Dependence of the optical density of samples (A_{670}) on analyte concentration (without the addition of AO).

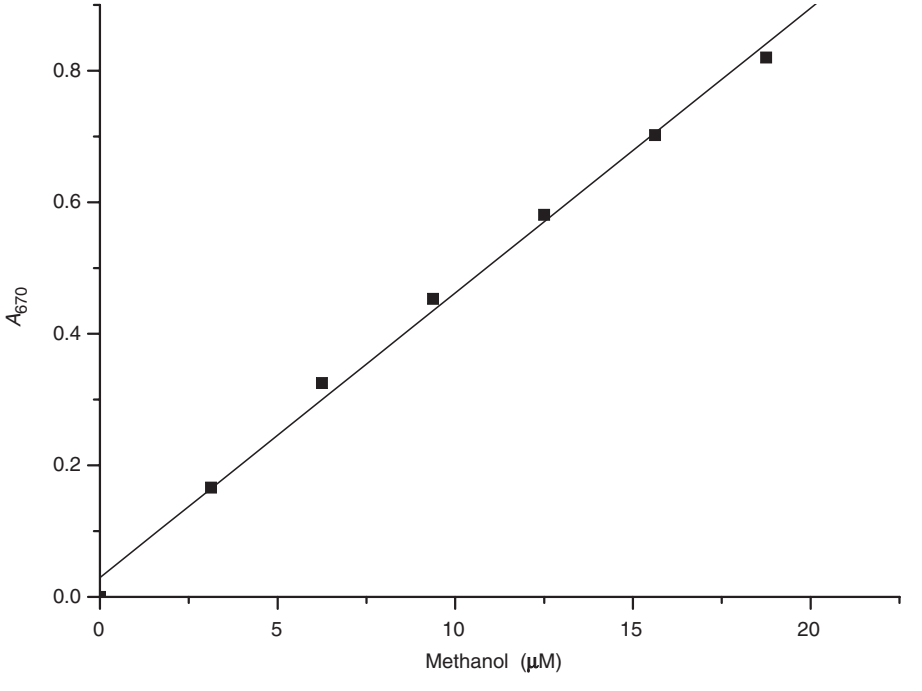

Figure 2. Dependence of the optical density (A_{670}) of samples on methanol concentration (in the presence of AO and MBTH).

these values confirms the practically complete oxidation of methanol to formaldehyde in the AO-catalysed reaction. As AO is also able to oxidize formaldehyde (see reactions 2 and 3), yielding formic acid which does not form a coloured product in the next reaction with ferric ions (see figure 1, line C), a positive colorimetric reaction in the system 'methanol + MBTH + AO → + Fe^{3+}' (figure 2) can be regarded as evidence that formaldehyde formed in enzymatic reaction is not oxidized any further by AO to formic acid. This fact can be explained by the formation of an azine adduct of formaldehyde with MBTH in the first reaction step, which, unlike free formaldehyde, is not oxidizable by AO but enters the next colorimetric reaction with ferric ions in the acid medium. This conclusion is confirmed by comparison of the following experiments: (1) formaldehyde was incubated for 30 min with AO in the presence of MBTH, variant 'formaldehyde + MBTH + AO → Fe^{3+}' (figure 3, line A); (2) formaldehyde was previously incubated with AO in buffer solution and then treated by a mixture of MBTH and ferric ions, variant 'formaldehyde + AO → MBTH + Fe^{3+}' (figure 3, line B). As shown in figure 3, the coloured product is accumulated only in the first experiment. The same pattern was observed for methanol as the dye was formed in the variant 'methanol + MBTH + AO → Fe^{3+}' (figure 2, line C) and not for the variant 'methanol + AO → MBTH + Fe^{3+}' (figure 3, line D).

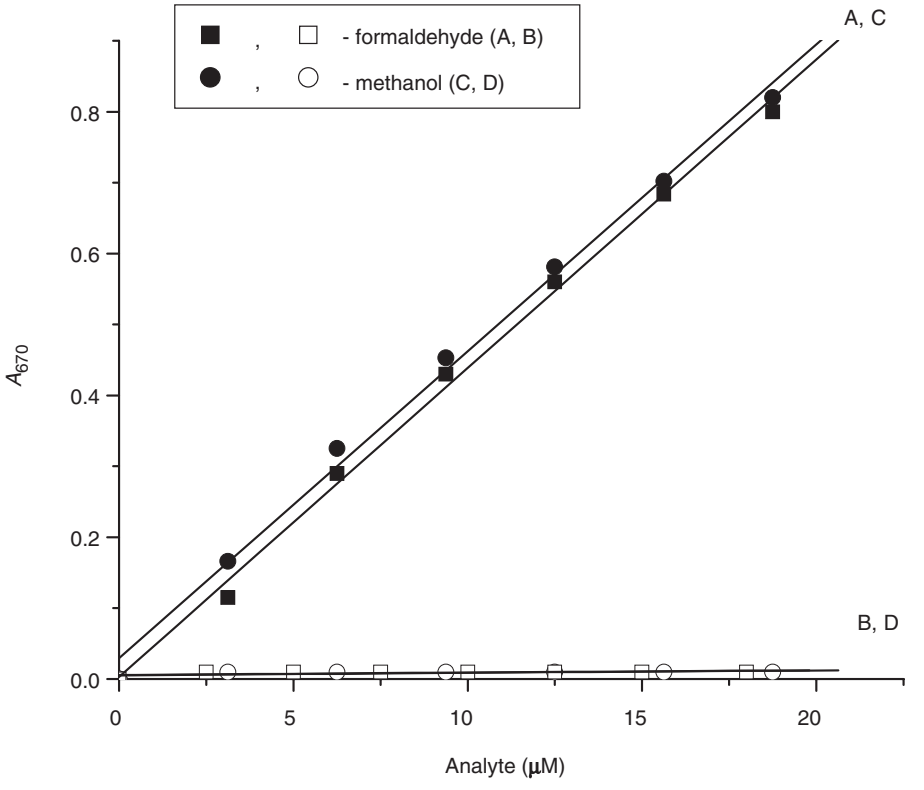

Figure 3. Dependence of the optical density of samples (A_{670}) on analyte concentration (in the presence of AO) in relation to the reaction step of MBTH addition. A, C: MBTH added in a mixture with AO at the first step of reaction; B, D: MBTH added at the second step of reaction (with solution of $FeCl_3$) after previous incubation of the analyte with AO.

From all the results obtained, we can conclude that MBTH in the first step of the analytical reaction serves as a formaldehyde-trapping reagent and thus prevents AO-catalysed oxidation of formaldehyde. MBTH is used also as a reagent for such an analyte in the next colorimetric reaction with ferric ions. This means that the addition of AO to the mixture with MBTH has no influence on the results of the pre-existing formaldehyde assay in the samples analysed.

These conclusions are confirmed by analysis of the model mixtures of methanol and formaldehyde added at different ratios (the total concentration of both analytes was 10 μM) without and with the addition of AO (figure 4, lines A and B, respectively). The results obtained allow us to make the following conclusions. First, methanol has no influence on the results of formaldehyde detection in the absence of AO. Second in the presence of AO, practically the same level of optical density was observed for the mixture of both analytes at their different molar ratio, but at constant total concentration. These data show a good additivity of the optical densities resulting from pre-existing and newly formed formaldehyde from methanol in the AO-catalysed reaction.

The enzymo-chemical method developed for differential detection of formaldehyde and methanol in their mixtures was used to analyse a real sample, a commercial product of formalin which is a concentrated formaldehyde solution containing methanol as a stabilizer inhibiting formaldehyde polymerization. The results of such an analysis shown in table 1 are in good agreement with data obtained by the chemical method and gas chromatography.

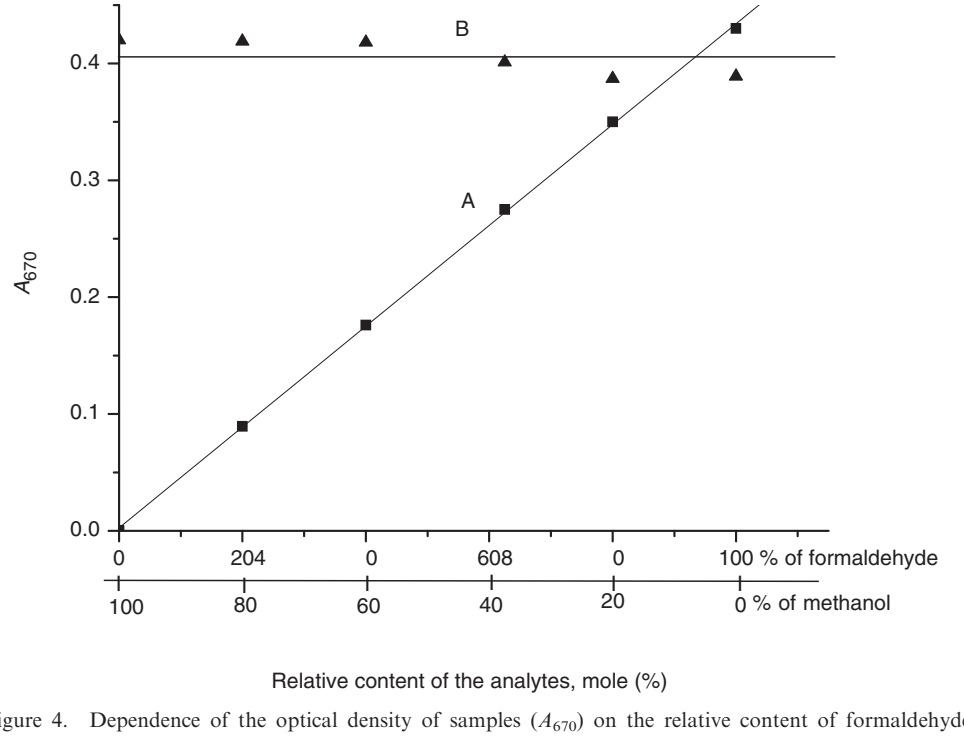

Figure 4. Dependence of the optical density of samples (A_{670}) on the relative content of formaldehyde and methanol in a 10 μM mixture of these analytes (in molar percent). A: without addition of AO; B: in the presence of AO. In both variants, MBTH was added at the first step of the reaction.

The enzymo-chemical method developed for a differential assay of formaldehyde and methanol in mixtures was also used to analyse real samples of industrial waste-waters (after their acid distillation). The results of such an assay for waste samples are shown in table 1. As can be seen from the methanol-content data, the results for the enzymo-chemical method are in good agreement with the data obtained with the chemical method and gas chromatography. A regression analysis of the analytical data obtained by the proposed method and by two reference methods is illustrated in figure 5(a) and (b). The correlation coefficient between the data for the enzymo-chemical method and the standard chemical method is 0.9974, and between the enzymo-chemical method and gas chromatography 0.9456. The worse correlation coefficient between the new method and gas chromatography can be explained by the difficulty in the analysis of the mixture of methanol and formaldehyde by gas chromatography [29].

Table 1. Assay of methanol and formaldehyde in diluted samples of acid distillates of waste-waters and technical formalin.

	Methanol (MeOH) and formaldehyde (FA) content, mg L^{-1} (M ± m, $n = 4$)				
	Enzymo-chemical method		Gas chromatography	Chemical method (chromotropic acid)	
Samples	MeOH	FA	MeOH	MeOH	FA
I	2.59 ± 0.19	4.36 ± 0.23	3.3 ± 0.5	2.7 ± 0.13	4.62 ± 0.11
II	4.61 ± 0.34	7.15 ± 0.37	5.39 ± 0.5	4.72 ± 0.27	7.27 ± 0.2
III	3.29 ± 0.38	6.95 ± 0.23	3.4 ± 0.5	3.01 ± 0.08	6.49 ± 0.28
IV	2.80 ± 0.32	6.23 ± 0.25	3.53 ± 0.5	2.70 ± 0.05	6.58 ± 0.33
V	0	1.72 ± 0.2	0	0	1.85 ± 0.1
VI	0	1.48 ± 0.13	0	0	1.73 ± 0.08
VII	3.77 ± 0.30	2.66 ± 0.16	3.13 ± 0.2	3.79 ± 0.12	3.82 ± 0.15
VIII	4.15 ± 0.32	2.14 ± 0.27	3.06 ± 0.5	3.93 ± 0.31	4.11 ± 0.13
Technical formalin (37.2% FA; 4–8% MeOH)	60.24 ± 6.88 or 5.59 ± 0.64%	417.6 ± 29.5 or 38.72 ± 2.74%	53.85 ± 5.35 or 5.0 ± 0.5%	56.5 ± 4.95 or 5.25 ± 0.46%	391 ± 40 or 36.3 ± 3.7%

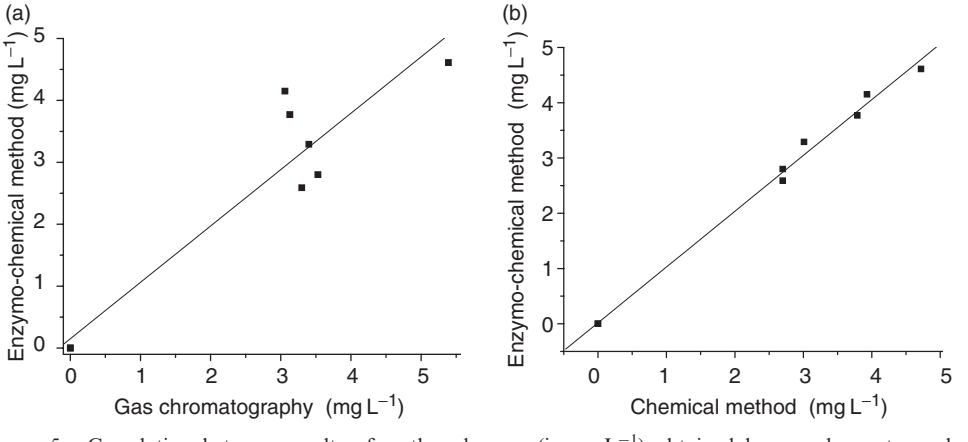

Figure 5. Correlation between results of methanol assay (in mg L^{-1}) obtained by gas chromatography (A) and the chemical method (B) related to the enzymo-chemical approach.

Generally, a good correlation was observed also between two methods of formaldehyde assay: the enzymo-chemical method and the method using chromotropic acid (table 1 and figure 6). For example, for formalin and waste-water samples I–IV, the difference between the formaldehyde contents measured by the two methods is not significant (Student's *t*-values are 0.927, 1.44, 0.404, 1.80, and 1.023, respectively). Only for samples V–VIII is this difference statistically significant (*t*-values for samples VII and VIII are 7.48 and 10.05; $p < 0.01$). The reason for this can be a low content of measured analyte in the tested samples (V and VI), as well as a different level of interfering compounds (phenols, etc.) in the waste-waters. A study of the possible interfering effects of different formaldehyde co-pollutants usually present in waste-waters from chemical plants and application of the developed method to test formaldehyde levels at different stages of waste-water purification will be the subject of a follow-up article.

The selectivity of enzymo-chemical AO-MBTH-based method for assay of methanol and formaldehyde is determined by selectivity of AO to the substrates and MBTH to aldehydes. AO can oxidize not only methanol but also primary alcohols and formaldehyde. As shown in this article and described by Anthon and Barrett [30], MBTH prevents oxidation of formaldehyde by AO, but the non-selectivity of oxidation of AO toward primary alcohols, first of all ethanol, is still a problem. A similar situation is with MBTH, which reacts with a broad variety of aldehydes [26]. Thus, the application of the developed AO-MBTH-based method for assay of methanol and formaldehyde is limited by the chemical nature of the tested samples. From our experience in testing waste-water from chemical plants using formaldehyde (formalin) in chemical synthesis, ethanol (or other primary alcohols) and aliphatic aldehydes

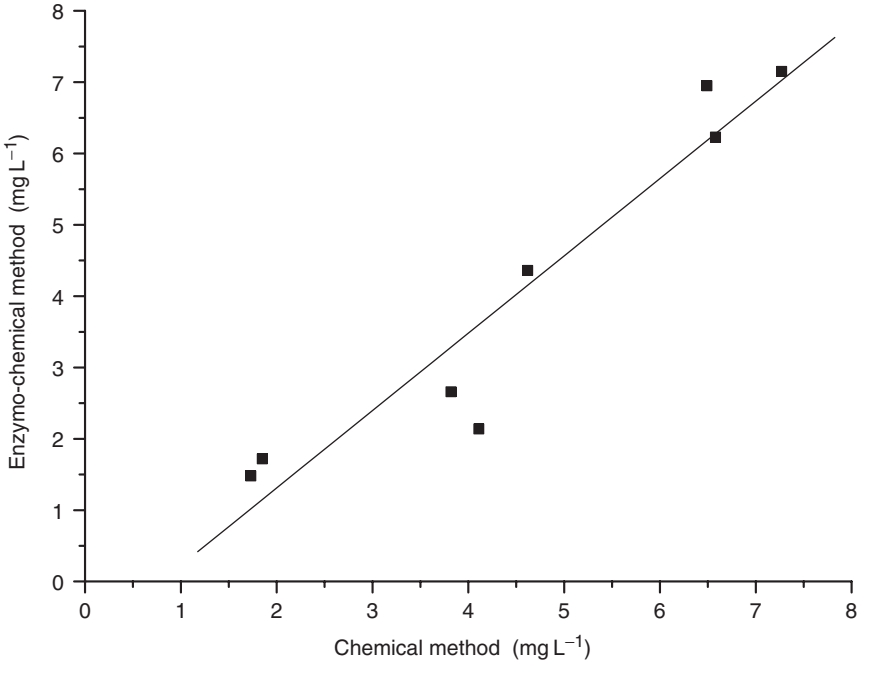

Figure 6. Correlation between the results of the formaldehyde assay (in mg L^{-1}) obtained by the chemical and enzymo-chemical method.

(besides formaldehyde) are usually absent and should not create any serious problems related to the selectivity of the proposed method. The development of the modified enzymo-chemical method with an enhanced selectivity based on the use of MBTH only as a formaldehyde-masking reagent and colorimetric reagents more selective to formaldehyde, for example, Purpald [31, 32], is in progress and will be the subject of another article.

In conclusion, we can assume that the novel enzymo-chemical method developed provides an opportunity to carry out a separate analysis of methanol and formaldehyde in mixtures as demonstrated on model mixtures, as well as on the commercial preparation of formalin and industrial waste-waters. The threshold sensitivity of the proposed method for both analytes is around 1 μM, which corresponds to 30–32 ng of analyte in 1 mL of reaction mixture, and is 3.2-fold higher compared with the chemical method using permanganate and chromotropic acid (figure 7).

Our previous investigations have shown the possibility of using AO or yeast cells with genetically adjusted metabolism as bio-recognizing elements of potentiometric biosensors, selective to methanol and formaldehyde [8, 11, 14–16]. Although such sensors are cheap and easy to prepare, their main drawback, which is common for practically all pH-FET-based sensors, is insufficient sensitivity to the analyte and dependence of the response on the samples' buffer capacity. Also, until recently, biosensors were not a major product in the world bioanalytic market and could not be a substitute for enzymatic kits. It is worth emphasizing that the sensitivity of the

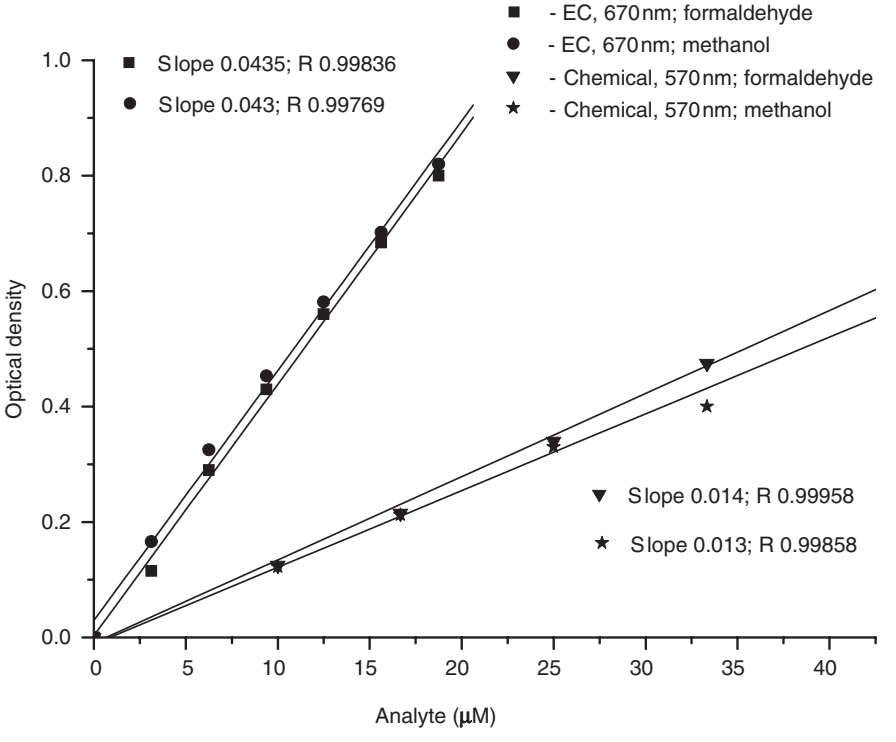

Figure 7. Comparison of the sensitivity of the chemical and enzymo-chemical (EC) methods for the assay of formaldehyde and methanol.

enzymo-chemical analysis proposed in this article is much higher (1000-fold) compared with pH-FET-biosensors [8, 11, 14, 15]. The linearity of the calibration curve is statistically significant (R is close to 0.998; $p < 0.0001$), and the standard deviation for parallel measurements for real samples does not exceed 7%. The proposed method, in contrast to the standard chemical approaches, does not require the use of aggressive chemicals (concentrated sulfuric, phosphoric, and chromotropic acids, and permanganate), and is simpler to use. We propose using this method for industrial waste-water control and for certification of formalin-containing industrial products.

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