SOUTHERN BRAZILIAN JOURNAL OF CHEMISTRY
Vol. 2, No.2, 1994

FLOW INJECTION ANALYSIS FOR METHANOL WITH ALCOHOL OXIDASE AND CHEMILUMINESCENT DETECTION

Andrei F. Dăneț[a#], *Mihaela Oancea*[b], *Silviu Jipa*[c] *and Tanța Setnescu*[c]

[a] Department of Analytical Chemistry, University of Bucharest, 13, Bd. Republicii, 70031-Bucharest, Romania
[b] Department of Biotechnology, ICECHIM, Bucharest, Romania
[c] Institute of Research for Electrotechnical Industry, Bucharest, Romania

ABSTRACT. A highly sensitive flow injection system was developed for the methanol determination. The system is based on the oxidation of methanol in the presence of alcohol oxidase to yield formaldehyde and hydrogen peroxide. The concentration of the hydrogen peroxide produced was determined by luminol chemiluminescence. A four-line flow injection assembly was used. The sample was injected into a flow of phosphate buffer at *p*H 9 that merged with a flow containing alcohol oxidase. The enzymatic reaction takes place in a coiled tube, 2 meters long and 0.8 mm i.d. Just in front of the chemiluminescence cell, the flow containing the sample merged with a flow of luminol (1.5 *m*M) and potassium ferricyanide (20 *m*M). The injected volume was 50 μL and the total flow rate 1.6 $mL\,min^{-1}$ The determination range was $5x10^{-4}$ - $1x10^{-2}$ %(v/v) methanol. The throughput was 40 samples per hour. From 20 tested substances, only *L*-cysteine generated a signal comparable with that of methanol. The others, at concentrations equal to those of methanol, either gave no signal at all (*n*-butanol, *cyclo*-hexanol, glycine, *L*-threonine, *L*-proline, copper sulphate) or gave signals much smaller than that of methanol.

RESUMO. Foi desenvolvido um método altamente sensível para para a análise de metanol na presença de álcool oxidase levando à formação de formaldeído e peróxido de hidrogênio. A concentração de peróxido de hidrogênio foi determinada usando a quimiluminescência do luminol.

KEYWORDS: methanol, flow injection analysis, chemiluminescence, alcohol oxidase.

To whom all correspondence should be addressed.

DOI: 10.48141/SBJCHEM.v2.n2.1994.106_1994.pdf

INTRODUCTION

It is well known that central nervous system disorders, particularly optic disorders, may occur from ingestion or inhalation of methanol, hence the development of simple and sensitive methods for the detection of methanol is important. Also, there is a lack of simple, specific methods for the rapid on-line monitoring of methanol in technological processes. Gas chromatography has been widely used for this purpose, but it requires a long time (at least 30 min) for each sample.

Biological methods using microorganisms or enzymes have also been reported.[1-6] A microbiological sensor consisting of an oxygen electrode covered with a membrane with immobilized microorganisms has been prepared[1]. However, this sensor may be difficult to handle, because the metabolic conditions of the immobilized microorganisms could be easily modified. In enzyme sensors for alcohols, alcohol oxidase (*EC* 1.1.3.13) (AOD) or alcohol dehydrogenase (*EC* 1.1.1.1) (ADH) have generally been used.[2-6] For methanol detection, AOD was used because ADH does not react very well with methanol. The specifications of enzymes given by manufacturers indicate that the response of AOD is about ten times higher for methanol than that for ethanol.

In a recently published paper,[7] however, a possibility was suggested for increasing considerably the sensitivity of the determination of methanol in the presence of ethanol by using a multienzymatic system which includes AOD.

Chemiluminescence (CL) has been widely used analytically because of its high sensitivity, wide dynamic range and simple instrumentation.[8,9] This detection technique presents some advantages

compared with other detection systems, such as amperometric methods,[10,11] for the determination of hydrogen peroxide. The difficulty of reproducing the physical and chemical conditions in CL can be eliminated by using a flow injection analysis (FIA) system.[12,13]

The purpose of our study was to develop a sensitive and automated FIA method for the rapid determination of methanol in fermentation broths, using on-line enzymatic conversion to H_2O_2 and CL detection of the H_2O_2 formed. We prefered to use a solution of alcohol oxidase instead of an immobilized enzyme, because, according to literature data, the activity of immobilized AOD decreases appreciably over time and, in the accidental presence of some poisons, the microreactor may be completely damaged.

EXPERIMENTAL PROCEDURE

Materials and Equipment

- Alcohol oxidase (*EC* 1.1.3.13), catalase-free, with an activity of 6.11 U/mL (1 U = 1 μM H_2O_2/min). It was extracted and then purified from methylotrophic yeast (*Hansenula polymorpha*) in the Biotechnological Department of the Institute of Chemical and Biochemical Energetics of Bucharest.

- 1.5 *m*M solution of luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) in 0.1 *m*M sodium carbonate.

- 20 *m*M solution of potassium ferricyanide in water.

- 1 *m*M solution of disodium phosphate, containing 1 *m*M of EDTA, having *p*H 9, used as a carrier stream into which the analyzed sample is injected.

- Methanol, absolute, from which water solutions with concentrations in the range $5x10^{-4}$ - 10^{-2} % (v/v) were prepared.

- Alcohols: ethanol, *n*-propanol, isopropanol , *n*-butanol, cyclohexanol, allyl and benzyl alcohols.

- Amino acids: glycine, *L*-threonine, *L*-tyrosine, *D, L*-serine, *L*-proline and *L*-cysteine.

- Glucose, copper acetate, zinc sulfate, manganese acetate, lead acetate, copper sulfate.

All reagents were of analytical grade. Doubly distilled water was used for preparing the solutions.

Figure 1 presents the flow injection analysis line. The carrier flows, those of the enzyme solution and of the reagents, were obtained by means of a 4-channel peristaltic pump (from IAUC - Bucharest), provided with 1.02 mm i.d Tygon tubes. A second, one-channel, peristaltic pump was used for filling up the injection loop with the sample solution and a 6-channel valve (Rheodyne-type, Modell 5051), was used for injection.

All tubes used for the FIA assembly presented in Figure 1 were of Teflon, with an i.d. of 0.8 mm. The carrier flow, into which the sample was injected, merges with the enzyme flow 40 cm down-stream from the injection valve. The enzymatic reaction with H_2O_2 generation takes place in the reaction loop L_1. The luminol and ferricyanide flows merge and mix in mixing loop L_2. The resulting flow merges afterwards with the flow in which the enzymatic reaction has taken place, just in front of the detector cell. In order to detect the chemiluminescence radiation, a coil-shaped cell, about 50 cm long, was built from a 0.8 mm i.d. Teflon tube. The flow cell was mounted directly in front of the photomultiplier tube (type FEU-19M, Russia) and was covered with an aluminium reflecting foil in order to improve the detection of the emitted light. The signals were measured with a

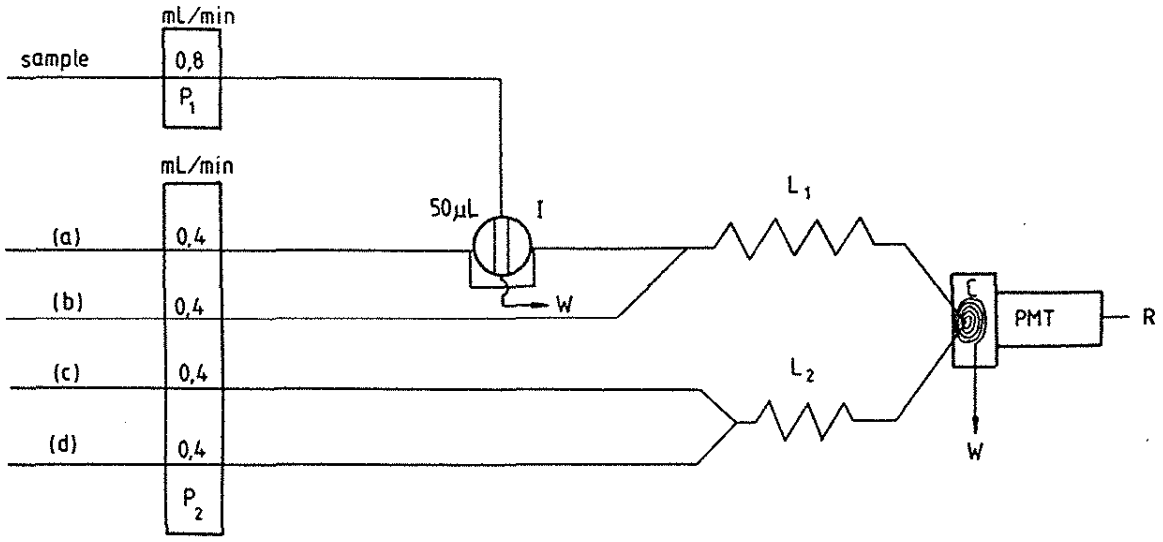

Fig. 1. Optimized flow injection manifold for methanol determination with chemiluminescent detection. P_1 and P_2 - peristaltic pumps; I -injection valve; L_1 *and* L_2 - mixing loops of 200 and 60 cm lengths, respectively; C - flow cell; *PMT*- photomultiplier tube; R - recorder; W - waste; (*a*) carrier; (*b*) - enzyme solution; (*c*) - 1.5 *m*M luminol solution; (*d*) - potassium ferricyanide solution.

y-t recorder (type Endim 621.02, Germany). All measurements were carried out at 19 ± 1 °C.

Recommended Procedure

The mixing loops L_1 and L_2 of the FIA manifold with optimized parameters are 200 cm and 60 cm long, respectively. One works with equal flow rates of the enzyme solution, of the reagents and of the carrier flow, i.e. 0.4 mL min^{-1}. The volume of the injected sample is 50 μL. The enzyme solution used has an activity of 0.12 U mL^{-1} (after a 1/50 dilution of the initial enzymatic solution). The compositions of the luminol solution and of the carrier flow, as well as that of the potassium ferricyanide solution, are those specified earlier (see the Materials and Equipment Section). The voltage applied to the PMT is 820 V. The measurement of the chemiluminescent signal was made in all cases by measuring the height of the recorded FIA peaks.

RESULTS AND DISCUSSION

The influence of the enzyme activity on the height of the signals recorded after injecting a 10^{-2} % (v/v) solution of methanol is shown in Figure 2. The experiments were carried out by observing the directions given in the Recommended Procedure Section. One can see from the figure that for enzyme activities higher than 0.12 U mL^{-1}, the recorded peak heights are practically equal, and for this reason subsequent experiments were carried out with enzyme solutions having an activity of 0.12 U mL^{-1} (which corresponds to a 1/50 dilution of the original enzyme solution in doubly-distilled water).

The parameters of the FIA system were optimized by modifying the flow rate, the volume of the injected sample, and the length of the

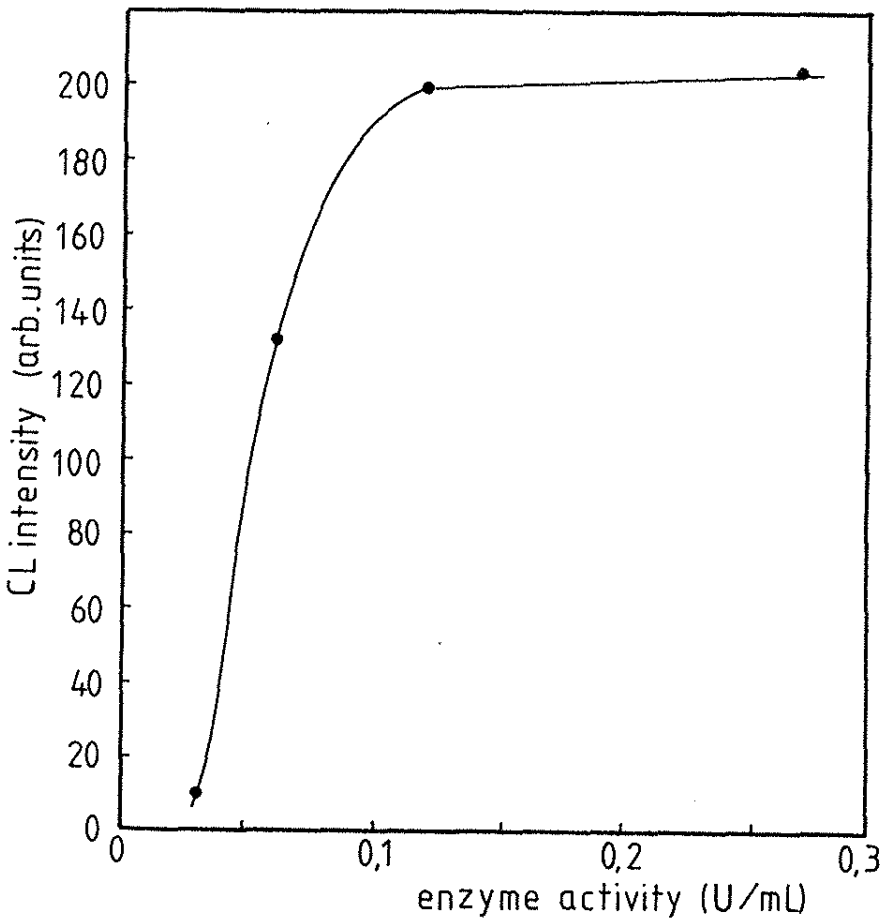

Fig. 2. The influence of the enzyme activity on the FIA output. $c_{methanol} = 10^{-2}$ % (v/v).

reaction loop L_1. The flow rate influences the intensity of the CL radiation by way of both the enzymatic and chemiluminescent reactions, since both these reactions have slow reaction rates. Samples of 5×10^{-3} % methanol solution were injected in portions of 50 μL, the total flow rate being varied between 1.6 and 10 mL min^{-1} (Fig. 3). The

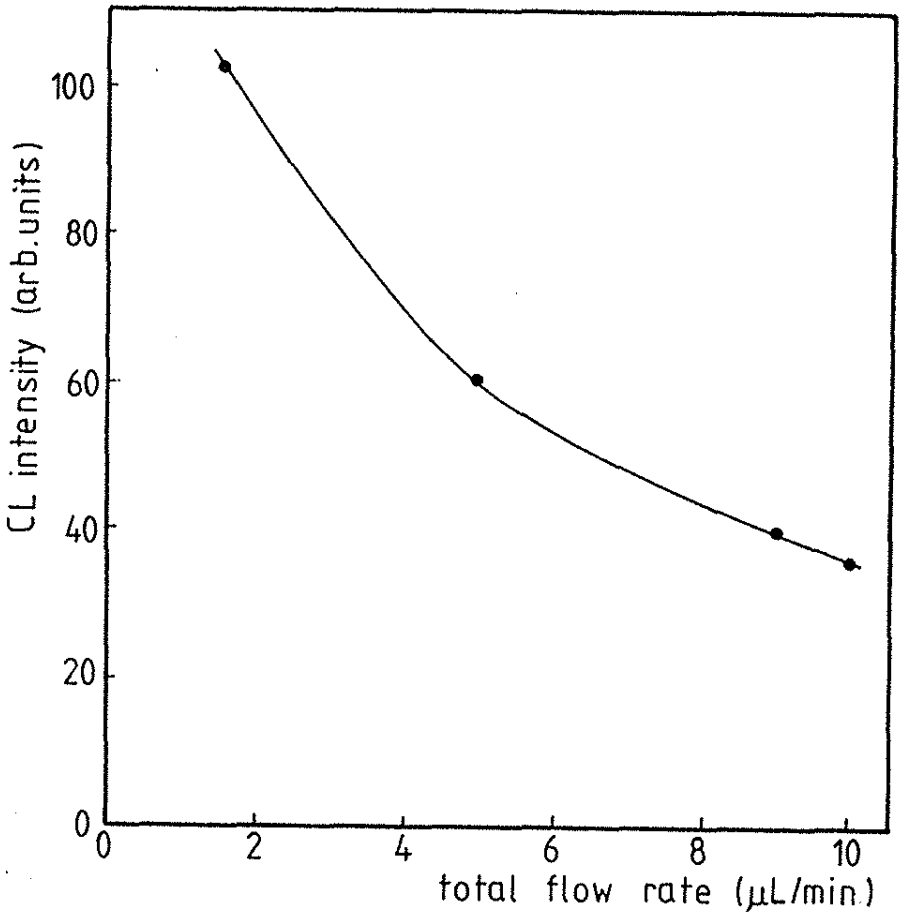

Fig. 3. The influence of the total flow rate on the FIA output. $c_{methanol}$ = $5x10^{-3}$ % (v/v).

flow rates in the four channels of the FIA installation were identical. When the flow rate was high, the peak height decreased appreciably, due to the short time available for the enzymatic reaction and also to the decrease of the time interval in which the CL radiation is measured. The experiments were therefore continued using a total flow rate of 1.6 mL min^{-1}, a flow rate which was considered optimal because the intensity of the CL radiation is then at its maximum value, the reagent

consumption is low and at the same time the duration of a single determination is reasonably short.

Calibration curves were drawn for various volumes of injected sample: 50, 100, 150, 200, and 250 μL. It was noticed that for small volumes of injected sample a linear dependence of the signal on the methanol concentration is obtained over a wider range. The value of 50 μL was thus selected as optimal.

In order to increase the reaction time, beside decreasing the flow rate, we increased the length of reaction loop L_1. Initially, the length had been set at 100 cm. If the length of the L_1 loop is doubled, one gets signals two times higher. The base line was very stable under these conditions. If very low methanol concentrations are sought, the length may be further increased or, alternatively, one can work in a stopped-flow manner.

The effect of the potassium ferricyanide concentration on the height of the recorded signals is illustrated in Figure 4. The intensity of the CL radiation increases linearly with the concentration of potassium ferricyanide, up to 10 *m*M, whereas at higher values, up to 20 *m*M, it remains constant. Therefore, an optimal concentration of 20 *m*M was chosen. The other parameters used have been mentioned in the Recommended Procedure Section.

The influence of the luminol concentration on the the height of the recorded signals is presented in Figure 5. For a luminol concentration higher than 1.5 *m*M, the intensity of the CL radiation no longer increases and for this reason the subsequent determinations were carried out by using the luminol solution at the above mentioned concentration. We should mention that it is necessary to work with

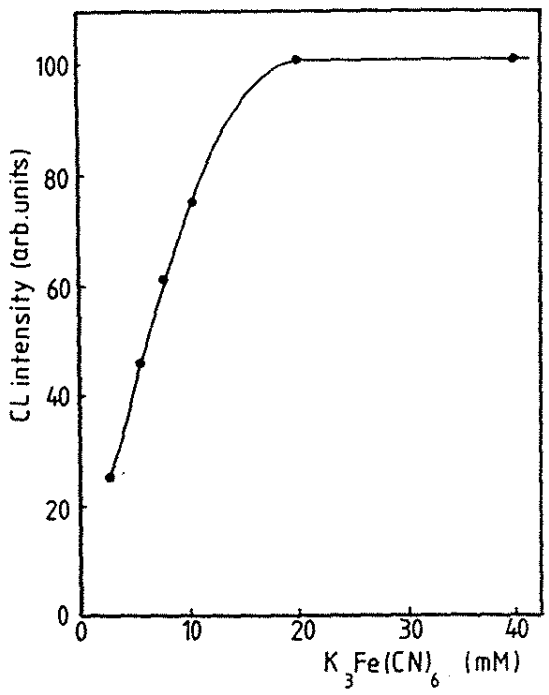

Fig. 4. The influence of the concentration of potassium ferricyanide on the FIA output. $c_{methanol}$ = 5x10^{-3} % (v/v).

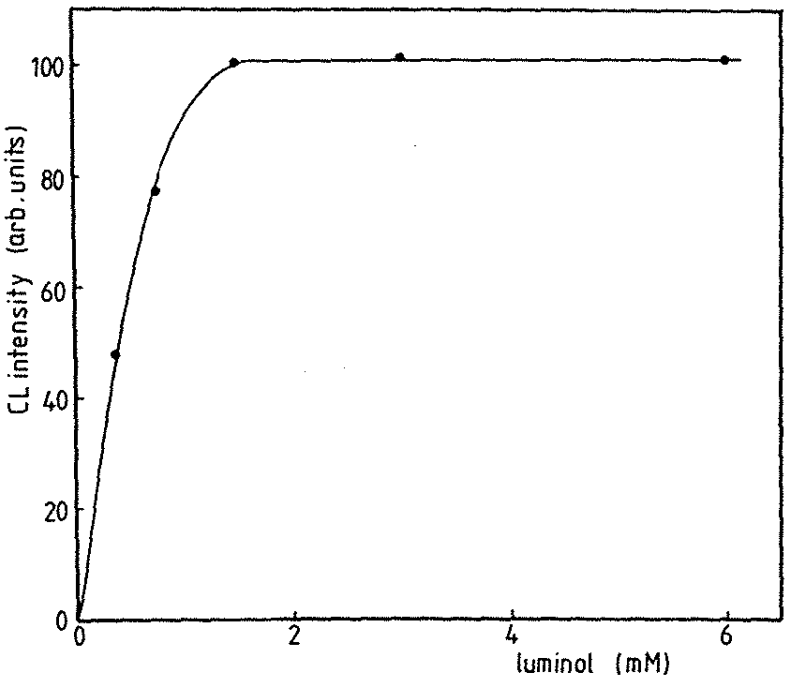

Fig. 5. The influence of the luminol concentration on the FIA output. $c_{methanol}$ = 5x10^{-3} % (v/v).

freshly prepared luminol solutions. Luminol solutions kept in the refrigerator preserve their activity over several days, but after some time, e.g., after 10 days, the height of the recorded signals for the same methanol sample decreased by half compared to the signal obtained with a fresh luminol solution.

System Performance

The calibration straight line obtained during the analysis of methanol-containing samples, in the $5x10^{-4}$- 10^{-2} % (v/v) concentration range, is shown in Figure 6. The experiments were carried out according to the directions described in the Recommended Procedure Section.

The equation of the calibration line was

$$y = 20{,}100c + 1.36$$

and the correlation coefficient 0.9998. The reproducibility of the method was checked by injecting 10 methanol samples of $5x10^{-3}$ % concentration. The relative standard deviation was 0.35%. The proposed

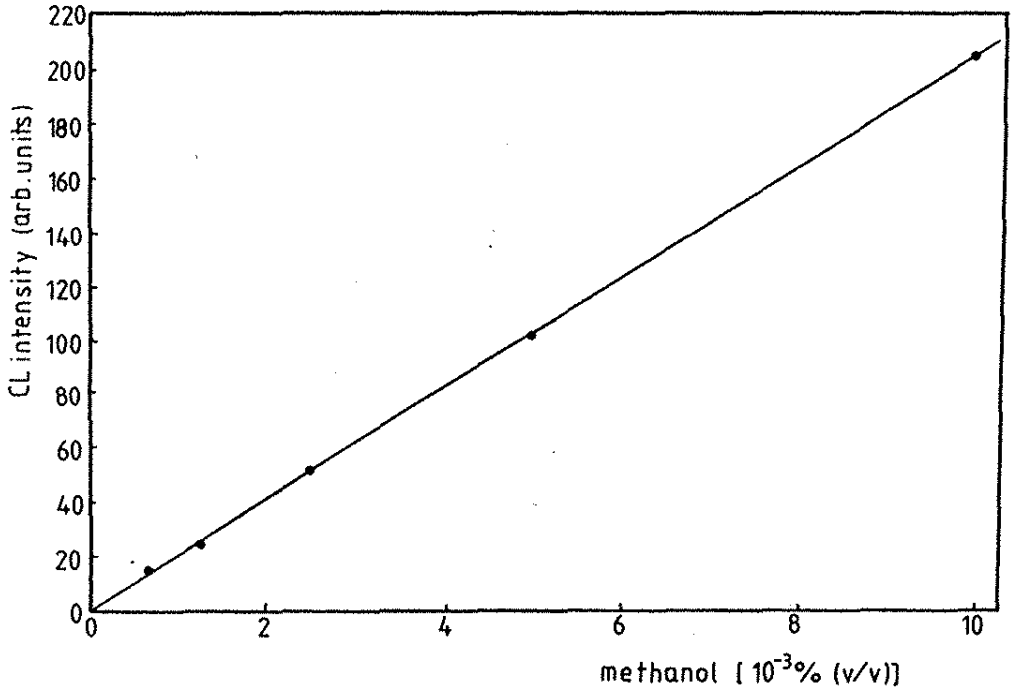

Fig. 6. Calibration curve for methanol, obtained by using the flow injection system.

analysis method allows for a throughput of about 40 samples per hour.

The Relative Activity of the Enzyme

Alcohol oxidase is specific for alcohols, but it is very selective for the length of the chain.[15] Due to differences existing between the specific activities for various alcohols, the amount of H_2O_2 produced by a given quantity of substance will depend on the nature of the alcohol.

The relative activity of the enzyme towards various alcohols, determined under the working conditions described above (injecting 50 μL samples) is shown in Table I. Solutions of various alcohols in doubly-distilled water, at concentrations of 10^{-2} % (v/v), were used. Methanol was taken as reference, considering that the enzyme has a specific activity of about of 100 % upon it.

Interferences

Starting from the idea of using the proposed method for monitoring the methanol concentration in the culture medium of a bioreactor, we have attempted to detect some possible interferences which may occur in a complex reaction medium.

The influence of some amino acids on the methanol determination was therefore investigated. Various amino acids were injected in concentrations of 10^{-2} % (m/v). The previously described procedure was strictly followed. A 10^{-2} % methanol solution was taken as reference. From the tested amino acids (glycine, threonine, tyrosine, serine, proline, cysteine), only cysteine gave a signal which indicated an interference of 54.1 % under the assumed conditions. It was noted that cysteine interferes due to a chemiluminescent reaction that takes place after its merging with the luminol and potassium ferricyanide flows.

Table I

The relative specific activity of AOD for various alcohols

Substrate (10^{-2} %, v/v)	Relative specific activity (%)
Methyl alcohol	100
Ethyl alcohol	36.6
n-Propyl alcohol	7
iso-Propyl alcohol	23.5
Allyl alcohol	22.6
n-Butyl alcohol	0
Benzyl alcohol	18
cyclo-Hexanol	0

Table II

The interference of some metal ions in the determination of methanol

Interferent ($5x10^{-3}$ M)	Percent modification of the signal, compared to a sample which does not contain interfering ions
Copper acetate	+22.3
Zinc sulfate	+28.7
Manganese acetate	+25
Lead acetate	+17.5
Copper sulfate	0

The alcohol oxidase has no influence upon this reaction. The interference of cysteine can be completely avoided by prior complexing with Cu^{2+} ions.

The influence of urea and glucose has also been checked, at concentrations of 10^{-2} % (m/v). Urea gave no signal at all, whereas glucose gave a signal which represented only 2.4 % of the height corresponding to samples of methanol with the same concentration.

The interference of some ionic metals is presented in Table II. Samples consisting of methanol samples ($2.5x10^{-3}$ %), containing metal ions in concentrations of $5x10^{-3}$ M, were injected. The $2.5x10^{-3}$ % methanol solution was taken as reference, and any modification of the height of the signals was related to it. All other measurements were carried out according to those specified in the Recommended Procedure Section.

Taking account of the fact that the concentrations of metal ions in culture media is lower than the tried concentrations (i.e. $5x10^{-3}$ M), and that, generally, for carrying out the analysis a dilution of about 1/100 of the samples is necessary, the interference of the metal ions mentioned in Table II, in the case of culture media, is practically negligible.

Application of the Proposed Analysis Method to the Determination of Methanol in Culture Media

The culture samples were collected from a bioreactor designed to produce the *Hansenula polymorpha* yeast; they were centrifugalized in order to separate the cells. The obtained supernatants were diluted in a ratio of 1/100 with doubly-distilled water, then were analyzed according to the indications given in the Recommended Procedure Section.

Samples resulting from culture media collected at different

biosynthesis time were analyzed. The range of methanol concentrations was 0.36 - 1.0 % (v/v). A relative standard error of 0.52 % was calculated by using t-test (n=10) for a concentration of methanol in culture medium of 0.56 % (v/v) and for a level of confidence of 90 %.

CONCLUSIONS

The described chemiluminescence/FIA enzymatic detection method for methanol determination is characterized by short analysis times and good reproducibility and sensitivity. The catalysts and luminol were injected simultaneously into two separate streams and then allowed to merge in a strictly reproducible manner before being mixed with the H_2O_2 produced in the enzymatic reaction. The proposed method is much faster then other methods of analysis for methanol in fermentation broths (e.g. gas chromatography). Adaptation of this system for monitoring other biotechnological processes based on the utilization of methanol seems promising.

REFERENCES

1. M. Hikuma, T. Kuba and T. Yasuda, *Biotech. and Bioeng., 21,* 1945-1950 (1979).
2. G. G. Guibault, B. Danielson, C. F Mandenius and C. Mosbach, *Anal. Chem.,* 55, 1582-1589 (1983).
3. J. A. Klavons and R. D. Bennet, *J. Agric. Food Chem., 34,* 597-602 (1986).
4. A. Maquieria, M. D. Luque de Castro and M. Valcarcel, *Microchem. J., 26,* 309-404 (1987).
5. V. J. Smith, R. A. Green and T. R. Hopkins, *J. Assoc. Off. Anal. Chem.,* 72, 30-35 (1989).
6. Y. Kitagawa, K. Kitabatake, I. Kubo, E. Tamiya and I. Karube, *Anal. Chim. Acta, 218,* 61-68 (1989).

7. Y. Sekine, M. Suzuki, T. Takeushi, E. Tamyia amd I. Karube, *Anal. Chim. Acta, 280,* 179-184 (1993).

8. K. Nakashima and K. Imai, *"Chemiluminescence"*, in: S. L.G. Schulman, Ed., *"Molecular Luminescence Spectroscopy"*, Part 3, Wiley, New York, (1993), Vol. 77.

9. M.A. DeLuca and W.D. McElroy, Eds., *"Bioluminescence and* Chemiluminescence", Academic Press, New York (1981).

10. K. Matsumoto, H. Matsubara, M.Hamada, H. Ukeda and Y. Osajima, *J. Biotech., 14,* 115-126 (1990).

11. J. Abdul Hamid, G.J. Moody and J.D.R. Thomas, *Analyst, 114,* 1587-1592 (1989).

12. B. A. Petersson, E. H. Hansen and J. Ruzicka, *Anal. Lett., 19,* 649-665 (1986).

13. P. J. Worsfold, J. Farrelly and M. S. Mathoru, *Anal. Chim. Acta, 164,* 103-109 (1984).

14. M. Garn, M. Gisin, Ch. Thommen and P. Cevey, *Biotech. and Bioeng., 34,* 423-428 (1989).

15. T. R. Hopkins and F. Muller, Eds., *"Microbial Growth on C_1 Compounds"*, Martinus Nijhoff Publishers, Dordrecht (1987), pp. 150-162.