Application of the Enzyme Thermistor for Determination of Mercury and Other Heavy Metals Using Free and Immobilised Alcohol Oxidase

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Abstract

This paper describes an enzymatic procedure for determination of mercury (I) and (II) based on the reversible competitive inhibition of free and immobilised alcohol oxidase Free enzyme was used in combination with immobilised catalase on controlled pore glass (CPG) coupled to a thermometric continous-flow system. Alcohol oxidase and methanol were injected into the system to give a temperature change corresponding to 100% enzyme activity. After inhibition by mercury, the enzyme activity and the corresponding temperature change were reduced. In order to improve the sensitivity, reusability and reduction of analysis time the alcohol oxidase was coimmobilised with catalase. Using free alcohol oxidase Mercury(II) was detected in the wide range between 25-1000 ng ml^{-1} with the linearity between 25 - 600 ng ml^{-1}, and with the detection limit 5 ng ml^{-1} and the relative standard deviation RSD $\leq$ 2.7%,. While using immobilised enzyme 17 times improvement in detection range (1-60 ng ml^{-1}), linearity (1 to 40 ng ml^{-1}) and 10 times better detection limit (0.5 ng ml^{-1}) with RSD $\leq$ 1.2 % has been achieved. Detection by mercury(II) can be overlap by Ag(I) present in ng ml^{-1} range.

A double reciprocal plot (Lineweaver-Burk) made for free and immobilised enzyme for varing methanol concentrations resulted in identical Km value (1 mM).

The alcohol oxidase was reversible and competitive inhibited by mercury(I), (II), and cupper(II) with K_i of 20, 17, and 190 nM, respectively as deduced from Dixon plot.

Keywords: mercury(II), immobilisation, alcohol oxidase, enzyme thermistor, heavy metals, catalase

Introduction

Heavy metals and its compounds are potentially the most toxic elements for the environment[1-3]. The presence of heavy metals in the chemical and waste products of modern society that are released into the environment leads to their participation in biogeochemical cycles. This, often results in interference and disturbance of natural systems. Various biological effects of heavy metals have been described [4,5].

Numerous enzymes were used for detect mercury by its inhibition effect, e.g. isocitric dehydrogenase [15], peroxidase [16,17], glucose oxidase [18], butyrylcholin-esterase [19], urease [20], invertase [21] and alcohol oxidase [22]. The inhibition of the activity of certain enzymes seems to involve the –SH groups of these proteins [6]. The mechanism of enzyme inhibition by heavy metals is based on the interaction of metal ions with exposed thiol- or methylthiol groups of protein amino acids often forming the active site of enzyme. The strongest interaction takes place in the case of mercury (II), therefore this metal exhibit the largest inhibition effect.

Classical techniques like atomic absorption spectrometry, anodic stripping voltammetry and inductively coupled plasma spectrometry [7] require sample pre-treatment and expensive instrumentation. Electrochemical and optical methods provide sensitivity and selectivity, their applications are limited because of interferences from electroactive species, ions and turbidity of the sample. In addition, transducers, such as electrodes, require frequent recalibration for long-term operation. Thermal biosensors [8,9] are therefore good alternatives to these devices. Mostly they involve a re-usable immobilised biocatalyst and analysis can be performed by a flow injection technique. Moreover, thermal biosensors such as enzyme thermistors are insensitive to the optical and electrochemical properties of the sample. Biosensors for heavy metals ions determination have been developed to measure the metal inhibitor effect on the activity of some oxidase enzymes [10-14].

In this paper we have presented a high sensitive method for the determination of mercury (I), (II) and other heavy metals based on the inhibition of alcohol oxidase activity. The alcohol oxidase was co-immobilised with catalase and used in a semi-continue FIA manifold coupled with thermometric detection. The advantages of this system are: reduced analysis time, the method is rapid with improved sensitivity and detection limit for mercury. Since the oxidase is co-immobilised with catalase the analysis can be easily automated and carry out continuously.

The storage stability of this immobilised enzymes kept in refrigerator has been already few years.

Experimental

Apparatus and equipment

The set-up for the thermometric assay consists in a peristaltic pump (Gilson Minipulse 2, France), an injection valve (type 50 from Rheodyne, Cotati, CA), the sensor device, a Wheatstone bridge equipped with a chopper- stabilized amplifier and a recorder (model 314). During the operation, the sensor was kept in an aluminium calorimeter blok insulated with polyurethane foam to minimize interferences from changes in the environmental temperature. The flow rate was 0,6 ml min^{-1}. The temperature change corresponding to the enzyme reaction taking place in the column was registered with the bridge. At maximum sensitivity this bridge produces a 100 mV change in the recorder signal for a temperature change of 10^{-3} ^{0}C.

Reagents

A 25 % aqueous solution of glutaraldehyde and, catalase (from *bovine liver*, 12 U/mg, EC 1.11.1.6) were purchased from Sigma Chemical CO (USA), alcohol oxidase (*Pichia Pastoris*, 1012 U/g, EC 1.1.3.13) was from ICN Biomedicals Inc.(Ohio, USA). Methanol, mercury(I) chloride, mercury(II) acetate and the glucose were from Merck, Darmstadt. Trosoperl controlled-pore glass (CPG) beads (particle diameter 125-140 nm, pore diameter 49.6 nm) were obtained from Schuller (Steinach, Germany). Silver nitrate and cupric acetate

were from Sigma (St. Louis, Mo. USA), lead acetate was from AnalaR (BDH Chemicals Ltd, Poole, England). All solutions were prepared with phosphate buffer (sodium phosphate dibasic + sodium phosphate monobasic) 0.1 mol l^{-1} at pH 7.0.

Standard solutions

Phosphate buffer 0.1 mol l^{-1}, pH 7.0, a standard solution of methanol of 1 mol l^{-1} and alcohol oxidase 1172 U ml^{-1} were prepared. A stock solution of 3 μg ml^{-1} mercury(II) and other used metals in phosphate buffer (0.1 mol l^{-1}, pH 7.0) was prepared.

The immobilisation of catalase on CPG

Catalase was covalently immobilized on CPG beads according to the following procedure: 350 mg of CPG was activated with 2.5% glutaraldehyde in phosphate buffer, 0.1 mol l^{-1}, pH 7.0. The reaction was allowed to take place under reduced pressure for at least 30 minutes (using a water aspirator) and then at normal pressure for 30 minutes. The product, which changed its colour to brick-red, was washed exhaustively on a Büchner funnel with distilled water and was left for 2 h under gentle shaking. Catalase 6.5 mg (78 U) dissolved in 400 μL, was added to the wet activated CPG after dialysed it. The coupling was allowed to proceed overnight in the cold under gentle shaking. The enzyme preparation was washed with buffer. Before packing into a column it was added 50 mg ethanolamine in order to terminate all the unreacted groups on the matrix, and then washed with phosphate buffer, 0.1 mol l^{-1}, pH 7.0.

The immobilisation of alcohol oxidase and catalase on CPG

Alcohol oxidase and catalase were covalently immobilized on CPG beads according to the following procedure: 170 mg of CPG were activated with 2.5% glutaraldehyde in phosphate buffer, 0.1 mol l^{-1}, pH 7.0. The reaction was allowed to take place under reduced pressure for at least 30 minutes (using a water aspirator) and then at normal pressure for 30 minutes. The product, which changed its colour to brick-red, was washed exhaustively on a Büchner funnel with distilled water and was left for 2 h under gentle shaking. Alcohol oxidase, 3 mg (3U) dissolved in 200 μL, and catalase, 3.5 mg (42 U) dissolved in 200 μL, were added to the wet activated CPG. The coupling was allowed to proceed overnight in the cold under gentle shaking. The enzyme preparation was washed with buffer. Before packing into a column it was added 25 mg ethanolamine in order to terminate all the unreacted groups on the matrix, and then washed with phosphate buffer, 0.1 mol l^{-1}, pH 7.0.

Procedure

The reactions which are involved in the process are:

$$CH_3OH + O2 \xrightarrow{A.O} CH_2{=}O + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{Cat} H_2O + 1/2O_2 + \Delta H \quad (2)$$

ΔH is the enthalpy change of the reaction. Reaction (1) is catalysed by alcohol oxidase which oxidises methanol to fromaldehyde and hydrogen peroxide. In reaction (2) catalase converts H_2O_2 into H_2O and O_2 and the enthalpy change. The signal due to the temperature change is correlated to the concentration of methanol and alcohol oxidase in solution. After the inhibition by mercury (II), the enzyme activity and the temperature change due to the reaction were reduced. The degree of inhibition was expressed by the ratio of the temperature peaks before and after mercury injection.

The flow diagram for the enzyme thermistor system is shown in (**Figure1**).

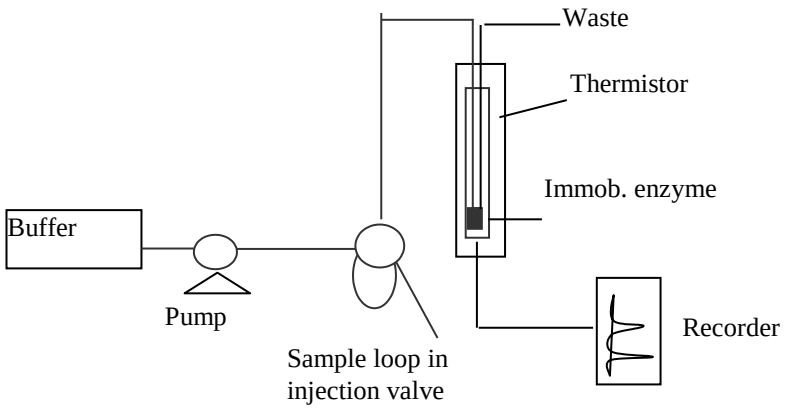

Figure 1. The flow diagram for the enzyme thermistor system.

A carrier buffer solution passes through the system, until a stable background line was reached when free enzyme was used. If free enzymes was used then it was injected together with methanol (85 μL) and the temperature peak in the absence of inhibitor was recorded.

In order to improve the sensitivity, to have shorter time for analysis and reusable enzyme alcohol oxidase was coimmobilised with catalase. Methanol was injected and the temperature change was recorded in the absence of inhibitor and then, the loop was filled with mercury or other heavy metals at different concentrations in order to obtain the temperature peak in the presence of inhibitor. The analysis time was 2 min. The degree of inhibition was calculated from the peak height with and without mercury (II) using the equation (1):

$$I(\%) = (\Delta T_1 - \Delta T_2)/ \Delta T_1 \times 100 \qquad (1)$$

where:

I = degree of inhibition

ΔT_1 = the temperature change in the absence of the inhibitor

ΔT_2 = the temperature change in the presence of the inhibitor

A flow rate of 0.6 ml min^{-1} was chosen as the best compromise in order to have a fast analysis time and a good signal.

The steps giving rise to the typical ΔT – time curves observed in the absence (a) and in the presence (b) of inhibitor are illustrated in (**Figure 2**).

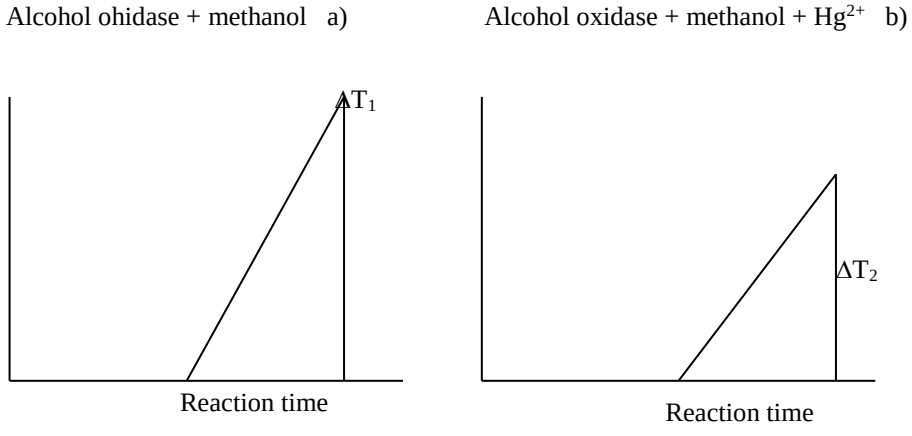

Figure 2. Typical ΔT – time curves in the absence (a) and in the presence (b) of inhibitor.

Results and Discussions

Earlier a number of oxidases were used for detection of mercury cations (**Table 1**) [20-24]. One of the oxidases was alcohol oxidase , which has been used in free and immobilised form [22].

Table 1. Various biosensors used for determination of mercury (II).

		The concentration range (μg ml^{-1})		Detection limit (μg ml^{-1})		
Enzyme	**Detection method**	**Free enzyme**	**Immob. enzyme**	**Free enzyme**	**Immob. enzyme**	**Ref.**
Invertase	Amperometric	0.01-0.06	-	0.001	-	21
Invertase	Thermometric	0.005-0.08	-	0.005	-	23
Alcohol oxidase	Amperometric	0.3-1	0.1-0.5	0.25	0.05	22
Alcohol oxidase	Thermometric	25-600	0.001-0.04	5	0.0005	this paper
Glycerol-3-P-oxidase	Amperometric	0.05-0.5	1-10	0.02	0.5	24
Lactate Oxidase	Amperometric	0.3-1.5	-	0.05	-	24
Lysine Oxidase	Amperometric	0.25-1.5	-	0.2	1.00	24
Choline Oxidase	Amperometric	1-10	2-10	0.5	-	24
Glutamate oxidase	Amperometric	0.5-2	-	0.2	-	24
L-amino Acid oxidase	Amperometric	2-10	-	0.8	-	20
Urease	Potentiometric	0.05-1	-	0.02	-	24

In order to follow the inhibitory effect of mercury and other heavy metals on the alcohol oxidize activity conditions for the enzymatic reaction has to been optimised.

The effect of methanol concentration on free and immobilised alcohol oxidase was studied. An increase of substrate concentration resulted in an linear increase of the temperature peak from 0.5 to 5 mM range, using alcohol oxidase in solution and from 0.125 to 2 mM, using immobilised enzyme (data not shown). 1 mM of methanol was selected for further work as the best compromise between a high signal and a low substrate requirement. According to the *Lineweaver Burk* plot the K_m = 1 mM for both free and immobilised enzyme was found **[Figure 3]**.

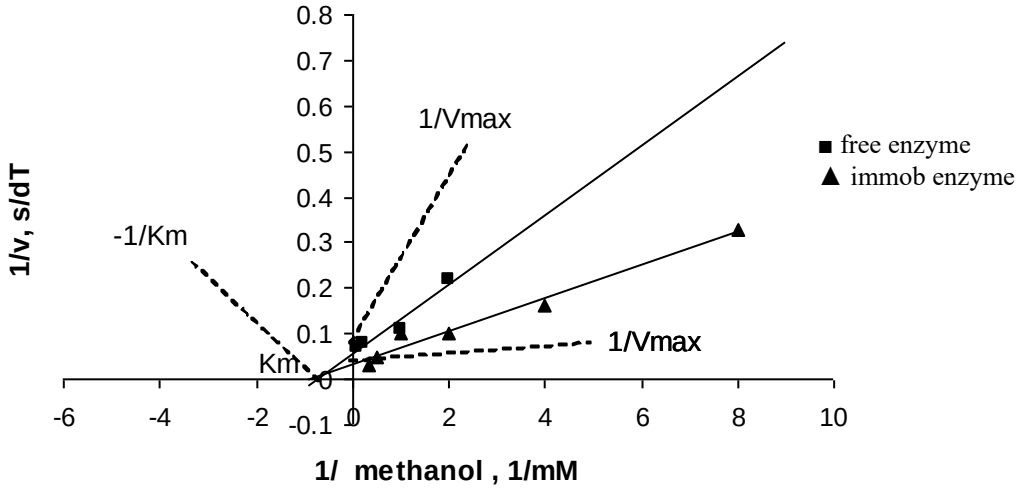

Figure 3. A double - reciprocal *Lineweaver Burk plot* for free and immobilised alcohol oxidase made for varying methanol concentrations (2 U/ml and 5 minutes of incubation) of free and (400 U) of immobilised alcohol oxidase was used. The flow rate was 0.6 ml/min. and injected sample volume was 85 μl.

Different degree of inhibiton by 100 ng ml^{-1} mercury(II) using 1mM methanol varying concentrations of free alcohol oxidase after 5 minute of incubation was found (**Table 2**). The degree of inhibition increases for lower concentrations of enzyme in solution. It means that the percent of inhibition is high for lower concentrations of the enzyme, and this could be manipulated to increase the sensitivity. On the other hand to low concentration of alcohol oxidase will oxidise less methanol and the signal will be too low. A concentration of 2 U/ml /injected units: 0.2) of free alcohol oxidase was chosen to provide a high sensitivity to inhibition and a good signal level.

Table 2. The inhibition effect of Hg (II) on different concentration of free alcohol oxidase. The measurements were performed with mercury (II) (100 ng ml^{-1}) at 5 minutes of incubation.

Alcohol oxidase (U/ml)	Inhibition (%)
0.5	32.5
1.0	27.0
2.0	22.5
4.0	15.0

Mercury(II) was detected in the range between 25-1000 ng ml^{-1} obtained with 2 U/ml alcohol oxidase and 1 mM methanol at different incubation time (2, and 5 min.), using free alcohol oxidase **[Figure 4]**. Linearity was found in the 25 –600 ng ml^{-1} range with RSD ≤ 2.7 %. The detection limit was 5 ng ml^{-1}. All measurements were done in triplicate and the data points represent the mean value of the determinations. The inhibition effect increases with increasing the incubation time.

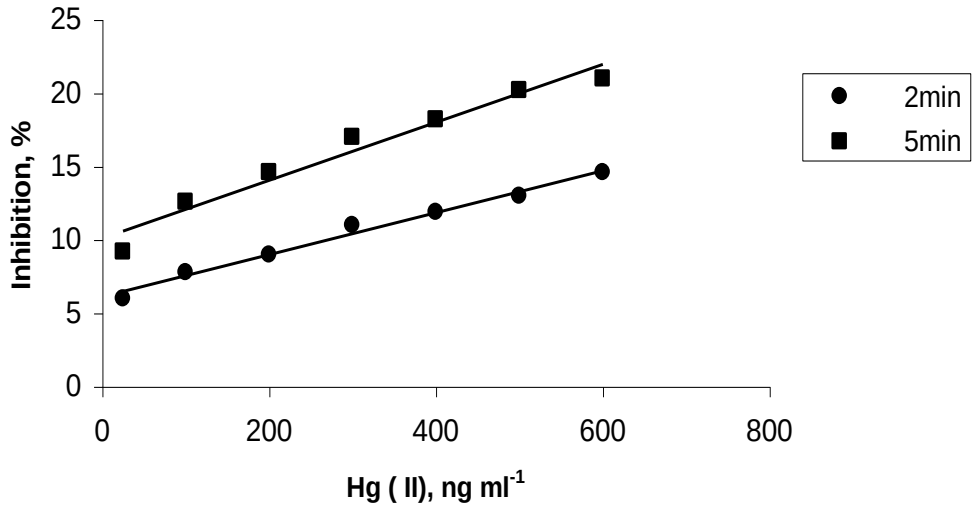

Figure 4. Calibration curve for mercury (II) based on the inhibiton degree of free alcohol oxidase (2 U) using 1 mM methanol at two different incubations time. The flow rate was 0.6 ml/min. and injected sample volume was 85 μl.

In order to reduce the analysis time, improve the sensitivity and reusability, alcohol oxidase was coimmobilised with catalase on CPG. The inhibition calibration curve for mercury (II) using 3 different concentrations of substrate (0.5, 1 and 2 mM methanol) is

shown in **[Figure 5]**. Mercury (II) was detected in 1-60 ng ml^{-1} range with a good linearity between 1-40 ng ml^{-1}. The detection limit was 0.5 ng ml^{-1} and RSD ≤ 1.2 %.

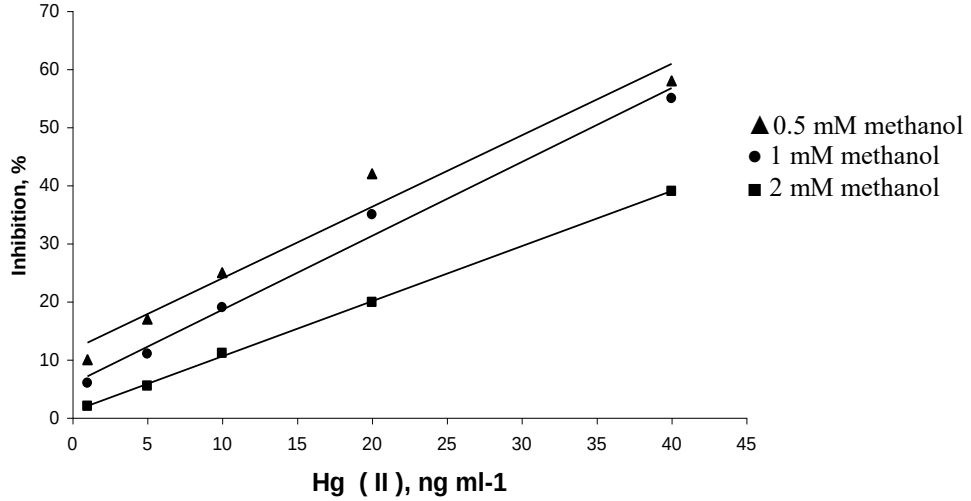

Figure 5. Calibration curve for mercury (II) based on the inhibiton degree of immobilised alcohol oxidase/catalase at three different concentrations of methanol. The flow rate was 0.6 ml/min. and injected sample volume was 85 μl..

Until now the best sensitivity towards mercury was obtained by the immobilised alcohol oxidase in our system. For the immobilise alcohol oxidase in comparison to the amperometric method [22] (**Table 1**) our sesnsitve has been improved by the factor of 100 times.

One of the reasons why the immobilised alcohol oxidase is more sensitive than free enzyme could be due to the stabilising role of immobilisation which protects the enzymes from conformational changes, but also the close contact of co-immobilised catalase which removes instantely the poisoning hydrogen peroxide and releases oxygen which is a limiting factor in oxidation reactions (see scheme 1).

In generally heavy metals are irreversibly inhibiting majority of enzymes, while alcohol oxidase was inhibited reversible. With a fixed amount of enzyme, and at two concentration of methanol (0,5 and 1 mM) the oxidase activity was assayed in the presence of increasing concentration of mercury. For the free enzyme 5-200 ng ml^{-1} and for the immobilised 1 to 40 ng ml^{-1}, mercury (II) was used. We also observed that inhibition caused by mercury (I) was of the same range as the one found for mercury (II). Therefore, in our system we would not be able to distinguish between mercury(II) and mercury(I) if both are present at the same concentration. The free and immobilised oxidase was competitively inhibited by mercury (II) with *Ki* of 210 nM and 17 nM, respectively **[Figure 6** and **7]** determinated from Dixon plot [25] for linear competitive inhibition. Mercury (I) has inhibited the immobilised enzyme (*Ki* =20 nM) in the same range as mercury (II) **[Figure 8]**. Applying steady state kinetic for reversible inhibition, the effect on the competitive inhibitor is to produce an apparent increase in *Km* by the factor 1+[I]/*Ki* (Guilbault,1984)[26] which increases without limit as [I] increases. The carefully chosen methanol concentration is one of the important factors when alcohol oxidase is involved for the determination of mercury.

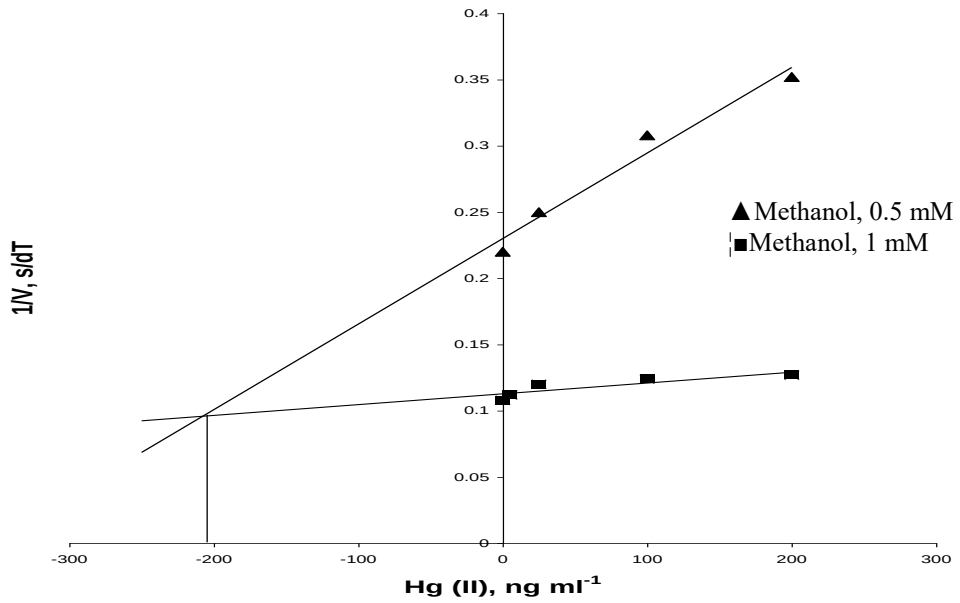

Figure 6. Dixon plot of 1/ v against [mercury] for linear competitive inhibiton of free alcohol oxidase (2 U) by increasing concentration of mercury (II) at two concentrations of methanol and 5 min. of incubation time. The flow rate was 0.6 ml/min. and injected sample volum was 85 µl. The concentration of mercury (II) is indicated on the abscissa. The inverse of the oxidase activity (1/v) is indicated on the ordinate.

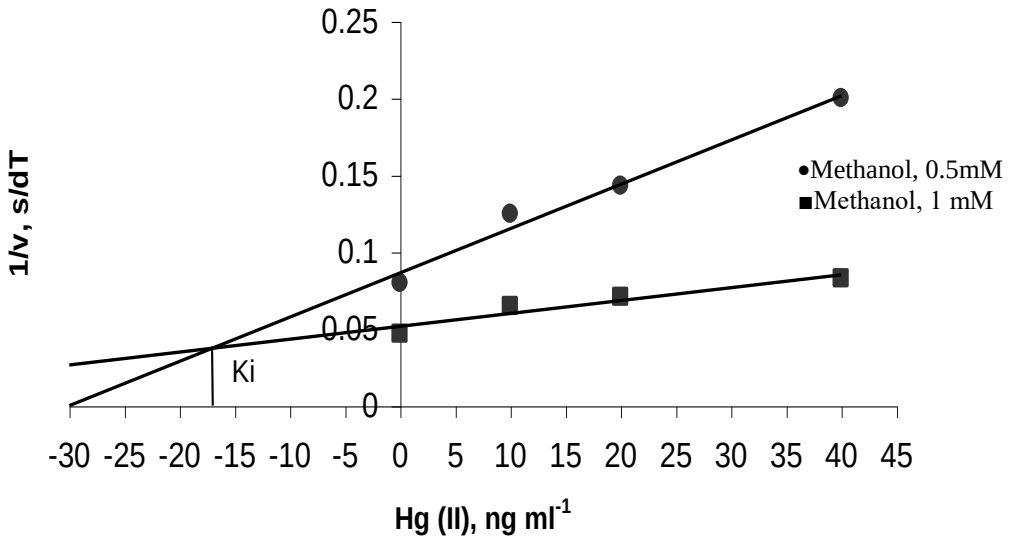

Figure 7. Dixon plot of 1/ v against [mercury] for linear competitive inhibiton of immobilised alcohol oxidase by increasing concentrations of mercury(II) at two different concentrations of methanol. The flow rate was 0.6 ml/min. and injected sample volum was 85 µl. The concentration of mercury (II) is indicated on the abscissa. The inverse of the oxidase activity (1/v) is indicated on the ordinate.

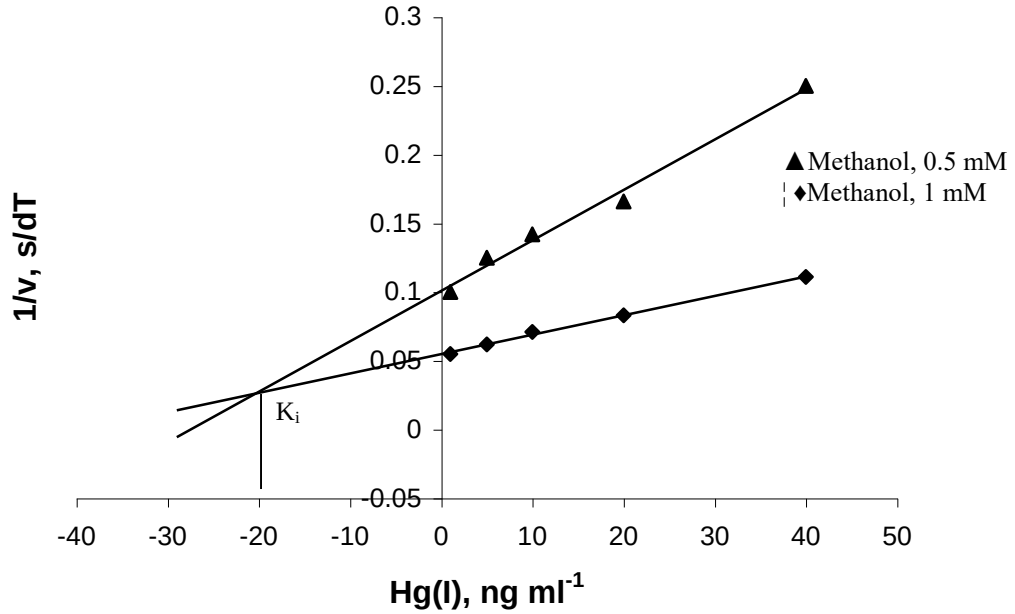

Figure 8. Dixon plot of 1/v against [mercury] for linear competitive inhibiton of immobilised alcohol oxidase by increasing concentrations of mercury(I) at two different concentrations of methanol. The flow rate was 0.6 ml/min. and injected sample volume was 85 µl. The concentration of mercury (II) is indicated on the abscissa. The inverse of the oxidase activity (1/v) is indicated on the ordinate.

Recovery test of mercury from real sample

Fresh sludge's collected from the municipal water purification system, Lund, Sweden, were analysed for mercury contents and other heavy metals. The sludge's were divided and part of them was spiked with known amount of mercury (II) and the recovery of the added mercury was followed and compared to a standard curve in our system. To 5ml volume of sludge's was added 10 ppb of mercury (II). The samples were mixed and kept for few minutes before centrifugation. After removing the solid particles of the sludge's, the supernatant was analysed in our system for mercury presence, with and without external added mercury (II). Since the sludge's were not diluted for the analysis, and we have not observed any inhibition of alcohol oxidise we presumed that no heavy metals were present there. We have recovered 100 % of the added amount of mercury (II) into the same sludge's. Therefore we can assume that when we made our analysis no reducing or chelating agent was active in the sludge's.

Inhibition by other metal ions

The degree of the inhibition effect of various metal ions is very different and depends on many factors like the use of free or immobilised enzyme, static or flow conditions or method of detection. Further inhibition studies of other heavy metals were tested with the immobilised alcohol oxidise. The literature data [22] show that some heavy metals ions like Cu (II) or V (V) can interfere and overlap with the determination of mercury (II) by alcohol oxidise. Therefore we additionally have tested the inhibition effect of Cu (II), Ag (I) and Pub (II) in our system. As we can see in **(Table3)** the degree of inhibition is not more than 40 % for the tested heavy metals ions studied at 1 mM concentration of methanol. The highest inhibition was found for Cu (II) (40 %) when testing 400 ng ml^{-1} of Cu (II). Also the linearity in the range between 100 to 400 ng ml^{-1} in this case was satisfactory. Likewise by mercury the immobilised oxidase was also competitively inhibited by Cu (II) with *Ki* of 190 nM **[Figure 9]**. Moreover, Pb (II) was detected in the 100 – 400 ng ml^{-1} range, but with lower degree of

inhibition. We can concluded that these two metals can't interfere in mercury(II) determination. In contrast to Cu(II) and Pb(II), Ag(I) was already determined in lower range from 20 to 200 ng ml^{-1} with 10 % inhibition for 20 ng ml^{-1} and 15 % for 40 ng ml^{-1}. For 20 ng ml^{-1} and for 40 nag ml^{-1} of mercury (II) the inhibition was 20 % and 40 %, respectively. Therefore, if Ag would be present in the samples with mercury it will interfere in determination of both mercury's.

Table 3. Heavy metal ions determination using immobilised alcohol oxidase at 1 mmol l^{-1} concentration of methanol.

Ion	Concentration range (ng ml^{-1})	Detection limit (ng ml^{-1})	Inhibition (%)
Hg(II)	1-40	0.5	6 -55
Hg(I)	1-40	0.5	10-55
Cu(II)	100-400	100	8.33 – 41.66
Pb(II)	100-400	100	5 –20
Ag(I)	20-200	20	10 -25

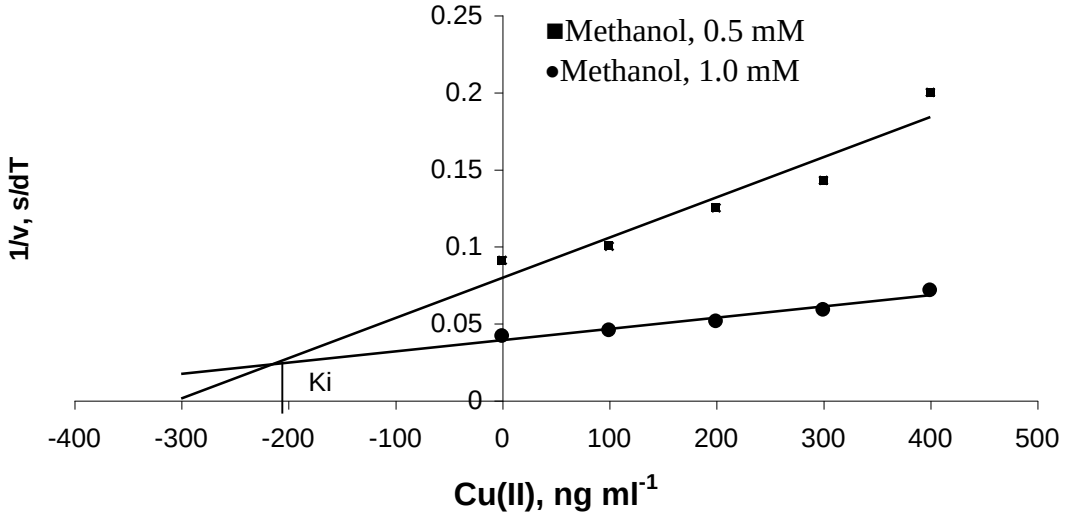

Figure 9. Dixon plot of 1/ v against [copper] for linear competitive inhibiton of immobilised alcohol oxidase by increasing concentrations of cupper(II). at two different concentrations of methanol. The flow rate was 0.6 ml/min. and injected sample volume was 85 µl. The concentration of copper (II) is indicated on the abscissa. The inverse of the oxidase activity (1/v) is indicated on the ordinate.

Most biosensors for the determination of heavy metals, when using immobilized enzymes, have to be regenerated after inhibition e.g., by adding a metal chelating agent such as EDTA or reducing agent. In the case of alcohol oxidase, the partially lost activity due to the competitive action of heavy metals is regenerated fully only by a fresh flash of methanol in buffer. This method being a continuous-flow method can be automated and using additional flow channels or multiple enzyme columns can increase the sample capacity.

Further more, thermal sensing can be applied to virtually any biological reaction. The temperature measurements as such do not require any calibration. The catalase and alcohol oxidase/cat. reaction column used here are extremely stable giving the same signal of the

initial value after 2 months being used 8-12 h per day. The columns can be stored in the refrigerator, and can be use for long time period giving very good reproducibility even after few years.

Conclusions

A thermometric assay for the determination of mercury (II) and (I), based on the inhibition of free and immobilised alcohol oxidase has been development. The method is based on the determination of ΔH produced by the enzymatic reaction. The temperature change is reduced by the presence of mercury inhibiting the enzyme activity. Mercury (II) was detected in the 25 - 1000 ng ml^{-1} range, using the free enzyme and 1-60 ng ml^{-1} range, using the immobilised enzyme.

Using free alcohol oxidase a good sensitivity and reproducibility have been obtained in the 25 -600 ng ml^{-1} range of mercury (II) with the detection limit at 5 ng ml^{-1}, and using the immobilised enzyme 1-40 ng ml^{-1} range with the detection limit at 0.5 ng ml^{-1}, which corresponds to the improvment in detection limit by the factor of 10. The inhibition was reversible easily regenerated without an any additional efforts.

The high sensitivity and easy handling operability of the method make it a good alternative to traditional methods for mercury (II).

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References

1. J.O. Nriagu, J.M.Pacyna, *Nature*, 1990, 333, 134-39.
2. J.O.Nriagu, Eds.: *Changing Metals and Human Health.* Springer-Verlag, Berlin, 1984.
3. E.Merian, Eds.: *Metals and Their Compounds in the Environment: Ocurrence, Analysis and Biological Relevance.*VHC; Weinheim: New York, Basel, Cambridge, 1991.
4. J. L.Webb, *Enzyme and Metabolic Inhibitors*, Academic Press: New York, 1966, vol 2, pp. 729-986.
5. J. T.MacGregor, T. W.Clarkson, in *Protein-Metal Interactions.* M.Friedman, Eds.: Plenum Press: New York, 1974, pp. 463-503.
6. J. L.Webb, *Enzyme and Metabolic Inhibitors*, Academic Press: New York, 1966, vol 2, pp. 635-653.
7. G.A.Drash, in *Handbook on Metals in Clinical and Analytical Chemistry*, Seiler, H.G.; Siegel, A.; Sigel, H., Eds.: Marcel Dekker: New York, 1994, pp. 479-493.

1. B.Danielsson, K.Mosbach, in *Biosensors: Fundamentals and Application.* A.P.F.Turner, I.Karube, G.S.Wilson, Eds.: Oxford University Press: Oxford, 1987, pp. 572.
2. B.Mattiasson, B.Danielsson, C.Hermansson, K.Mosbach, *FEBS Lett*, 1978, 85, 203.

3. J.C.Gayet, A.Haouz, A.Geloso-Meyer, C.Burnstein, *Bios. Bioel*. 1998, 8, 177-82.
4. C.Preininger, C.; Wolfbeis, O. *Bios. Bioel*. 1996, 11, 981-90.
5. S.Fennouh, V.Casimiri, A.Geloso-Meyer, C.Burnstein, *Bios. Bioel*.,1998, 13, 903-909.
6. D.C.Cowel, A.A.Dowman, T.Ashcroft, *Bios. Bioel*. 1995, 10, 509-516.
7. T.Danzer, G.Schwedt, *Anal. Chim. Acta*. 1996, 318, 275-86.
8. B.Kratochvil, S.L.Boyer, G.P.Hicks, *Anal. Chem*. 1967, 39, 45-51.
9. I.F. Dolmanova, T.N. Shekhovtsova, V.V., 1987, Assay of the enzyme effectors, *Talanta*, 34, 201-205.
10. T.N.Shekhovtsova, S.V.Muginova, N.A.Bagirova, *Anal. Chim. Acta*. 1997, 344, 145-151.
11. A.M.Donlan, G.J.Moody, J.D.R.Thomas, *Anal. Lett*. 1989, 22, 1873-1896.
12. L.Campanella, R.Cocco, M.P.Sammartino, T.Tomassetti, *Sci. Total. Environ*. 1992, 123/124, 1-16.
13. T.K.vel Krawczyk, M.Malgorzata, M.Trojanowicz, *Biosens. Bioelectron*. 2000, 15, 681-691.
14. A. Ciucu, S.Pîrvuţoiu, *Batch and FIA Determination of Mercury(II) Based on*
15. *Enzyme Inhibition and Biosensor Detection* in *Biosensors for Environmental*
16. *Monitoring. A Practical Guide for Students*, Ed.by A.Ciucu, Ed. Ars Docendi,
17. Bucharest, 2000,pp 93-95.
18. D.Compagnone, A.S.Lupu, A.Ciucu, V.Magearu, C.Cremisini, G.Palleschi, *Anal. Lett*. 2001, 34, 17-27.
19. S.Pirvutoiu, S.E.Dey, I.Surugiu, A.Ciucu, V.Magearu, B.Danielsson, *The Analyst*, 2001, 126, 1612-1616.
20. D.Compagnone, G.Palleschi, P.Imperialli, G.Varallo, *Enzyme inhibition based detection of heavy metals using H_2O_2 electrochemical probes*., Artif. Nat. Percept., Proc. Ital. Conf. Sens. Microsyst., 2nd, 1997, 74-78.
21. D.V.Roberts, in *Cambridge Chemical exts. Enzyme Kinetics*. D.T.Elmore, A.J.Leadbetter, K.Schofield, Eds.: *Cambridge University Press: Cambridge*, 1997, pp. 49-59.
22. G.Guilbault, Eds.: in *Analytical Uses of Immobilized Enzymes*. Marcel Dekker INC.: New York, 1984, pp. 24-38.