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Rapid alcohol determination in plasma and urine by column liquid chromatography with biosensor detection

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Abstract

An enzyme based amperometric biosensor used as a selective and sensitive detection unit in column liquid chromatography for the determination of ethanol and methanol in biological fluids such as plasma and urine is described. The reagentless enzyme electrode is based on the co-immobilisation of alcohol oxidase and horseradish peroxidase in carbon paste. The selectivity of the biosensor was found to vary when four various alcohol oxidase enzyme preparations from *Candida boidinii*, *Pichia pastoris*, and *Hansenula polymorpha* were used in the biosensors described. High sensitivity could be obtained for a number of alcohols, organic acids, and aldehydes. Optimisation regarding the sensitivity and selectivity of the four alcohol oxidase co-immobilised biosensors are outlined. A fast and reliable liquid chromatographic separation system with a PLRP-S polymer based separation column used with a phosphate buffer as the mobile phase was optimised using the best biosensor which was based on alcohol oxidase from *P. pastoris* and which showed the highest turnover rate for alcohols, as the detector for the determination of ethanol and methanol in human urine and plasma samples. The selectivity and stability of the biosensor were retained by working at an applied potential of -50 mV versus Ag/AgCl, the optimal operational potential, and by the casting of a protective membrane on the electrode surface. High selectivity of the enzyme electrode was also found towards other easily oxidisable interfering species normally present in biological fluids. It was found that stable and reliable determinations of ethanol and methanol in plasma and urine could be performed with only a simple dilution and centrifugation step prior to injection into the liquid chromatographic system. An analysis time of 4 min was required for the assay, with a sample throughput of 13 samples h^{-1}.

Keywords: Alcohol determination; Biosensor; Alcohol oxidase; Peroxidase; Liquid chromatography; Plasma; Urine

1. Introduction

In our society, ethanol is the most common social drug. When abused, the necessity arises for the fast determination of acute intoxication as well as of suspected drunk driving. Methods with

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high sensitivity and selectivity are required in order to distinguish between ethanol and methanol intoxication. Pharmacological effects are obtained at blood ethanol levels of approximately 10 mM whereas lethal levels are 10 times this concentration [1]. Methanol requires a much smaller intake for toxic effects and patients with an excess of 15 mM in the blood must immediately undergo treatment with hemodialysis and ethanol [2].

Lately, biological recognition has been successfully utilised in analytical flow techniques for enhanced selectivity [3] where the biomolecules responsible for the recognition are enzymes [4–10], receptors [11], or antibodies [12,13]. Enzymes as derivatisation reagents have been used most extensively for detection purposes in flow injection (FI) and column liquid chromatography (CLC), both in the pre- and in the post-column mode [14,15]. Recently, enzymes have also been successfully used as solid state reagents in capillary electrophoresis [16]. The enzymes are most commonly immobilised either on a solid support contained in a small column, or bound onto an electrode to be used as a biosensor with electrochemical transduction [14,17].

Selective and sensitive detection in FI and LC of methanol and ethanol can be achieved by the use of immobilised alcohol oxidase (AOD) or alcohol dehydrogenase using several approaches [6,7,10,18–26]. Other amperometric biosensors for alcohol determinations based on immobilisation of whole cell [27] or tissue [28] into carbon paste have been reported by Wang and co-workers. Reversed phase separations in combination with immobilised enzyme reactors with both silica and polymer based supports have been made for applications in plasma samples [5,29–31].

We report here on the characterisation of enzyme based amperometric biosensors by co-immobilisation of horseradish peroxidase (HRP) with two different AOD preparations from *Candida boidinii*, one from *Pichia pastoris*, and one from *Hansenula polymorpha*. Optimisation of the catalytic performance of the enzymes in the pastes were investigated with respect to sensitivity and selectivity of the four types of enzyme electrodes in a FI system. The objective of this study was also to take advantage of the biocatalytic detection in combination with fast CLC separation for the determination of ethanol and methanol in biological fluids such as plasma and urine. This rapid determination was achieved with an optimised CLC system using the *P. pastoris* based biosensor.

2. Experimental

2.1. Biosensor preparation

Plain carbon paste (CP) was prepared by thorough mixing of 40 μl paraffin oil (Fluka, Buchs, Switzerland, cat. no. 76235) with 100 mg graphite powder (Fluka, cat. no. 50870) in an agate mortar. Plastic syringes (1.0 ml syringe, Once, Asik, Denmark) were filled with the plain CP paste leaving about 3 mm at the tip empty to be filled with chemically modified carbon paste. Electrical contact was provided by inserting a silver thread into the paste.

Three of the AOD preparations were immobilised by adsorption onto graphite powder according to A–C as presented below. Further, AOD from *P. pastoris* was covalently immobilised as described in D below. The amount of enzyme used in A–D was adjusted so that each graphite paste contained 137 U AOD (EC 1.1.3.13) and 395 U HRP (EC 1.11.1.7) [21]. In an additional study as described below under E, an AOD preparation from *H. polymorpha* was immobilised to the graphite powder through adsorption according to the amounts specified.

(A) To 100 mg heat treated graphite powder [18], 1.37 mg HRP (Sigma, St. Louis, MO, cat. no. P-8375, 288 U mg^{-1} solid) dissolved in 200 μl 0.1 M phosphate buffer at pH 7.0 were added along with 113 μl of AOD (from *C. boidinii*, obtained as a solution in phosphate buffered 60% sucrose (Serva, cat. no. 12085, 30.3 U mg^{-1} protein)). While stirring, 200 μl 0.32% aqueous solution of polyethylenimine (PEI, Sigma, cat. no. P-3143, 50% aqueous solution) in 0.1 M phosphate buffer at pH 7.0 were added. The mixture was stirred for 16 h at 4°C and thereafter dried in a desiccator under reduced pressure for 4.5 h.

Finally, 40 μl of phenylmethylsilicon oil (Silicone DC 710, Alltech, Arlington Heights, IL) [18] were thoroughly mixed with the enzyme modified graphite in an agate mortar to form a uniform paste.

(B) The same immobilisation procedure was followed as in A, but using 115 mg AOD from *C. boidinii* (obtained as a lyophilised powder, Sigma, cat. no. A-0763, 10 U mg^{-1} protein) dissolved in 100 μl phosphate buffer and HRP dissolved in 150 μl of the same buffer. Because of the large protein content incorporated into the paste (see Section 3), a second batch (B2) was prepared containing one third of the AOD content. The paste with the lower AOD content was used in the study.

(C) 125 μl AOD from *P. pastoris* (obtained as a solution in phosphate buffered 60% sucrose, Sigma, cat. no. A-2404, 24 U mg^{-1} protein) were used. Apart from the different AOD source, the immobilisation procedure was the same as described in A above.

(D) AOD from *P. pastoris* was also covalently immobilised and crosslinked in CP. The same procedure was used as in C except that the heat treated graphite powder was replaced with carbodiimide activated graphite powder [21]. Also, 2.5 μl 25% glutaraldehyde solution (GA, Sigma, cat. no. G-5882) was added to the enzyme–graphite mixture prior to mixing for 16 h.

(E) AOD from *H. polymorpha* was co-immobilised with HRP by adsorption of the enzymes onto the graphite powder. The ratio between the amount of HRP to AOD was varied, keeping the total protein constant (15 mg:100 mg graphite). To 100 mg heat treated graphite, varying amounts of HRP (Boehringer Mannheim, Mannheim, Germany, cat. no. 814 407, 1000 U mg^{-1}) as specified in Section 3, and 400 μl 0.05 M phosphate buffer (pH 7.0) were added and stirred at 4°C for 30 min. To this mixture, varying amounts of AOD from *H. polymorpha* (obtained as a solution, 730 U ml^{-1}, a gift from Dr T.D. Gibson, University of Leeds, UK) was added and the mixture was further stirred for 1.5 h. The mixture was dried in a desiccator under reduced pressure for 2.5 h. For some batches (E2–E7), different additives or combinations of additives were also incorporated into the pastes. PEI, GA, polymeric dialdehyde (PD, Sigma, cat. no. P-9265), lactitol (a gift from Dr T.D. Gibson) and DEAE–dextran (Pharmacia Køge, Køge, Denmark) were the additives used. They were incorporated into the graphite–enzyme mixtures immediately after the addition of the AOD.

Aliquots of the chemically modified CPs in A–E above were packed into the tips of the plastic syringes as described above. The electrode surface was smoothed by gently rubbing it on a glass surface resulting in a surface area of 0.053 cm^2.

Some electrodes were further surface modified by the deposition of membranes by the following procedure. After rinsing the electrode carefully with distilled water, the surface of the electrodes were covered with an electropolymerised layer of *ortho*-phenylenediamine (*o*-PDA, Fluka, cat. no. 78412). The *o*-PDA film was applied by dipping the electrode into a 0.5 M acetate buffer solution at pH 5.2 containing 5 mM *o*-PDA and running a series of cyclic voltammograms (5 sweeps, scan rate: 50 mV s^{-1}) between 0 and + 650 mV versus a saturated calomel electrode (SCE) [7,18]. The electrode was then covered with 6 layers of a cation exchange membrane, Eastman AQ-29D, as described previously [7,32]. After the last dip the electrode was dried for 1 h before use. In all cases, the electrodes were kept in a dry state at 4°C when not in use.

2.2. Flow systems

2.2.1. Flow injection analysis

The prepared biosensors were mounted in a flow-through amperometric cell of the wall-jet type [33] connected to a three electrode potentiostat (Zäta Elektronik, Lund, Sweden). A platinum wire was used as the counter electrode and a Ag/AgCl served as the reference with 0.1 M KCl as the reference solution.

The cell was connected to a single line FI system using an automated sample injection analyser (Ismatec, Glattburg-Zürich, Switzerland). The injection loop volume was 50 μl. Connections between the various parts in the FI system were made with Teflon tubing, 0.5 mm i.d. and Altex screw couplings.

2.2.2. CLC

A liquid chromatographic pump (model 2150, LKB, Bromma, Sweden) delivered the mobile phase, phosphate buffer (50 mM, pH 8) with a flow rate of 0.7 ml min^{-1} to an injector (model 7000, Reodyne, Cotati, CA) with an injection loop volume of 50 μl. The PLRP-S column material (particle size 5 μm, pore size 100 Å) was packed in a column (150 × 0.4 mm i.d., Church Stretton, Shropshire, UK) and used as the analytical column. In the CLC system, PTFE tubing with 0.25 mm i.d. were used throughout in the system. The mobile phase was degassed with helium.

2.2.3. Sample handling in CLC

A urine sample from a volunteer pregnant woman was diluted with water and thereafter directly injected into the CLC system. Blood samples were taken in 1 ml tubes and allowed to clot for 10 min. To 1 ml serum, 100 μl perchloric acid were added and the sample was centrifuged at 4000 × *g* for 15 min. The supernatant was decanted and stored at −20°C until used for analysis. Prior to analysis, the samples were diluted and spiked with stock solutions of ethanol and methanol and thereafter injected directly into the CLC system.

2.3. Chemicals

Ethanol was of highest quality (99.5%, spectrographic grade, Kemetyl, Stockholm, Sweden). Methanol, acetic, malic, lactic, citric, and formic acid, formaldehyde, acetaldehyde, and hydrogen peroxide were obtained from Merck (Darmstadt, Germany). All other standards were of analytical grade and purchased from Sigma.

2.3.1. Standard solutions

Stock solutions of methanol and ethanol, aldehydes, organic acids, and other sample and model compounds (0.1–10 mM) were prepared in phosphate buffer based on Millipore water obtained from a Millipore (Bedford, MA) Milli-Q water purification system.

3. Results and discussion

3.1. Characterisation of the biosensors

The co-immobilisation of AOD from *C. boidinii*, *P. pastoris*, or *H. polymorpha* together with HRP, interfaced with an amperometric transduction will result in a coupled enzymatic and electrochemical reaction sequence for the detection of some aliphatic alcohols, aldehydes, and organic acids according to the reaction scheme in Fig. 1.

Enzymatically produced hydrogen peroxide from AOD (in its oxidation step) is detected by the use of HRP in a series of reactions. The hydrogen peroxide reacts with native HRP in a single two-electron transfer step forming water and an oxidised form of the enzyme (compound I). The reduction of compound I back into native HRP is made possible by donation of electrons from the electrode material in two separate single-electron steps with the formation of an intermediate form, compound II, by an apparent direct electron transfer [34].

Special care must be taken to optimise the sensor in such a way that the enzymes, which are physically confined or localised in the organic micro-environment, will retain their catalytic activity and operational stability. The source and preparation procedure for any enzyme is very important for its stability and catalytic property. These critical criteria have also been found by others, especially Woodward [35] who studied the enzyme mechanisms of different types of AODs in more detail. AOD belongs to the group of oxi-

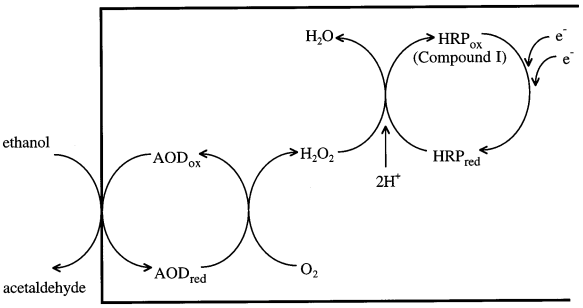

Fig. 1. The reaction scheme for the AOD/HRP biosensor operated at − 50 mV vs. Ag/AgCl reference electrode.

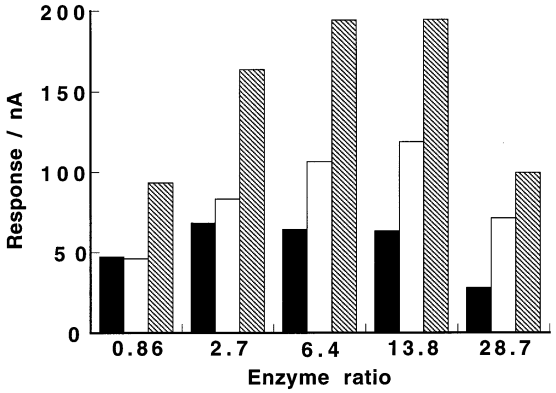

Fig. 2. The response characteristics of the alcohol sensor based on AOD from *H. polymorpha* for 0.1 mM H_2O_2 (■), 0.5 mM ethanol (□), and 0.5 mM methanol (hatched box) in 0.1 M phosphate buffer (pH 7.0) at −50 mV vs. Ag/AgCl, with varying AOD to HRP ratios in the carbon paste.

dases with a flavin adenine dinucleotide as its bound cofactor. It comprises eight subunits, each with a molecular weight of approximately 74000 Da [36] and the enzyme is catalytically active only when the subunits are kept together.

3.2. Optimisation of enzyme ratio

From previous results with the HRP preparation from Sigma, 1.35 mg HRP with 100 mg graphite was found to be optimal [21]. This amount was taken for all CPs based on AOD from *C. boidinii* and *P. pastoris* (A–D in Section 2). The AOD from *H. polymorpha* was co-immobilised with a different preparation of HRP than in the case of the other AODs and thus the optimal ratio of AOD to HRP (w/w) was investigated keeping the total protein content at 15 mg:100 mg graphite (E in Section 2). The responses to ethanol and methanol was highest when the AOD to HRP ratio was 13.8 (w/w), while that to hydrogen peroxide was almost at a maximum compared with the other CPs (Fig. 2). Increasing the ratio further resulted in a decrease in response for all three analytes indicating a restriction in the turnover of HRP.

3.3. Selectivity

The choice of the source of AOD and enzyme preparation procedures were found to have a big impact on the selectivity of the alcohol sensor, as illustrated in Tables 1 and 2. The Tables show the relative responses of the sensors when operated in FI mode for a large number of alcohols, aldehydes, organic acids, and related functional structures. This broad selectivity has been reported previously [35,37]. As can be seen in Table 1, there were large differences in selectivity when using various preparations and immobilisation techniques of AOD. Table 2 shows the high selectivity toward ethanol compared with possible interferents present in complex biological or biotechnological samples. This is a most important feature, for instance, when monitoring fermentation processes where the sensing unit may be exposed to a variety of compounds present in complex matrices and which are difficult to separate from the analytes of interest [10].

AOD from *C. boidinii* from Serva exhibited selectivity towards very few compounds, showing little or no activity towards aldehydes and acids. The other AODs showed much broader selectivity, where several acids and aldehydes were detected. Butyraldehyde proved to be the most readily detected aldehyde despite its long carbon backbone, which is noteworthy, since increasing chain length is generally connected with decreasing activity for alcohols [35]. The CP electrodes with covalently bound AOD from *P. pastoris* (D in Section 2) showed a different selectivity compared with the electrodes with adsorbed enzyme. For instance, the covalently bound enzyme did not show any activity toward acetic or propionic acid, but readily catalysed the oxidation of acetaldehyde. The sensor based on the *C. boidinii* preparation from Sigma (B in Section 2) had a very high background current due to the high protein content in the paste. Sensors containing only one third the amount of oxidase (B2) were therefore prepared and used in the remaining investigations. Along with the CP containing AOD from *H. polymorpha*, it showed the broadest substrate specificity towards the analytes investigated.

In general, it can be stated that the oxidases exhibit broad selectivity, short aliphatic alcohols being the most favourable substrates. As a result, AOD from one source may be more suitable than another depending on the intended application. Even though the sensor operates at −50 mV, certain chemical species that can compete with the electrode as electron donors to compounds I and II, e.g. phenolics, may interfere in the sensing chemistry, producing radicals which are electrochemically active [38,39].

3.4. pH dependence

The pH dependence was studied for the four AOD/HRP preparations immobilised through adsorption. Ethanol and hydrogen peroxide solutions at pH 5, 6, 7, 7.5, 8, and 9 were tested in FI in order to differentiate the pH effect on both enzymes, as well as to estimate the conversion efficiency of ethanol with that of hydrogen peroxide. Between each pH, solutions with pH 7.5 were injected in order to normalise the forthcoming signals for any deactivation caused by the wide pH range. The paste preparations with AOD from *C. boidinii* (Serva) as well as from *P. pastoris* demonstrated a pronounced maximum response for ethanol at pH 8 as seen in Fig. 3a. This is consistent with the results of Woodward [35] for AOD in aqueous solutions. AOD from *H. polymorpha* has a broad pH optimum in aqueous solutions (7–11), but for the present bienzyme electrode, the optimum was found to be at pH 7.

Fig. 3b shows the variation of the response for hydrogen peroxide with pH and reflecting the activities of HRP revealed a broad optimum. The

Table 1
Interferents to AOD immobilised in carbon paste

Substrate	Relative response (%)				
	C. boidinii (Serva[a])	*C. boidinii* (Sigma)[a]	*P. pastoris*[a]	*P. pastoris*[b]	*H. polymorpha*[a]
Ethanol	100	100	100	100	100
Hydrogen peroxide	75	866	189	162	43
Methanol	540	252	282	340	153
Formaldehyde	22	58	99	141	78
Acetaldehyde	<1	10	<5	196	3
Propionaldehyde	15	163	94	<2.5	7
Butyraldehyde	29	378	652	97	4
Formic acid	<1	<7	<5	—	46
Acetic acid	<1	34	19	<2.5	3
Propionic acid	7.1	51	56	<3.5	25
Butyric acid	1.4	44	41	—	4
Monochloroacetic acid	<1	60	11	—	—
Citric acid	<1	<7	<5	—	5
Lactic acid	<1	<7	<5	5	4
Malic acid	<1	31	<5	—	—
Pyruvic acid	<1	<7	<5	—	4
IP_3	151	217	159	—	—
Allylalcohol	68	46	55	130	62
2-Butenol	79	377	470	—	—
2-Chloroethanol	22	15	24	31	—
Dihydroxyacetone	4.6	57	57	<2.5	6
Ethanol amine	—	—	—	<2.5	—

Sample concentrations were 0.5 mM except hydrogen peroxide (0.1 mM).
—, Not determined.
[a] Immobilisation through adsorption.
[b] Covalent immobilisation.

Table 2
Interferents to AOD immobilised in carbon paste

Sample	Relative response (%)		
	C. boidinii (Serva)	*C. boidinii* (Sigma)	*P. pastoris*
Ethanol	100	100	100
Adenosine	<9	<14	<15
Glucose	<9	37	<15
Lactate	<9	62	<15
L-Phenylalanine	<9	<14	<15
Phenol	<9	<14	<15
m-Cresol	<9	<14	<15
Benzaldehyde	<9	<14	<15
Furaldehyde	<9	<14	<15
5-HMF	<9	<14	<15
BSA	137	82	141

Sample concentrations were 0.5 mM. Enzymes immobilised through adsorption.

only bienzyme system that showed a markedly decreased response to hydrogen peroxide at pH 8 was the CP containing AOD from *H. polymorpha*. In all further experiments using enzyme preparations from *C. boidinii* and *P. pastoris*, phosphate buffer set to pH 8 was used, whereas for *H. polymorpha* a pH of 7 was chosen.

3.5. Optimisation of the flow injection system

In flow systems such as FI and CLC, it is imperative to optimise various flow parameters for rapid determination in analytical applications. At the same time, the compatibility of the detection unit in the flow systems must be taken into consideration so that the enzymatic reaction sequence followed by the electrochemical regeneration (Fig. 1) will reflect the composition of the analyte in the sample plug. The flow rate and the distance between the inlet and electrode in the wall-jet cell were optimised to minimise the band broadening effects caused by the flow system and evaluated as the peak widths measured at half the peak height.

3.5.1. Flow rate

The variation of the response peak with the flow rate was investigated in FI. The peak widths were measured at half the peak height while simultaneously increasing the paper feed rate of the chart recorder by the same factor as the flow rate in the FI system. As depicted in Fig. 4a, the signal observed for methanol using the alcohol sensor from *C. boidinii* (Serva) was greatest at flow rates below 0.5 ml min^{-1} and decreased steadily with increasing flow rate while the peak widths remained constant, indicating kinetic restrictions in the substrate turnover of the oxidase in the sensor. The FI system was found to have an optimum flow rate of 0.7 ml min^{-1} at which the current signal was close to the maximum and band broadening was only slight.

The relationship between the peak height for ethanol and the flow rate in FI was reversed for the *P. pastoris* based sensors as shown in Fig. 4b. The peak height increased with increasing flow rate, while the peak width showed a slight minimum at 0.7 ml min^{-1}. This indicates a high turnover rate for the AOD from *P. pastoris*, allowing for rapid analysis. This is also apparent in Table 3 where the responses to hydrogen peroxide, ethanol and methanol, along with the corresponding peak widths for all AOD preparations, can be found and will be discussed further in Section 3.9.1 regarding applications to plasma and urine. The curve in Fig. 4b levels out at the higher flow rates and is probably due to increased dispersion. Even though the optimum flow rate was 1.2 ml min^{-1}, near optimum conditions were found at 0.7 ml min^{-1}. For the sake of simplicity,

0.7 ml min^{-1} was used as the flow rate in all measurements in FI for all the electrodes described below.

3.5.2. Inlet–electrode distance

The electrode distance denotes the distance the sample travels when it flows from the cell inlet, through the bulk solution in the cell, until it reaches the electrode surface [33]. The maximum current for ethanol was observed at a distance of 4 mm and shorter, as seen in Fig. 5, whereas the peak width continuously decreased with decreasing distance until it reached the lower limit at 2 mm. At longer distances, the sample plug disperses into the bulk solution causing the analyte concentration to dilute, and also decreases the mass transfer at the electrode surface, resulting in lower signals as well as broader peaks indicating a greater effective dead-volume. For practical purposes, such as checking the electrode surface for air bubbles, 3 mm was chosen as the inlet–electrode distance instead of 2 mm where the minimum peak width was registered.

3.6. Calibration

Fig. 6a shows the calibration curves as log–log plots of the FI responses for hydrogen peroxide, ethanol, and methanol from the electrodes containing adsorbed AOD from *H. polymorpha* with the optimal enzyme ratio but without any additives incorporated into the paste. Methanol gave higher response currents than equal concentrations of ethanol. This is expected, as for all AODs, the product formed when oxidising methanol is formaldehyde, which in itself is a substrate for AOD also yielding hydrogen peroxide, in combination with the effect of different Michaelis–Menten constants for ethanol and methanol [35,37]. Hydrogen peroxide, the substrate for HRP, had the steepest calibration curve for each enzyme modified CP, as expected. At the electrode surface, the substrates for AOD produce hydrogen peroxide, which diffuses in all directions from the electrode surface unless further reacted with HRP. Less than 100% trapping of the hydrogen peroxide produced is thus expected. The amount of trapped and converted hydrogen peroxide is expressed as the conversion and is calculated as the ratio between the responses to equal concentrations of ethanol and hydrogen peroxide. The conversion for all AODs was less than 0.15. More efficient trapping of hydrogen peroxide would increase the conversion and lower the detection limits.

A linear current response was observed for hydrogen peroxide from 2 μM to 0.2 mM, $R = 0.996$, for ethanol 30 μM–2 mM, $R = 0.999$, and for methanol 8 μM–0.5 mM, $R = 0.998$. Higher concentrations above 0.4 mM hydrogen peroxide were not investigated because of the risk of the formation of compound-III, an inactive form of

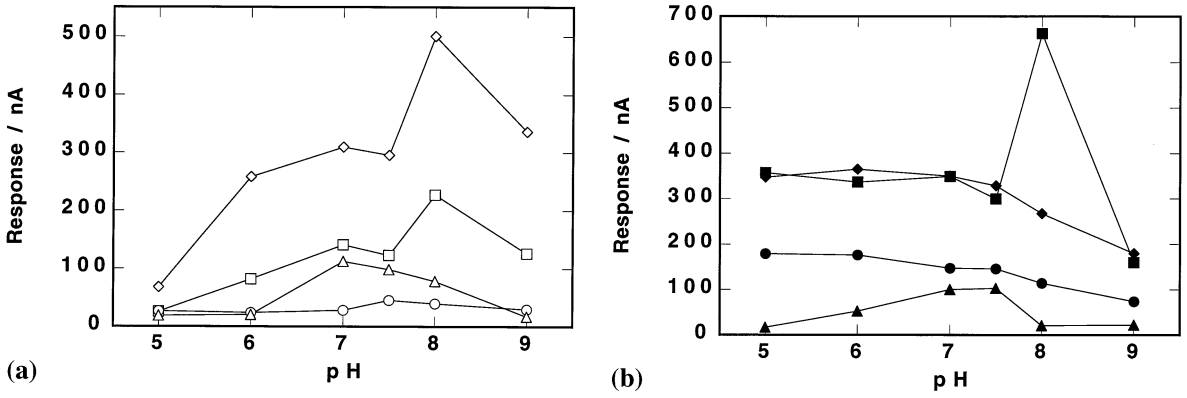

Fig. 3. The pH dependence for the four AOD/HRP CPs in FI. (a) The response to 0.5 mM ethanol for the AOD sources *C. boidinii* (Serva) (□), *C. boidinii* (Sigma) (○), *P. pastoris* (◇), and *H. polymorpha* (△); and in (b) the electrode responses (filled symbols, respectively) to 0.1 mM hydrogen peroxide.

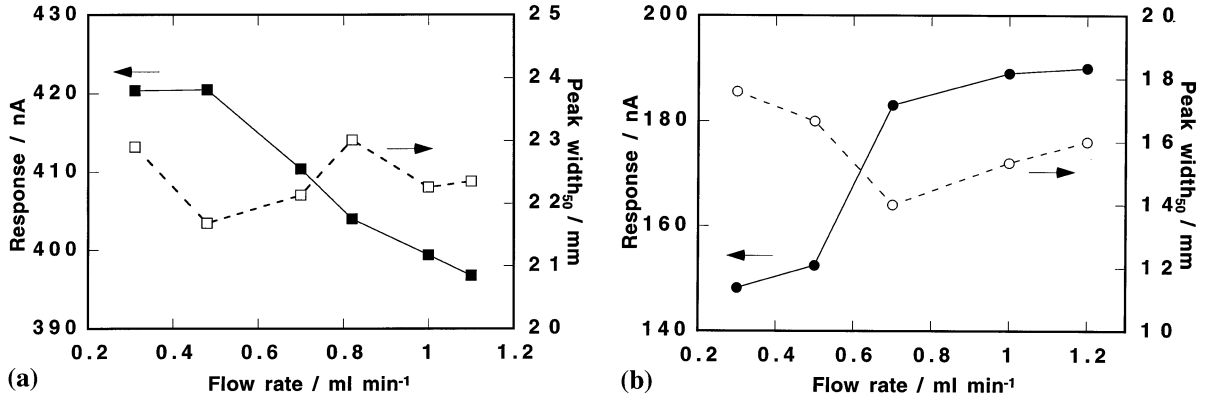

Fig. 4. Flow rate optimisation in FI for AOD from (a) *C. boidinii* (Serva) and (b) *P. pastoris*. The responses to 0.5 mM methanol in (a) and to 0.5 mM ethanol in (b) and the corresponding peak widths (at half the peak height, unfilled symbols) are plotted against the flow rate.

HRP. The slope of the log–log plot for hydrogen peroxide, ethanol, and methanol were 0.77, 0.96, and 0.91, respectively. This is in accordance with previously published data that the slope of the log–log calibration for co-immobilised oxidase–peroxidase deviates from the ideal value of unity for hydrogen peroxide but is much more close to unity for the oxidase substrate [21]. The reason for this remains unclear. Apparent kinetic parameters, Michaelis–Menten constant (K_m^{app}) and I_{max}, were evaluated using the Eadie–Hofstee approach and found to be 0.35 mM and 902 nA for hydrogen peroxide, 9.9 mM and 2505 nA for ethanol, and 1.57 mM and 1727 nA for methanol, respectively. As the sensor response for the alcohols is based on two consecutive enzyme reactions coupled to an electrochemical reaction, the K_m^{app} values only reflect the entire sensor characteristics and thus cannot be used for comparison with results from other sensor types.

The linear regions for the sensor containing covalently bound AOD from *P. pastoris* when investigated in the CLC system were 5–600 μM, $R = 0.997$ for methanol, and 20 μM–2 mM, $R = 0.995$ for ethanol. The slope of the log–log plots were 0.89 and 0.996, respectively, once again close to unity. In Fig. 6b and c, the log–log plots for the calibration of methanol and ethanol are shown for an electrode before and after application of the electropolymerised *o*-PDA membrane.

K_m^{app} was determined for the immobilised enzyme configuration using Eadie–Hofstee and Lineweaver–Burk plots. Linearity was obtained in the latter. However, the Lineweaver–Burk analysis may result in an overemphasis on lower concentration points [40]. Despite poor correlation coefficients in the Eadie–Hofstee plots, these were used for the K_m^{app} approximations. The K_m^{app} observed were 0.91 mM for methanol and 1.42 mM for ethanol. I_{max} was found to be 3511 nA for methanol, and 1268 nA for ethanol. Compared with the sensors with AOD from *H. polymorpha*, the K_m^{app} values were lower for the sensors with *P. pastoris* indicating faster kinetics for this enzyme source.

A comparison of the peak widths of five enzyme configurations when operated in FI is summarised in Table 3. The transport of the substrate from the electrode surface to the AOD molecule, the oxidation of the substrate by the enzyme, and the release of a hydrogen peroxide molecule are three steps where the AODs may differ. *C. boidinii* (2) showed greater tailing of the peaks for all substrates investigated indicating a lower turnover rate. Also, the signal for hydrogen peroxide is markedly lower than for *C. boidinii* (1) and *P. pastoris*[a] despite the same amounts of HRP in these pastes. Differences in enzyme purification may be the cause leading to varying micro-environments experienced by the peroxidase. The peak widths of the responses to hydrogen perox-

Table 3
Electrode responses and peak widths in FI for four preparations of AOD

Alcohol oxidase	Response (nA)			w_{50} (s)			w_{10} (s)		
	H_2O_2	Methanol	Ethanol	H_2O_2	Methanol	Ethanol	H_2O_2	Methanol	Ethanol
C. boidinii (1)[a]	500	692	128	10.6	11.2	11.0	18.6	18.0	23.0
C. boidinii (2)[a]	206	237	94	11.4	13.2	13.8	19.5	22.8	24.6
P. pastoris[a]	422	813	275	10.9	11.3	11.7	19.2	20.1	22.8
P. pastoris[b]	555	1135	336	11.2	11.6	11.8	20.0	20.4	20.8
H. polymorpha[a]	187	422	120	6.0	10.2	10.8	12.0	18.0	20.4

Measurements performed on a 0.1 mM H_2O_2 solution and 0.5 mM alcohol solutions.
w_{50}, Peak width at half the peak height; w_{10}, peak width at 10% of the peak height.
[a] Immobilised through adsorption to graphite powder.
[b] Immobilised covalently to graphite powder.

ide are consistently lower than those to the alcohols, partly due to the lower concentrations of hydrogen peroxide but also due to the turnover rate of HRP being higher than for AOD. This is especially apparent for the AOD/HRP paste from *H. polymorpha* and *C. boidinii* (2).

The fourth paste in Table 3 (*P. pastoris*[b]) was prepared by covalent attachment of the enzymes and was found to be the best CP with high a turnover rate for ethanol and high enzyme activity. The peak widths are similar to the CPs based on AOD from *P. pastoris*[a] and *C. boidinii* (2) but the current signals are amplified compared to immobilisation through adsorption. The CP containing *H. polymorpha* was immobilised with a different peroxidase than the other CPs and exhibited a much faster turnover rate for the peroxidase as seen by the narrower peak widths in the responses to hydrogen peroxide. The turnover for the alcohols was also faster for this paste due to the peroxidase alone or to a combined effect with the AOD, but the activity of the enzyme configuration was rather low as seen by the low responses to the analytes.

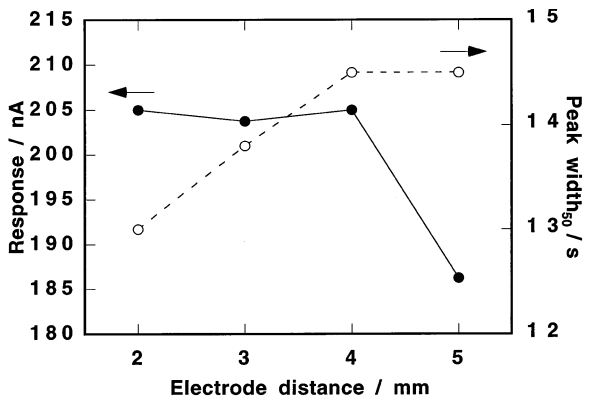

Fig. 5. Optimisation of the inlet–electrode distance for *P. pastoris* based electrode. Current signals for 0.5 mM ethanol (●) and the resulting peak widths at half the peak height (○) are plotted vs. the electrode distance.

3.7. Additives

Previous investigations have shown that the electrode response and the response time can be drastically improved by the addition of polyelectrolytes and/or sugar derivatives to the enzyme modified CP [18,25,41–43]. Addition of PEI in the paste mixture improved the response characteristics for ethanol of an electrode based on co-immobilised AOD (from *C. boidinii*) and HRP in CP [18]. Positive results on the addition of PEI have also been reported in other sensor systems [21,26,44,45].

Both the sensitivity and the stability of the sensors are increased in the presence of certain additives in the CP. Different additives were therefore incorporated into CPs containing co-immobilised HRP and AOD from *H. polymorpha*, to study the effect on the electrode response. From our previous studies [41,43], the additives were selected as shown in Fig. 7a–c. A blank electrode was prepared in all cases as a reference without

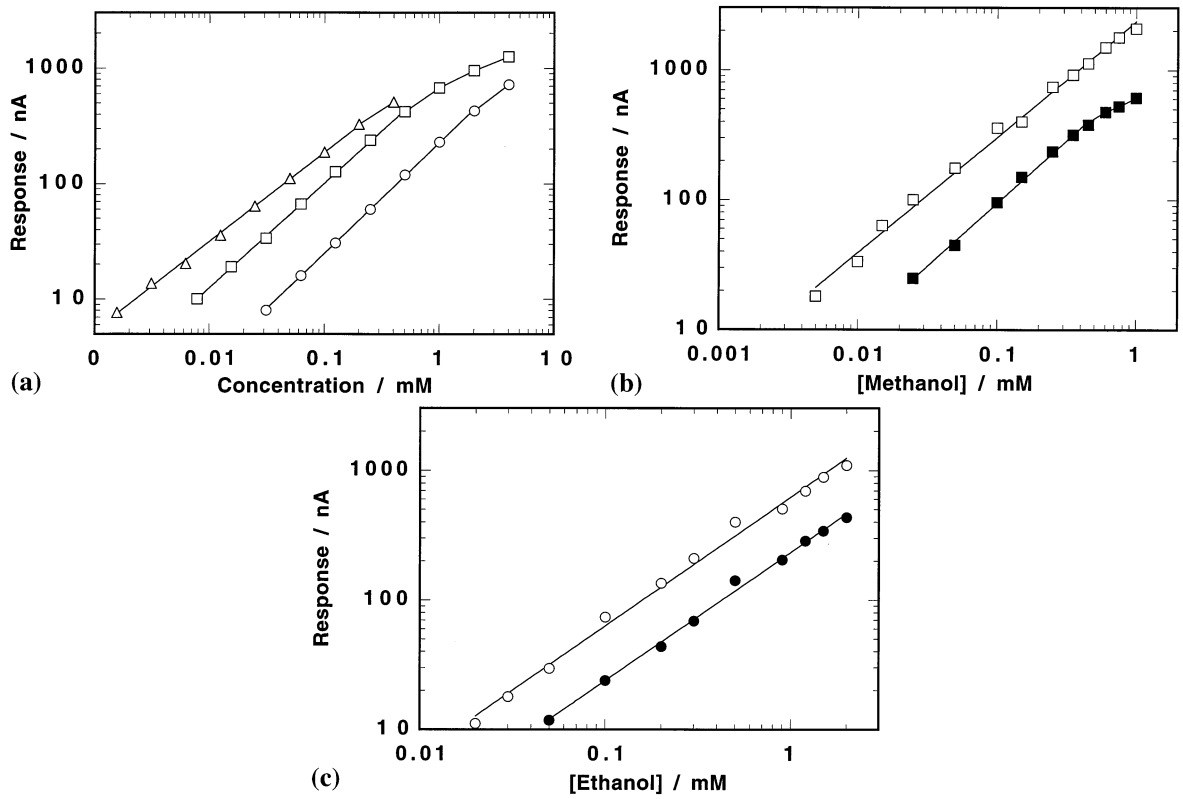

Fig. 6. Log–log plots of calibration curves for an alcohol electrode from (a) *H. polymorpha* (no additives in the paste) in FI for hydrogen peroxide (△), ethanol (○), and methanol (□). (b), (c) The log–log calibrations plots for methanol and ethanol, respectively, in CLC for electrodes based on covalently bound AOD from *P. pastoris*. The unfilled symbols depict the electrodes without surface membranes and filled symbols depict the electrodes with electropolymerised *o*-PDA on the surface.

any additives. From the experiments, it was observed that the addition of GA reduced the electrode response drastically for ethanol and methanol, though the response for hydrogen peroxide was not affected in any way. This could be due to the denaturing of AOD in the presence of GA. The addition of PEI did not have much effect on the electrode response either to ethanol, in contrast to previous results with AOD from other sources [7,21], or to hydrogen peroxide. The addition of lactitol (10 mg:100 mg graphite), a sugar alcohol, showed increased response for all three substrates. This is in good agreement with previous investigations [25,43,46], where the addition of lactitol was shown mainly to have a beneficial effect on the peroxidase. PD (1 mg:100 mg graphite) did not show much effect on the sensor, though in combination with lactitol, an improved response was observed. The amounts of lactitol and PD in the paste needs further optimisation. A combination of lactitol and DEAE–dextran [46,47] showed the greatest effect on the electrode with an increased response and higher conversion factors for ethanol and methanol. The dextran derivative is positively charged and was shown in previous investigations to have a stabilising effect on AOD [47,48].

3.8. Stability

The stability of a biosensor is of course important for long-term applications. A stability test was performed using an electrode with covalently immobilised AOD from *P. pastoris* and HRP with

GA and PEI added to the paste. No protective membrane was added to the electrode surface. The operational stability for the immobilised AOD is depicted in Fig. 8a. The figure shows the electrode's response to repeated injections of a standard solution of ethanol every hour over a period of one week, resulting in a 71% loss in activity. The decrease was not linear, though. After a large initial activity loss during the first 50 h, the electrode response levelled off into a more linear curve with a less steep slope. The electrode response half-life was 52 h.

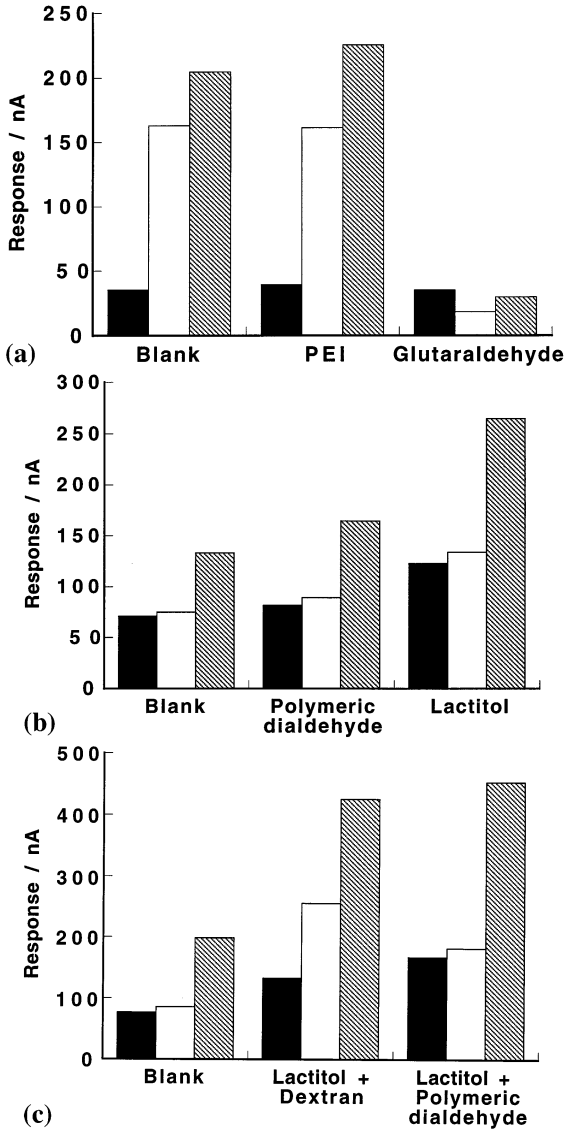

Fig. 7. (a), (b), (c) Show the effect of different additives on the sensors based on AOD from *H. polymorpha*. The analytes were 0.1 mM H_2O_2 (■), 0.5 mM ethanol (□), and 0.5 mM methanol (hatched box) measured in FI.

When mounted into the flow system, the loss in the electrode response could be accounted for by surplus enzyme that was not covalently bound to the graphite and which would therefore leak from the CP at the electrode surface into the bulk solution. Also, the enzyme molecules at the electrode surface may be deactivated with time from exposure to the mobile phase. When using the sensor for long-term measurements, it would be advantageous to place the sensor in a flow system for two days prior to use to eliminate non-linear activity loss. Also, the addition of membranes to the electrode surface would prevent enzyme molecules from leaking into the bulk solution.

To determine whether the electrode instability was due to the peroxidase or the oxidase, the conversion was calculated at different times during the stability test. The conversion calculated as the ratio between the responses to 0.5 mM ethanol and 0.1 mM hydrogen peroxide are shown in Table 4. The operational stability was studied as well as the stability when the electrodes were stored in a dry and cool state. The initial conversion was approximately 0.6 and decreased to 0.5 during continuous operation indicating that the decrease in the enzymes' activities were similar. The stored electrodes actually showed an increase in the conversion during the initial 25 h which means that HRP initially exhibited the largest loss in activity. After 120 h, the conversions were below the original values and even lower after 200 h. In the long run, the response to ethanol decreased to a greater extent than that to hydrogen peroxide, possibly reflecting that AOD is less stable in the paste than HRP.

The storage stability for electrodes from *H. polymorpha* that had not been modified by additives were also investigated. The sensors were followed for a period of 4 days. Between measurements, the sensors were refrigerated at 4°C, either in a desiccator or in 0.1 M phosphate buffer solution at pH 7.0. Storage in a desiccator reduced the response by 90% while the buffer stored

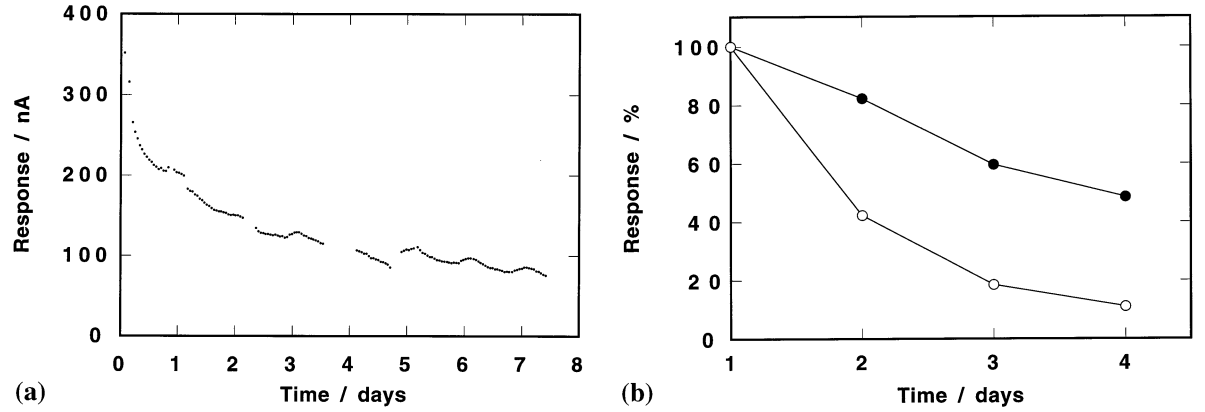

Fig. 8. (a) The operational stability of a covalently bound *P. pastoris* based sensor to repeated injections of 0.5 mM ethanol every hour in FI. (b) The storage stability of *H. polymorpha* based sensors stored at 4°C in buffer (●) or in a desiccator (○).

sensors showed better stability characteristics with a 50% loss in response as shown in Fig. 8b. The loss of activity in the sensors stored in the desiccator could be due to the repeated hydration and dehydration of the electrode surface, during measurement and storage, respectively.

It is of great importance to improve the enzyme stability. As discussed previously, the *P. pastoris* based electrodes used in this stability study contained PEI, which had earlier been shown to improve electrode performance and stability [18]. Various membrane configurations have been examined in an earlier study [7], thus gaining a greater understanding of the type of electropolymerised membrane that is the most suitable for electrode stability and selectivity when used in the analysis of complex samples. A recent approach to overcome drifts in biosensor performance is by the use of chemometrics [49].

3.9. Applications to plasma and urine

The alcohol sensor based on covalently bound AOD from *P. pastoris* was chosen as the most suitable sensor to be interfaced with a CLC separation. Of the alcohol sensors investigated, this sensor exhibited the highest turnover rate leading to quick responses which is important in chromatography in order to minimise peak tailing and to enhance resolution. The interfacing of the biosensor to the CLC separation for the separation and detection of ethanol and methanol in spiked urine and plasma samples was optimised.

3.9.1. Membranes

Extensive sample handling and sample preparation of the plasma and urine samples in the analysis can be avoided by the use of the discriminating nature of an enzyme based detection unit. However, the proteins, peptides and other interfering compounds must be made non-interactive with the electrode surface and other parts of the chromatographic system. Therefore, increasing attention is devoted to the use of protective membranes. In this application, different membranes were cast on the electrode surface in order to increase the operational stability and to improve the selectivity of the biosensor when applied to

Table 4
Changes in conversion with relation to time for electrodes based on AOD from *P. pastoris* during continuous operation or stored in a dry and cool state

Time (h)	Conversion[a]	
	Continuously operated	Stored
0	0.60	0.58
25	0.59	0.71
120	0.54	0.48
200	0.49	0.32

[a] Calculated as the quotient between responses in nA to 0.5 mM ethanol and 0.1 mM hydrogen peroxide.

the analysis of biological samples. Experience gained from earlier investigations of protective membranes [7] made us choose the use of a mixture of *o*-PDA electropolymerised onto the surface and Eastman AQ, an anionic membrane. No significant difference could be found in the recovery of either ethanol or methanol values when 10 repetitive separations of plasma were made.

When investigating the kinetic parameters of the membrane covered electrodes containing covalently bound AOD from *P. pastoris*, the K_m^{app} observed were 1.45 mM for methanol and 1.73 mM for ethanol. The I_{max} were found to be 1541 nA and 579 nA, respectively. These higher values for K_m^{app} and lower values for I_{max} compared with the electrodes without membranes (found in Section 3.6) were expected due to the increased limitations in the diffusion to the electrode surface. The slopes of the log–log plots of the calibration curves for the membrane covered electrodes (filled symbols in Fig. 6b and c) were 0.99 and 0.97 for methanol and ethanol, respectively, which were even closer to unity than for the bare electrodes.

The protecting layer on the electrode and its biocompatibilty was found to be of the utmost importance since the sample handling step developed, along with the fast CLC separation, in fact gave us a more complex sample in comparison with the use of disposable solid phase extraction cartridges (SEP-PAK, C-18). However, we also found that the recovery for both methanol and ethanol in spiked plasma samples were 50–65% using SPE clean-up (data not shown). By elimination of the SPE step, an increase in sensitivity by a factor of around two could be gained without clogging the analytical columns or fouling the biosensor.

3.9.2. Injection volume

Varying the injection volume in the CLC set-up resulted in maximum peak heights for methanol and ethanol using a 50 μl loop as seen in Fig. 9. The peak width at half peak height was at most 7 s broader than the minimum widths at smaller injection volumes. On the other hand, a 50 μl loop resulted in much larger current signals which outweighed the negative effects of peak broadening.

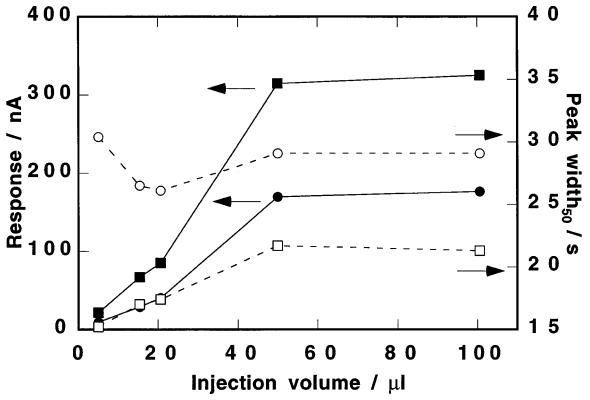

Fig. 9. Optimisation of the injection volume in CLC for *P. pastoris*. The current signals for 0.5 mM methanol (■) and 1.0 mM ethanol (●) and their corresponding peak widths (at half the peak height, unfilled symbols) are plotted as a function of the volume of the injection loop.

3.9.3. Flow rate

In optimising the flow rate for the chromatographic separation of methanol and ethanol, as described in Section 2, important factors to be considered are good separation for fast analytical determination, yet maintaining optimal conditions for the detection unit. The optimum flow rate for *P. pastoris* based sensors for the analytes ethanol and methanol, 1.0 ml min^{-1}, can be seen in Fig. 10, where the maximum signal as well as the minimal band width was obtained. Baseline separation between the two analytes was obtained at the same flow. Lower flow rates improved the resolution due to increased stagnant mobile phase mass transfer (number of interactions between the analytes and the stationary phase) and a higher plate number in the analytical column. However, sufficient resolution was observed at 1.0 ml min^{-1} for the separation of methanol and ethanol.

3.9.4. Chromatographic separation

The urine and plasma samples were diluted and spiked with methanol and ethanol as described in Section 2. Typical chromatograms are shown in Fig. 11 where the early eluting matrix components (peak 1 in sample b in Fig. 4) are separated from the methanol and ethanol peaks (peaks 2 and 3, respectively). The matrix does not interfere with the other two retarded peaks in the chro-

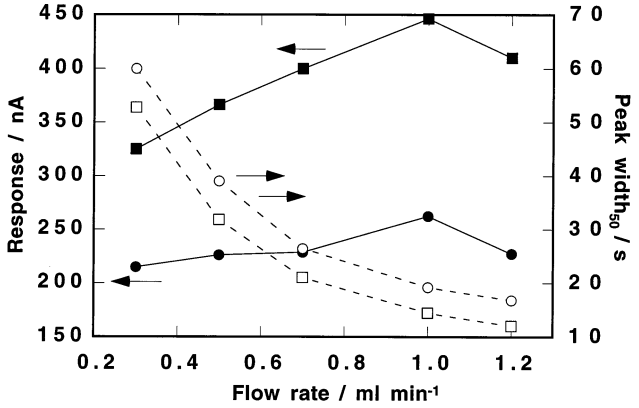

Fig. 10. Flow rate optimisation in CLC for a sensor based on AOD from *P. pastoris*. The responses to 0.5 mM methanol (■) and 1.0 mM ethanol (●) and the corresponding peak widths (at half the peak height, unfilled symbols) are plotted against the flow rate.

matogram. The chromatographic separation was quick; less than 5 min from the point of injection to detection resulting in 13 injections h^{-1}.

If necessary, faster assays can be performed with retained chromatographic resolution (data not shown) by minimising the inner diameter of the analytical column to 2.0 mm × 150 mm. The column flow rates are subsequently decreased to 0.2–0.4 ml min^{-1} to keep, approximately, the same linear velocity and resolution. In this way, the separation times can be lowered to a total run time of 2 min with a total throughput of 30 samples h^{-1}.

Earlier studies have been made to ensure the repeatability of the CLC separations. It was found that reversed phase columns such as silica based C18 (Nucleosil) column material gave good resolution for the separation of methanol and ethanol [7]. However, since the working pH was 8.0, it was found that not only did the separation factor and the plate numbers decrease, but we also found a decrease in the stability of the biosensor. Probably, there is hydrolysis of the silica phase which causes the silanol rests to adsorb onto the electrode surface and foul the electrode. We therefore developed a separation on a polymer based separation column (PLRP-S), used throughout the experiments, which was found to be almost as efficient in terms of separation but with improved stability. This analytical column material was also found to operate at its optimum when flushed with acetonitrile–water (30:70) over night every third day.

3.9.5. Recovery studies

The plasma samples were spiked with varying levels of methanol and ethanol and thereafter diluted 10-, 50-, and 100-fold prior to separation. Three spiked plasma samples of different dilution are illustrated (samples b, c, and d in Fig. 11) and are to be compared with the standard mixture

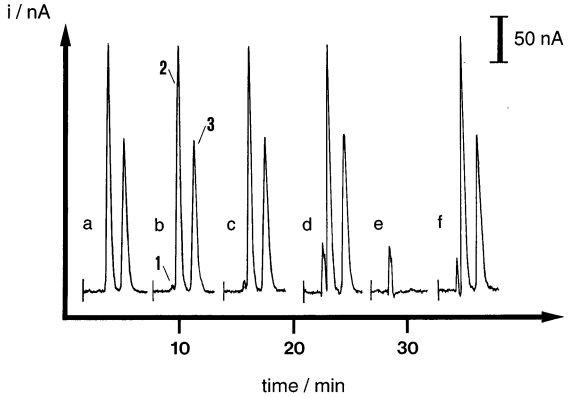

Fig. 11. Detection by a covalently bound *P. pastoris* based sensor of LC separations of 0.5 mM methanol (peak 2) and 1.0 mM ethanol (peak 3) from matrix compounds (peak 1) in spiked plasma (samples b–d having 100, 50, and 10 × matrix dilution, respectively) and urine (sample f, 10 × dilution). The chromatograms are to be compared to a standard solution of methanol and ethanol (sample a) and unspiked plasma of 10 × dilution (sample e).

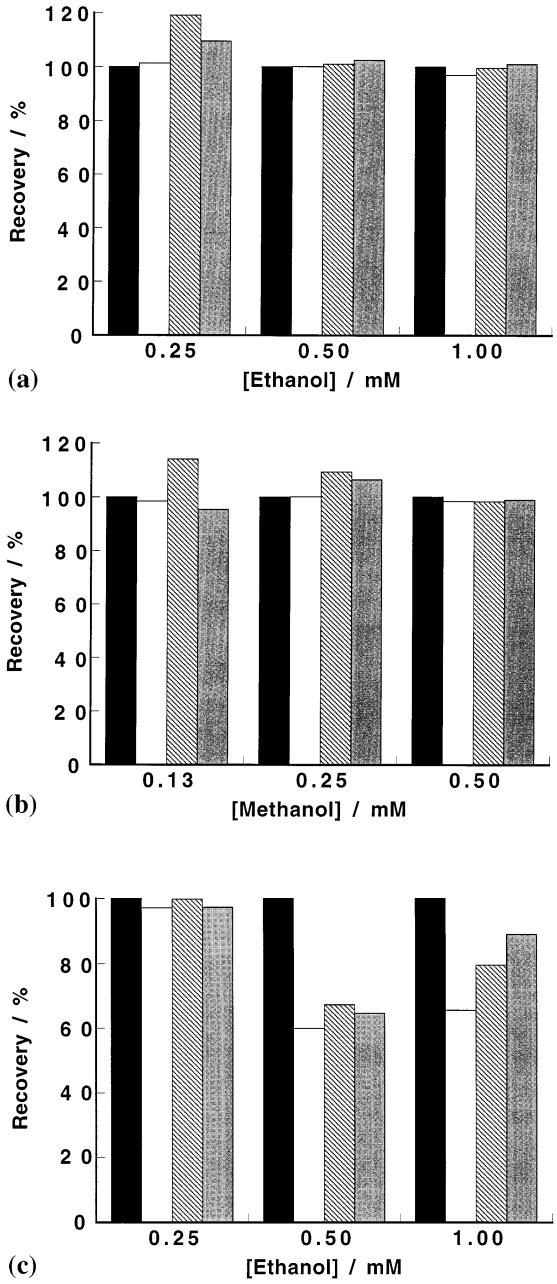

Fig. 12. The relative recovery of ethanol in plasma and urine in (a) and (c), respectively, and of methanol in plasma in (b) for varying concentrations after separation in the CLC system and detection by a covalently bound *P. pastoris* based sensor. The plasma and urine matrices were diluted 100 (□), 50 (hatched box), and 10 times (shaded box) and the recovery of the analytes were compared to the response to standard solutions (■).

(same concentration; methanol 0.5 mM and ethanol 1 mM, sample a) and the plasma blank (sample e). No compounds from the plasma blank are detected at the retention times of the two analytes. The recovery values for three concentrations of ethanol and methanol in three different dilutions of plasma samples are depicted in Fig. 12a and b, respectively. All recovery values were very close to 100% (97–109%) except for 0.25 mM ethanol and 50 times dilution (119%, Fig. 12a) and 0.12 mM methanol with the same dilution (114%, Fig. 12b), which means that regardless of the concentration level of the analytes and matrix concentration, the bioselective CLC-detection system gives good qualitative and quantitative results.

The urine sample taken from a pregnant women was found to behave somewhat differently from that of the plasma samples. The blank injection of 10 times diluted urine (not shown) was found to be similar to that of the plasma blank (sample e in Fig. 11). The separation of methanol and ethanol in a 10 times diluted urine sample is seen in Fig. 11f, where the analytes elute without any interference from matrix compounds present in the urine. However, greater scattering in recovery values were found for spiked urine samples (60–100%) than for plasma as can be seen for ethanol in Fig. 12c. Surprisingly, the largest deviations in recovery were found for samples with higher concentrations of spiked analyte. Also, the most diluted urine samples with ethanol concentrations of 0.50 and 1.0 mM exhibited recovery down to 60%. It would be expected that the most dilute samples would be the least affected by matrix interference.

4. Conclusions

The CLC-biosensor set-up for analysis of ethanol and methanol in biological samples involves many steps from the point of injection to the electrochemical transduction leading to amperometric detection. The analytes are first sepa

rated from interfering matrix components and from one another in the analytical column, transported to the wall-jet cell, through the bulk solution, before reaching the electrode surface. The analytes must then diffuse through the membranes covering the surface of the electrode before reaching the AOD molecules immobilised onto graphite in the organic media.

The four preparations of AOD examined showed large variations in selectivity when immobilised in carbon paste indicating that the type of application should determine which AOD should be used. In this study, biosensors with AOD from *P. pastoris* had the highest turnover rate for alcohols and was also found to be the best sensor for the detection unit in CLC applications. The stability for a bare electrode was investigated and illustrated the necessity for protective membranes on the surface of the electrode to minimise fouling and increase the electrode lifetime.

The set-up described in this paper for the detection of ethanol and methanol in spiked biological samples exhibited the selectivity required to eliminate interference from compounds found in the plasma matrix. The urine matrix did not interfere either, although some problems were experienced in the quantification step. The detection limits for ethanol and methanol in blood samples lie in the range for sample pre-treatment and detection. The analysis time was less than 5 min per sample, but this time could be reduced further if other column dimensions are used.

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