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Enzyme-based biosensor as a selective detection unit in column liquid chromatography

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ABSTRACT

A reagentless enzyme electrode based on co-immobilized alcohol oxidase and horseradish peroxidase was used as the working electrode in an amperometric flow-through cell connected to a column liquid chromatographic (CLC) system for the selective detection of methanol and ethanol. The enzymes were covalently immobilized in carbon paste (graphite–phenylmethylsilicone oil) in the presence of polyethylenimine. Electrodes prepared from the enzyme-modified carbon paste were optimized with respect to their sensitivity and selectivity. Different membranes were cast or electropolymerized directly on the surface of the electrode to increase the long-term stability of the biosensor. The compatibility with the reversed-phase chromatographic system was established. A PLRP-S polymer-based separation column was used with phosphate buffer as the mobile phase. The selectivity of the enzyme electrode was also determined by injecting some easily oxidizable and possibly interfering species normally present in biological samples. The enzyme electrode was also used in an on-line system, consisting of a microdialysis probe as the sampling unit, the CLC system and the biosensor detection device, for the selective following of the ethanol produced when a paper pulp industrial waste water was fermented with *Saccharomyces cerevisiae*.

INTRODUCTION

Derivatization techniques are widely used in column liquid chromatography (CLC) in both the pre- and postcolumn modes in order to enhance detectability and/or increase sensitivity [1–4]. Most commonly the reagents taking part in the reaction detection are solid-phase reagents immobilized on a liquid support. Both silica and polymeric supports are used and the coupling of the reactive modifier to the support is commonly achieved by physical adsorption, covalent binding or coating through precipitation. Solid-phase reagents offer advantages such as more efficient derivatization yield due to an improved chemical stability of the immobilized reagents, increased reaction capacities due to the high local concen-

tration of the derivatization reagent, and in many cases the derivatization reactions are subject to fewer side-reactions [5]. Biological recognition is used to improve selectivity further by the use of enzymes or antibodies mainly immobilized in solid-phase reactors. Enzymes are by far the most commonly used catalytic derivatization reagents in CLC. They are easily made compatible in various flow systems and are therefore nowadays common and acknowledged as bioselective detection units in CLC and flow injection (FI), determining solutes in a selective and sensitive way [6–9]. It is predicted that highly selective immunological interactions between antibodies and antigens will have a great impetus in future selective detection systems in both CLC and FI [10–12]. The increased commercial availability of various enzymes and antibodies will be of great importance in this field. Immobilized enzymes on solid supports are already commercially available from several manufacturers, also supplying chromatographic col-

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umns and optimized integrated systems for applications in various fields.

The most commonly studied field of biosensors is the enzyme electrode. Clark and Lyons [14] predicted the construction of an enzyme electrode in 1962 [13] and in 1967 Updike and Hicks [14] were the first to report on the construction and use of a glucose electrode by physically entrapping glucose oxidase by a dialysis membrane close to a Clark-type oxygen electrode. Since then, there has been rapid progress in the development of enzyme electrodes and more than 1200 original papers have appeared on glucose determinations alone. The enzyme electrode is based on the immobilization of the enzyme in close proximity to, *e.g.*, in a membrane, or directly on or in the electrochemical transducer, most commonly consisting of noble metals or various carbonaceous-type electrodes including carbon paste. Enzyme electrodes have most often been developed for batch analysis or in combination with non-chromatographic flow systems. Enzyme-based detection in CLC has, however, most often been effected by the use of immobilized enzyme reactors (IMERs), where the enzyme is bound on an inert solid support [8,9]. Only a few papers have reported on the use of such materials in detectors in conjunction with CLC [15], mainly because the sensors studied were developed for the specific analysis of one analyte only. However, Yao and Wasa [16] illustrated in a very elegant way the possible use of enzyme electrodes in conjunction with CLC by using a double electrochemical flow cell approach in which L- and D-amino acid oxidase were immobilized on two different electrodes for the stereoselective analysis of L- and D-amino acids. Both of these enzymes are group- rather than one substrate-specific enzymes [16–20]. This makes it possible to use common liquid chromatographic supports and separations in comparison with highly specific chiral stationary phases.

Ethanol and other alcohols are important compounds to be determined in a number of different fields, such as in medicine, the food industry and biotechnology. A variety of methods for their determination already exist. However, the simultaneous determination of, *e.g.*,

the sugar and alcohol contents in biotechnical processes would benefit greatly if the same (on-line) system could be used for their sampling and separation but using selective detection devices reflecting their individual concentrations. With respect to alcohol measurements, the selective and sensitive detection of methanol, ethanol and other short-chain aliphatic alcohols in FI and CLC can be achieved by the use of either alcohol oxidase (AOD) or alcohol dehydrogenase (ADH) [21–33], both enzymes being group- rather than absolute substrate-specific. As with all redox enzymes, AOD and ADH depend on a co-factor for activity. In the case of AOD, the redox cofactor is flavin adenine dinucleotide (FAD) strongly bound within the enzyme structure, whereas ADH depends on nicotinamide adenine dinucleotide (NAD^+) acting as a soluble co-substrate. Both of these enzymes have been studied in amperometric biosensor configurations and in immobilized enzyme reactors with both silica- and polymer-based supports [34]. There are pros and cons with both of these enzymes. AOD needs molecular oxygen as the natural reoxidation agent (electron–proton acceptor) resulting in the production of hydrogen peroxide:

$$\text{alcohol} + O_2 \xrightarrow{AOD} \text{aldehyde} + H_2O_2 \qquad (1)$$
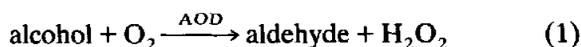
The equilibrium of the reaction strongly favours the product side, owing to the strong oxidizing power of oxygen. The low solubility of molecular oxygen and the difficulties encountered with following electrochemically either the decrease in oxygen or the production of hydrogen peroxide are the main obstacles to be overcome. Additionally, AOD has been reported to suffer from a restricted long-term stability [35,36].

Some problems with restricted long-term stability of enzymes can be solved with their immobilization. Of particular interest is the reported increase in operational stability when the enzyme is immobilized into an organic phase [37]. Organic phase electrochemical transducers are found within the group of carbon paste and composite electrodes [38]. These electrodes are easily bulk modified compared with solid electrodes only allowing surface modification. Enzymes, stabilizers, activators, necessary cofac-

tors, mediators, etc., can be introduced and homogeneously distributed in the electrode material [19,20,27–30,32,33,37,38]. The coupling of charge-transfer reactions between redox enzymes and electrochemical transducers can be greatly facilitated by the use of redox mediators shuttling the electrons between the conducting material and the cofactor of the redox enzymes at substantially decreased overvoltages.

Recent reports on the further stabilization of a series of redox enzymes including AOD by adding polyelectrolytes and sugar derivatives suggest AOD rather than ADH to be a good candidate for further studies for the construction of reliable amperometric biosensors for alcohol measurements [27,35,36]. As a step in that direction, we report here on the use of a reagentless amperometric alcohol biosensor based on co-immobilized alcohol oxidase (AOD) and horseradish peroxidase (HRP) in carbon paste. Increased stabilization of the enzymes in the paste and improved response characteristics of the sensor are obtained by a proper choice of immobilization reagents and other additives to the paste. The biosensor was used as a selective detector in CLC for the analysis of short-chain aliphatic alcohols. Further optimizations were made in order to improve the stability of the sensor by covering the electrode surface with different membranes. The biosensor detection unit–CLC system was implemented in an on-line system using a microdialysis sampling unit to follow the ethanol production in a fermentation based on a technical lignocellulose hydrolysate as substrate.

EXPERIMENTAL

Chemicals

Ethanol of the highest quality (99.5%, spectrographic grade; Kemetyl, Stockholm, Sweden) was used as received. All solutions, the mobile phase of the chromatographic system and the perfusion medium in the microdialysis unit were prepared by using a Milli-Q water purification system (Millipore, Milford, MA, USA). The mobile phase used in the chromatographic system consisted of either 50 mM phosphate buffer (pH 7.5) or 4 mM sulphuric acid prepared by

dilution of concentrated sulphuric acid (Merck, Darmstadt, Germany), depending on the chromatographic column used (see below). The mobile phase and the perfusion medium used in the microdialysis probes were first filtered through 0.22-μm sterile filters (Millex HA, Millipore) and then degassed with helium. o-Phenylenediamine (o-PDA), phenol, aniline, pyrrole, m-phenylenediamine (m-PDA) and resorcinol were of analytical-reagent grade and used as received. Eastman AQ-29D was a gift from Dr. W. Waeny, Eastman Chemical International, Zug, Switzerland. All other chemicals were of analytical-reagent grade and used as received.

Preparation of the enzyme electrode

Alcohol oxidase (AOD) (EC 1.1.3.13, from Candida boidinii, Serva, 30.3 U mg^{-1}) and horseradish peroxidase (HRP) (EC 1.11.1.7, Sigma, 270 U mg^{-1}) were used as received. Chemically modified carbon paste (CMCP) electrodes were prepared according to a previous investigation [27]. Graphite powder (Fluka) was heated at 700°C for 15 s in a muffle furnace and cooled to ambient temperature in a desiccator. Next, the graphite powder was activated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulphonate ("carbodiimide") (Janssen Chimica). A 4.2-mg amount of carbodiimide was dissolved in 300 μl of 0.05 M acetate buffer at pH 4.8 per 100 mg of graphite and allowed to react for 2 h at ambient temperature. The activated graphite was then rinsed with pure water seven times and dried under vacuum for 4.5 h before addition of 1.5 mg (ca. 400 U) of HRP and 4.5 mg (ca. 140 U) of AOD dissolved in 200 μl of 0.1 M phosphate buffer (pH 7.0). The immobilization reaction mixture also contained 0.12% of glutaraldehyde (Sigma, 25% aqueous solution) added to the enzyme solution just prior to mixing with the graphite powder. The glutaraldehyde was purified from polymers by centrifugation with active carbon. Finally, 200 μl of a 0.32% solution of polyethyleneimine (PEI) (Sigma, 50% aqueous solution) were also added before allowing the mixture react for 16 h at 4°C. Subsequently, the reaction mixture was dried under vacuum before addition of the pasting liquid, 40 μl of phenylmethylsilicone oil

(silicone DC 710; Alltech Associates, Arlington Heights, IL, USA) to produce the final CMCP.

Plain graphite–paraffin oil paste (40 μl of paraffin oil were mixed with 100 mg of graphite powder) was filled into plastic syringe holders (1.0-ml syringe, Brunswick 81/79J03, with a tip of 7.0 mm O.D. and 1.8 mm I.D.), leaving about 3–4 mm empty at the top to be filled with the CMCP to produce the final electrode. Electrical contact was made by inserting a gold wire in the CP. After the tip of the syringe had been filled with enzyme-modified carbon paste, the end was gently rubbed on glass to produce a flat, shiny electrode surface with an area of about 0.024 cm^2. A schematic diagram of the electrode is shown in Fig. 1.

The surface of some electrodes was covered with an electropolymerized layer of poly-o-phenylenediamine, polyaniline, polyphenol, polypyrrole or a copolymer obtained through the combination of m-phenylenediamine and resorcinol. The poly-o-phenylenediamine film was made by dipping the electrode into a 0.5 M acetate buffer solution (pH 5.2) containing 5 mM o-PDA [39] and running a series of cyclic voltammograms (scan rate 50 mV s^{-1}) between 0 and +650 mV vs. a saturated calomel electrode (SCE) [27]. The electropolymerized layers of the other monomers were similarly obtained with some alterations to the experimental conditions. Polypyrrole was formed in 5 mM pyrrole dissolved in 1 M KCl [40]. Polyaniline or poly-

phenol was formed in solutions containing 5 mM of the monomer dissolved in 0.1 M phosphate buffer (pH 7.0) [41,42]. The electropolymerized layer of the combination m-PDA and resorcinol was formed in 0.1 M phosphate buffer (pH 6.5) also containing 1.5 mM of each monomer [43]. Cyclic voltammetry was employed using a platinum gauze as the counter electrode and an SCE, as the reference electrode in these experiments. Additionally, some electrodes were further surface modified through the following procedure. After rinsing the electrode carefully with distilled water, the electrode was covered with an Eastman AQ 29D cation-exchange membrane by dipping the electrode into a solution containing 0.5% of the cation exchanger dissolved in water and letting the electrode dry for at least 20 min between dippings [21,27,44,45]. This procedure was repeated five times. After the last dipping the electrode was dried for 1 h before use. In all instances, when not in use the electrodes were kept in a dry state at 4°C.

Apparatus

The final electrodes were mounted in a flow-through amperometric cell of the confined wall-jet type [46] under three-electrode potentiostatic control using a Princeton Applied Research Model 174A instrument. A platinum wire and Ag/AgCl (0.1 M KCl) served as the counter and reference electrodes in the cell. The detector was connected to either a single-line flow-injection (FI) system or a column liquid chromatographic (CLC) system. The FI system consisted of a peristaltic pump (Gilson Minipuls 2) and a pneumatically operated valve (Cheminert, Type SVA) with an injection volume of 50 μl. Connections between the different parts of the FI system were made of Teflon tubing of 0.5 mm I.D. and with Altex screw couplings. The flow buffer, consisting of 0.1 M phosphate buffer (pH 7.5), was pumped at a flow-rate of 0.8 ml min^{-1}. The chromatographic system is depicted in Fig. 2 and consisted of a Model 2150 HPLC pump (LKB, Bromma, Sweden), a CMA/160 on-line injector (CMA/Microdialysis, Stockholm, Sweden) fitted with a 20-μl injection loop (Valco CGW-K; Vici, Zug, Switzerland), and one
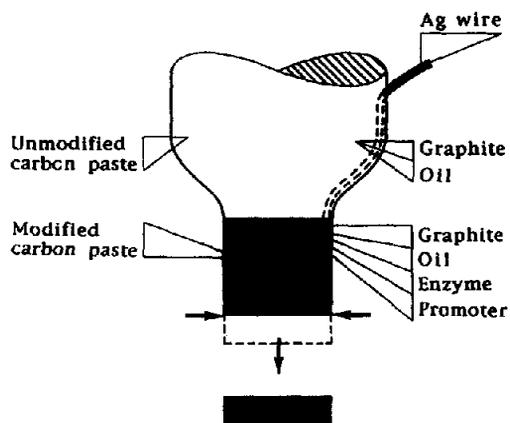

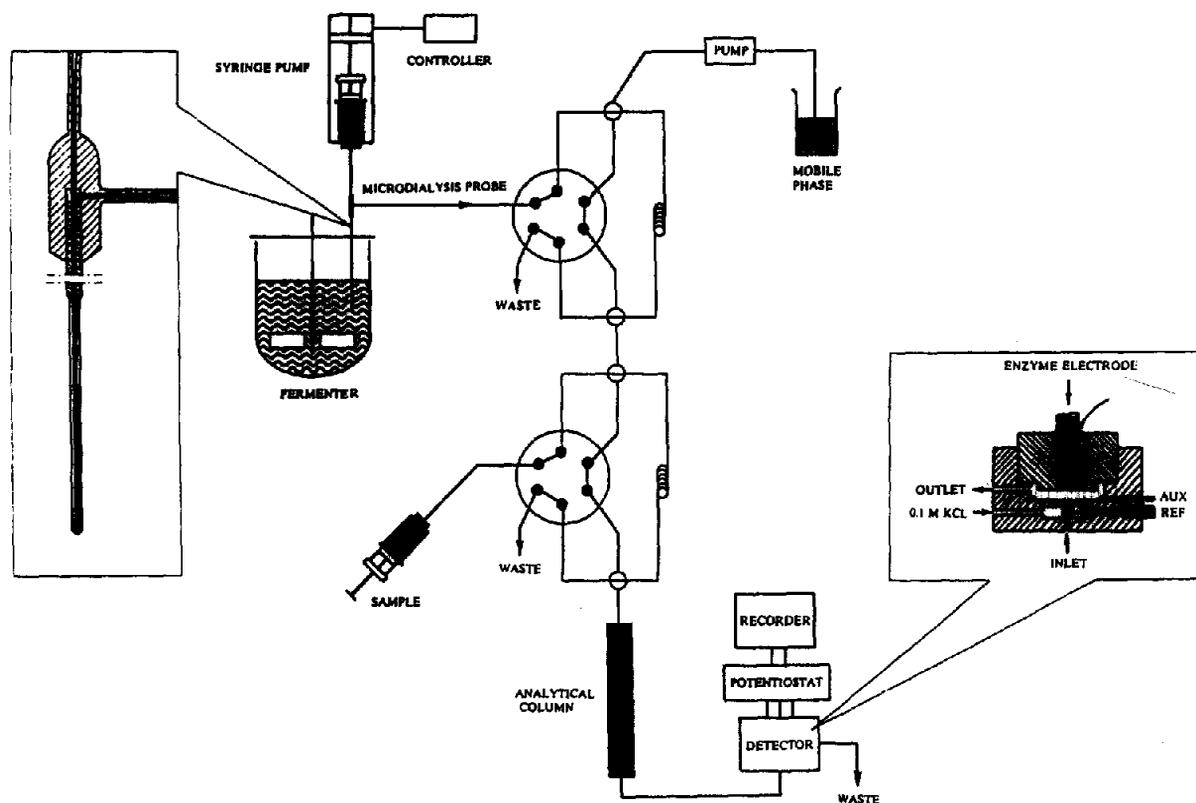Fig. 1. Schematic diagram of the enzyme-modified carbon paste electrode.

Fig. 2. Set-up for on-line analysis of fermentation broth including microdialysis sampling probe, CLC system and the electrochemical flow-through cell incorporating the biosensor.

Model 7045 six-port switching valve (Rheodyne, Cotati, CA, USA) equipped with a 19.6-μl injection loop, a polymer-based analytical column (50 × 4.0 mm I.D.) (PLRP-S, Polymer Labs., Church Stretton, Shropshire, UK), the same amperometric flow cell as described above, a potentiostat (Zäta Elektronik, Lund, Sweden) and a Model 2077 chart recorder (LKB). With the chromatographic system 50 mM phosphate buffer (pH 7.5) was used.

Another CLC system was also used (not shown) consisting of the pump, one injector and the recorder as above but with a polymeric cation-exchange analytical column in the hydrogen form (Model HPX-87H; Bio-Rad Labs., Richmond, CA, USA) operated at an elevated temperature of 40°C in a column oven (Model 2055, LKB) with sulphuric acid (4 mM) used as the mobile phase, and a refractive index detector (model 2142, LKB).

The fermentation substrate, spent sulphite liquor (SSL), used for ethanol production was a waste water from the pulp industry. It originally had a pH of 3.2, which was adjusted by addition of sodium hydroxide (5 M) to a final pH of 6.0. SSL was obtained from a paper pulp plant (MoDo, Örnsköldsvik, Sweden). Baker's yeast, *Saccharomyces cereviseae* (Jästbolaget, Stockholm, Sweden), was first dissolved in 5.0 ml of water and then added to the substrate to give a final concentration of 80 g l^{-1}. A beaker (10 ml) was used, sealed with a rubber screw fitting, for the ethanol fermentation broths. Microdialysis probes (CMA/10, CMA/Microdialysis) containing a polycarbonate membrane (0.5 mm O.D., length 10 mm, M_r cut-off 20 000) were used in conjunction with a syringe pump incorporating a control unit (CMA/100 microinjection pump, CMA/Microdialysis) and coupled on-line with the CLC system as indicated in Fig. 2.

RESULTS AND DISCUSSION

The direct electrochemical conversion of hydrogen peroxide suffers from too high an applied potential at the working electrode, which may open up the detection system for other possibly present compounds that are easily oxidizable and would thus contribute to the response signal and counteract the use of a selective enzyme in the detection system. Much work has been focused on trying to make it possible to operate oxidase-based electrodes at less extreme potentials, either by the use of chemically modified electrodes incorporating catalysts for electrochemical conversion of hydrogen peroxide or by the use of artificial mediators exchanging molecular oxygen as the electron acceptor in the enzymatic process and also facilitating the charge transfer to the electrode at substantially lower applied voltages. Recently, several papers have described the use of electrodes on which peroxidase has been immobilized, acting as a very efficient catalyst for electrochemical reduction of hydrogen peroxide [27,32,33,47–50]:

$$H_2O_2 + 2H^+ + 2e^- \xrightarrow{\text{peroxidase}} 2H_2O \qquad (2)$$
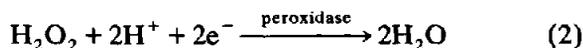
Reaction 2 starts at about +600 mV vs. Ag/AgCl and its rate increases as the applied potential is made more negative. Maximum response currents are obtained within the optimum working potential range for an amperometric biosensor, between about −150 and 0 mV vs. Ag/AgCl, where the background current switches signs, resulting in low noise and background current levels and where molecular oxygen and easily oxidizable compounds contribute not at all or very little to the response signal. In most cases, a direct electron transfer exchange between a redox enzyme and an electrode is obscured by steric or kinetic limitations, and the use of artificial mediators has often been used to facilitate electric communication between the enzyme and the electrode [51]. In the case of immobilized peroxidases on preferentially carbonaceous electrodes, it seems as though a direct charge-transfer process can be accomplished. HRP in its native form reduces hydrogen peroxide in a single two-electron step whereby water and an

oxidized form of the enzyme denoted compound I are formed. Compound I is re-reduced to the native form in two separate one-electron steps with the intermediate formation of compound II as described previously [25].

A series of different reducing agents are able to donate these necessary electrons [52]. Here, the carbon material in the electrode can obviously function as a source of electrons without the deliberate addition of any other reducing agent, thus making it possible to produce a reagentless sensor for hydrogen peroxide. The mechanism, however, has been much debated [27,47,50].

The alcohol oxidase sensor consists of two enzymes, alcohol oxidase (AOD) and HRP, co-immobilized in a carbon paste electrode material. AOD, a non-substrate-specific enzyme, oxidizes its substrates to form H_2O_2 according to reaction 1 using molecular oxygen as the electron acceptor. In a following step, H_2O_2 is electrochemically reduced through the action of HRP. The entire reaction sequence utilizing the coupled enzyme reactions in the amperometric biosensor is illustrated in Fig. 3.

Membrane deposition and its impact on the selectivity of the biosensor performance

Membranes cast directly on or formed through electropolymerization of electroactive monomers on electrode surfaces have been used for different purposes in conjunction with enzyme-based amperometric biosensors [25,27,34,39–45,53]. Enzymes can be immobilized within the membranes, an increased stability has been observed of enzymes immobilized on/in the membrane-covered electrodes, and membranes can act as size- or charge-exclusion barriers to prevent possible interfering compounds in the sample
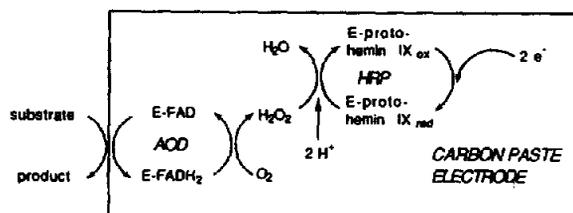
Fig. 3. Reaction sequence of the biosensor incorporating co-immobilized AOD and HRP.

matrix from reaching the true electrode surface and also prevent enzymes from leaking out from bulk modified electrode material. A membrane can also act as an additional diffusion resistance for the substrates of the enzymes to reach the electrode surface. The noticeable effect will be that the linear part of the calibration graph will be shifted towards higher concentrations of the substrate as an effect of an increase in the apparent Michaelis–Menten constant, K_M^{app}. This may be advantageous in biosensor systems when using enzymes with low K_M values, as the linear part of the calibration graph will be extended even though the sensitivity will decrease.

A series of different membranes were therefore investigated for this alcohol sensor in order to increase the operational stability and extend the linear calibration characteristics. In order to determine the substrate specificity of the biosensor, some possible substrates were investigated on a naked uncoated electrode surface (see Table I). Five different types of electropolymerized membranes were thereafter investigated, poly-o-PDA, polyphenol, polypyrrole, polyani-

line and a copolymer obtained from m-PDA and resorcinol. Additionally, covering with a cation-exchange membrane formed through evaporation of the aqueous solvent directly on the electrode surface was also investigated. The procedures for making electropolymerized membranes have been described previously [39–43]. Essentially the same procedures were used in this work with the exception that the concentration of the monomers when forming the polyaniline, polypyrrole and polyphenole membranes was decreased from the molar to the millimolar level. Fig. 4 shows some cyclic voltammograms obtained when forming these electropolymerized membranes. Included are background voltammograms obtained in the absence of the electroactive monomers (the lower flat-looking voltammograms) and also the voltammograms registered after five voltammetric cycles. For all monomers (A, o-PDA; B, phenol;

TABLE I

RELATIVE RESPONSE FOR SOME POSSIBLE SUBSTRATES FOR THE ALCOHOL BIOSENSOR IN A FI SYSTEM

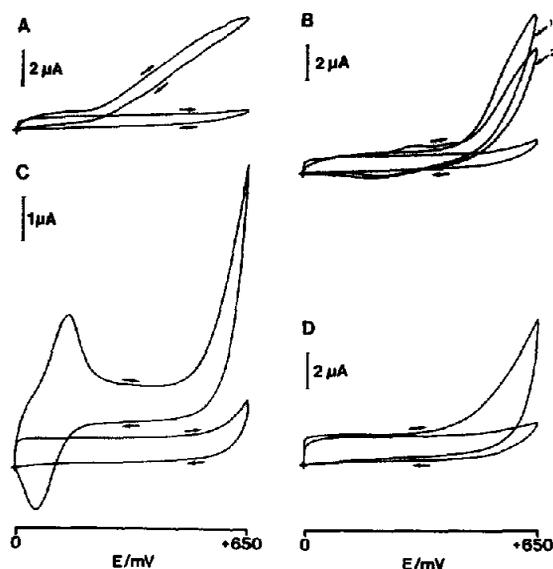| Compound	Relative response (%)
Ethanol	100
Methanol	240
Formaldehyde	170
Acetaldehyde	<0.2
Propionaldehyde	<0.2
Butyraldehyde	<0.2
Formic acid	<0.2
Acetic acid	<0.2
Propionic acid	<0.2
Butyric acid	<0.2
Monochloroacetic acid	<0.2
Citric acid	1.8
Lactic acid	11
Malic acid	0.4
Pyruvic acid	8.4
Inositol triphosphates	0.9
Dihydroxyacetone	2.6

Fig. 4. Cyclic voltammograms obtained when electropolymerizing (A) poly-o-PDA, (B) polyphenol, (C) polyaniline and (D) polypyrrole on the biosensor surface. The cyclic voltammograms shown were obtained after running five cycles, except in (B) where both the first (1) and the fifth (2) cyclic voltammograms are shown as indicated. The lower cyclic voltammograms also included in all figures are those obtained in the absence of monomers in the contacting solution. The arrows indicate sweep direction and + the starting potential. For further details, see Experimental.

C, aniline; and D, pyrrole) a drastic increase in the anodic current is seen, at potentials determined by the monomer, reflecting the oxidation of the monomers to form reactive radicals which in turn will react with unoxidized monomers forming the membrane on the electrode surface. In Fig. 4B is also the first voltammograms in the presence of phenol, included to illustrate the difference in wave form compared with the fifth scan. What can be noticed is that the anodic wave will successively decrease as a result of partial blocking of the electrode surface by membrane formation preventing phenol from reaching the electroactive surface. What is also noticed on comparing the different voltammograms is that a second electroactive species is formed when electropolymerizing phenol (B) and aniline (C), but not for o-PDA (A) and pyrrole (D), reflected by the reversible voltammetric waves appearing at around +150 V for polyaniline and +300 mV for polyphenol. The structures in these films will incorporate redox functionalities in their respective reduced states basically of p-quinone (polyphenol) or p-phenylenediamine structures known to acts as electron donors for compounds I and II (discussed above). Even though an increased diffusional barrier has been created at the electrode surface, the FI response to hydrogen peroxide will increase after forming the initial polyaniline and polyphenol membranes, reflecting the electroactivities of these membranes (see Table II). This is not observed for the other electropolymerized membranes lacking electroactivity. In Table II are shown the effect on the response to both ethanol and hydrogen peroxide for two equivalently prepared electrodes obtained in the FI mode as a result of various thicknesses of the electropolymerized layers.

Recently, a series of papers have appeared describing electrodes covered with a new anion-exchange polymer, which, contrary to another very popular electrode anion-exchange modifier, Nafion [54], can be obtained in an aqueous soluble form [27,44,45]. A series of premade polymers, denoted AQ, containing negatively charged groups covalently bound within the polymeric backbone are available from Eastman Chemical. This special kind of polymer suits the

TABLE II

SIGNAL RESPONSE FOR VARIOUS ELECTRO-POLYMERIZED MEMBRANES ON THE ALCOHOL BIOSENSOR IN A FI SYSTEM

Membrane	Number of CVs	Response, i (nA)		Relative response (%)	
		EtOH	H_2O_2	EtOH	H_2O_2
Polypyrrole	0	571	806	100	100
	2	374	716	65	89
	5	338	660	59	82
	20	208	586	36	73
	0	510	722	100	100
	2	392	630	77	87
	5	364	618	71	86
	20	344	548	67	76
Aniline	0	436	605	100	100
	2	511	761	117	126
	5	430	721	99	119
	20	233	597	53	99
	0	577	676	100	100
	2	584	844	101	125
	5	502	824	87	122
	20	260	666	45	99
Phenol	0	588	584	100	100
	2	504	592	86	100
	5	446	462	76	79
	20	213	182	36	35
	0	645	613	100	100
	2	639	611	99	89
	5	548	484	85	82
	20	305	214	47	73
o-Phenylene-diamine	0	632	633	100	100
	2	519	477	82	75
	5	503	494	80	78
	20	420	317	66	50
	0	525	503	100	100
	2	447	371	85	74
	5	444	390	85	78
	20	376	288	72	57
	0	647	737	100	100
m-Phenylene-diamine and resorcinol	2	486	326	75	44
	5	407	249	63	34
	20	228	53	35	7
	0	698	652	100	100
	2	511	379	73	58
	5	391	211	56	32
	20	178	41	35	6

purpose of casting an ion-exchange membrane directly on an enzyme-covered electrode surface very well [27,44,45]. Because of the charged

groups, the polymer can be dissolved in water at an elevated temperature (95°C). This polymer–water solution will stay in a homogeneous state at room temperature, but once dried it is no longer water soluble. The pH of the polymer–water solution is between 5 and 6, causing no serious effects when applied to an electrode surface containing biochemical ingredients. There are obvious advantages of casting the membrane directly on the electrode surface compared with covering an electrode with a premade membrane. Thinner membranes can be made and direct adhesion of the membrane to the electrode surface is possible, etc. The AQ membrane was shown to have excellent properties with both oxidases [27,44] and dehydrogenases [21,45] and also with HRP with respect to stability (see below). Membranes of this type will certainly be of continuous use in further studies of enzyme electrodes. The possibility of using the negative charges within the membrane as possible immobilization sites for enzymes has already been investigated [55]. The effect on the use of the AQ membrane on this AOD/HRP electrode was therefore also investigated. As expected, after membrane formation the response to ethanol and hydrogen peroxide de-

creased by about 10–20% depending on the membrane thickness (data not shown).

The selectivity of the alcohol sensor is shown in Table III and IV for a number of possible electroactive interfering compounds and well known solutes in biological fluids and biotechnological samples, respectively. The values given are the mean values obtained after at least five injections of each compound. The relative responses for ethanol of the different electrodes after exposure to these compounds are also included for comparison. For the uncovered electrode, some decrease in the response to ethanol is obtained, whereas the stability-enhancing effect on the response to ethanol is shown for the covered electrodes. A drastic decrease in the relative response to ascorbate is registered for the polypyrrole- and Eastman AQ-covered electrodes, as expected by the negatively charged AQ membrane.

Table IV shows some additional results obtained for some other possibly interfering compounds not necessarily electroactive within the applied potential range but known to foul the electrode surface. For all electrodes, including the uncovered type, virtually the initial response remains after exposure to these compounds.

TABLE III

MEMBRANE CHARACTERISTICS OF ALCOHOL BIOSENSOR WHEN EXPOSED TO WELL KNOWN BIOMEDICAL INTERFERENTS (EXPERIMENTS PERFORMED IN THE FI SYSTEM)

Analyte	Relative response (%)						
	Without membrane	Eastman AQ-29D	Polypyrrole	Polyaniline	Polyphenol	Poly-o-PDA	Copolymer of m-PDA and resorcinol
Ethanol	100	100	100	100	100	100	100
Adenosine	0.4	0.7	1.4	1.8	0.8	1.4	1.3
Ascorbate	−806	−134	−70	−552	−249	−995	−453
Glucose	0.6	0.4	<0.2	1.7	0.4	1.9	0.9
Lactate	15	15	17	13	14	16	13
Paracetamol	0.8	0.7	1.0	2.5	0.4	1.9	1.3
L-Phenylalanine	0.4	<0.4	0.2	2.1	0.6	1.6	0.9
Terbutaline sulphate	1.7	<0.4	1.4	1.4	0.9	2.2	1.3
Ethanol		82	115	95	97	100	112

TABLE IV

MEMBRANE CHARACTERISTICS OF THE ALCOHOL BIOSENSOR WHEN EXPOSED TO POSSIBLE ELECTROAC-TIVE BIOTECHNICAL INTERFERENTS IN THE FI SYSTEM

Analyte	Relative response (%)				
	Without membrane	Eastman AQ-29D	Poly-o-PDA	Polyphenol	Polyaniline
Ethanol	100	100	100	100	100
Phenol	<0.1	<0.3	<0.1	<0.2	<0.1
m-Cresol	<0.1	<0.3	<0.1	0.2	<0.1
Benzaldehyde	<0.1	<0.3	<0.1	<0.2	<0.1
Furfuralaldehyde	<0.1	<0.3	<0.1	<0.2	<0.1
5-Hydroxymethyfurfural	1.7	2.8	1.8	0.8	0.3
Saccharomyces cerevisiae	0.2	<0.3	0.8	<0.2	<0.1
Bovine serum albumin	130	96	115	117	142
Ethanol	104	95	101	83	94

What is clear is that the AQ membrane has the most strongly excluding effect for BSA and yeast cells.

The results in Tables III and IV suggest that a combination of first forming one electropolymer-ized membrane on the electrode surface followed by an additional covering with a layer of the Eastman AQ would give the most suitable and versatile protection of the electrode surface. A series of electrodes were therefore made with the combination of poly-o-PDA (five cyclic voltam-mograms) and Eastman AQ 29D (dipping three times). These electrodes were used in conjunc-tion with the on-line measurement of ethanol production in the fermentation experiments de-scribed below.

Optimization of CLC system for optimum performance of the biosensor

Optimization of different parameters of the CLC systems has to be taken into consideration when an enzyme-based detection system is util-ized. Enzymes are inherently made to work in an aqueous-based medium as they are most often found not to be very stable in organic–aqueous mobile phase mixtures. However, the stability of the enzyme might vary depending on the enzyme and the conditions under which the enzyme-based detection system is operated. It was found, *e.g.*, when using this alcohol sensor as a detec-tor, that it has a very high operational stability using acetonitrile–50 mM phosphate buffer (pH

7.0) (20:80) as the mobile phase in a CLC system over one day [56].

The flow dependence of the alcohol sensor incorporated in a reversed-phase separation sys-tem was investigated with respect to retention times, signal response and band-broadening ef-fects ($w^{1/2}$). Ethanol and methanol were chosen as the test analytes in this investigation. As can be seen from Table V, the flow-rate has a great influence on all the parameters investigated. A short analytical column was chosen in order to perform fast separations preferentially within 5 min. This was done in order to be able to follow

TABLE V

VARIATION OF SOME RESPONSE CHARACTERIS-TICS OF THE BIOSENSOR FOR METHANOL AND ETHANOL WITH THE FLOW-RATE OF THE MOBILE PHASE

Flow-rate (ml min^{-1})	Methanol			Ethanol		
	t_R (min)	$w^{1/2}$	i (nA)	t_R (min)	$w^{1/2}$	i (nA)
0.1	31.7	7.5	735	47.7	6.8	620
0.2	16.7	3.6	775	24.0	3.4	635
0.4	8.3	1.7	820	12.3	1.7	620
0.6	5.7	1.3	775	8.3	1.3	575
0.8	4.7	1.0	750	6.5	1.0	590
1.0	3.5	0.9	735	5.3	0.9	570
1.2	2.8	0.8	725	4.8	0.8	550

continuously the change in biotechnical fermentation processes (see below). The PLRP-S stationary phase was found to fulfil these chromatographic requirements. The column was also found to have a better operational stability than different types of silanized C_{18} phases such as LiChrosper 100 RP-18 (Merck, Darmstadt, Germany) and Nucleosil C-18 (5 SY-308; Bioseparation Technology, Budapest, Hungary). The instability of these silica columns with time was probably due to hydrolysis of the silica support. On opening these columns it was found that the front of the column with the packing had partly gone. The various capacity factors at the different flow-rates were mostly acceptable with respect to times of analysis. This was made possible by choosing a small reversed-phase separation column, but with a sufficient length to give the separation needed. The amperometric signal peak response generated by the catalytic activity of the enzymes was found to change only slightly with the flow-rate. The amperometric signal reflects both the enzyme kinetics and the mass transport of solutes to/from the electrode surface. Generally, the higher transport of solutes to the electrode surface at higher flow-rates results in an increase in the current registered. This is also the case for hydrogen peroxide, the substrate for HRP, the second enzyme in the reaction sequence of the sensor, when the response for this substrate was investigated in the FI mode. However, as argued above, the combined effect of hydrogen peroxide being produced by AOD at the electrode surface from injected samples containing alcohol and the turnover kinetics of AOD being low results in higher currents being registered at lower flow-rates than at increased flow-rates.

The band-broadening effect determined as the peak width at half-height ($w^{1/2}$) is more than doubled on comparing peaks obtained for flow-rates of 1.2 and 0.4 ml min^{-1}, and becomes much broader at very low flow-rates (see Table V). In order to see any possible additional band broadening effect given by the biosensor reflecting any kinetic limitations in the reaction sequence outlined in Fig. 3, a separate investigation was made with the same set-up, exchanging the biosensor for a refractive index detector. Similar $w^{1/2}$ values were found at these flow-

rates, indicating that the additional band-broadening effect from the sensor itself was not a critical factor.

The dependence of the injection volume on the peak height was investigated, choosing a flow rate of 0.8 ml min^{-1}. The results are shown in Table VI, where the effect for ethanol was found to be much more pronounced than for methanol. The current is almost three times higher for ethanol on increasing the injection volume from 15 to 200 μl but only about twice for methanol. The effect seen is due to two different reasons. The K_M of AOD for methanol is lower than that for ethanol [57] and, as stated above, formaldehyde produced in the enzymatic oxidation of methanol is also a substrate for AOD. At the flow-rate chosen it will partly disappear from the electrode surface before it is trapped by AOD in a second reaction cycle. A typical separation of methanol and ethanol using a 20-μl injection volume with a flow-rate of 0.8 ml min^{-1} is shown in Fig. 5. It can be clearly seen that the biosensor performance is well adapted for use as a detection device in conjunction with CLC.

Determination of alcohols in fermentation broths and substrates

The development of new sample handling techniques that can offer higher selectivity with larger capacity has grown over the last 10 years. This rapid development is due to an increasing demand for the characterization and quantification of progressively smaller amounts of compounds in complex biological matrices. There is

TABLE VI

VARIATION OF THE RESOLUTION (R_s) BETWEEN THE PEAKS OBTAINED FOR METHANOL AND ETHANOL AND THEIR MAXIMUM RESPONSE VALUES WITH THE INJECTION VOLUME

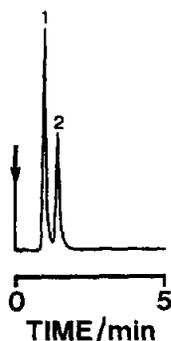| Injection volume (μl)	i (nA)		R_s
	Methanol	Ethanol	
5	430	160	3.3
15	440	215	2.0
20	450	240	2.4
50	740	575	0.8
200	775	650	0.6

Fig. 5. Chromatographic recording of the separation of methanol (peak 1) and ethanol (peak 2) using the set-up shown in Fig. 2, omitting the microdialysis unit and the switching valves. Conditions: mobile phase, 50 mM phosphate buffer (pH 7.5); flow-rate, 0.8 ml/min; injection volume, 20 μl; operational potential, 50 mV $vs.$ SCE.

also a need for the development of automated systems for the screening of large numbers of samples and a trend towards increasing the selectivity in the sample clean-up procedure and/ or in the detection step in order to reduce the chromatographic requirements. This will result in the need for shorter analytical columns with simpler and faster separations. The use of biological recognition in detection systems for the analysis of highly complex samples is easily rationalized by the need for selectivity when analysing such samples. The number of papers on utilizing biosensors for on-line monitoring of fermentation processes has increased lately and thus reflects this general trend in the development of highly sophisticated and selective detection methods.

Microdialysis can be used as an online sampling unit in continuous *ex vivo* and *in vivo* monitoring in combination with CLC. This method has been used, *e.g.*, in rat brain for choline and acetylcholine monitoring with a detection limit of *ca.* 2–5 fmol [58] and for the determination of glucose in serum [59].

In a previous report study we investigated the potential use of a microdialysis probe as a sampling unit for the analysis of industrial waste waters from the pulp industry, which have been investigated as possible fermentation substrates for ethanol production [31,60].

The stability and reproducibility of the microdialysis unit in the industrial waste water (a lig-

nocellulose hydrolysate, spent sulphite liquor, SSL) were investigated in an earlier study for 24- and 36-h continuous sampling in stirred shake-flask experiments with excellent results showing no drastic decrease (<5%) of the dialysis factor of the sampling device for ethanol [60]. The recovery value will differ depending on the type of dialysis membrane and on the structure of the actual analytes. The naturally occurring carbohydrates in the waste water were used as analytes in these experiments and recoveries above 90% were obtained, reflecting no serious clogging effects or other interactions taking place on or close to the polycarbonate membrane surface interacting in the diffusion process. Although these fermentation substrates and broths have been shown to contain a wide range of lignin poly-, oligo- and monomers [61,62]. The recovery for ethanol was *ca.* 92%, and was stable over a period of typically 10 h. One should consider these data bearing in mind that the ethanol present in the broth makes the solubility of less polar compounds increase, *i.e.*, the complexity of the broth for hydrophobic compounds has increased.

On the basis of these earlier studies, an on-line system was used as shown in Fig. 2, consisting of the microdialysis probe, the CLC system and the AOD/HRP electrode. The microdialysis unit is positioned in the fermenter with the membrane-covered probe immersed in the fermentation solution. The perfusion solution is pumped through the capillary in the probe down to the tip of the probe. From there it is transported upwards through a laser-drilled hole in the capillary wall. The perfusion liquid that is transported upwards on the inner side of the dialysis tubing now also contains representative amounts of each diffusable compound present in the fermentation solution. The flow reaches the T-connection (on the top of the probe) and is then pumped and trapped in the switching valve (see Fig. 2). Next, the valve is positioned in the inject position, whereby the dialysed sample is introduced into the analytical column, separated and then detected at the enzyme electrode that is mounted in the flow cell. Continuous monitoring of ethanol production was made when adding *Saccharomyces cerevisiae* to a sample of SSL

contained in an aseptic and continuously stirred flask. The process was followed for 10 h with injections of the microdialysate into the CLC system every 5th min the first 2 h, and thereafter every 30th min. The set-up in Fig. 2 also gives the possibility of intermittently injecting standard samples of ethanol into the chromatographic system through the second injector, permitting the control of possible variations in the separation and in the detection performance.

The pH of the waste water was adjusted to 6.0 prior to the start of each fermentation in order to minimise contamination from bacteria.

The difference in selectivity using one of the most commonly used monosaccharide CLC separation systems utilizing a polymer-based cation-exchange column and refractive index detection and the on-line system described is demonstrated in Fig. 6. The difference in analysis time for ethanol measurements is also clearly demonstrated in Fig. 6. The retention times for other possible analytes that are detectable with this system were investigated (data not shown). These retention data did not cause any co-elution problems with the ethanol fermentation process.
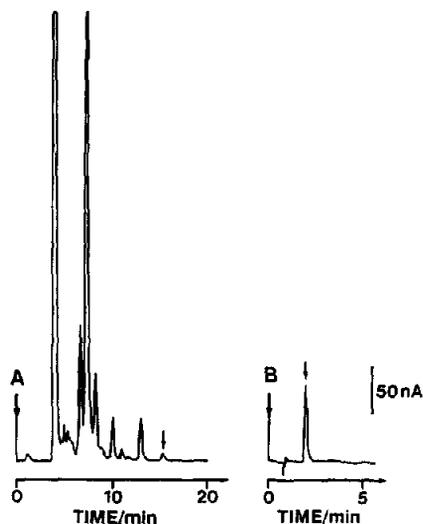

Fig. 6. Chromatograms obtained when injecting the microdialysate into two different chromatographic systems. (A) Cation-exchange column (mobile phase, 4 mM sulphuric acid) with refractive index detection; (B) PLRP-S column [mobile phase, 50 mM phosphate buffer (pH 7.5)] using the biosensor for enzymatic–electrochemical detection. For further details, see Experimental.

The current system differs from an earlier on-line monitoring system [31] in that the current system does not involve further sample handling other than the sampling itself (dialysis). In earlier work with a cross-flow filtration sampling unit, solid-phase extraction was essential and it was made by a coupled column clean-up system [63]. Increased selectivity in sample handling makes the system less complex with respect to instrumentation, i.e., the need for additional switching valves and columns. One drawback in future might be some adsorption on the analytical column with time, which might influence the stability and the reproducibility of the system. However, during the 10-h fermentations made in this study, this was not a problem, although minor colouring in the front of the separation column was noticed.

The decline in the catalytic performance of the biosensor with time in these complex biotechnological substrates can easily be adjusted for by renewal of the electrode surface. Discarding the outer layer of the electrode is achieved by cutting, exposing a fresh and fully active surface. This is also illustrated in Fig. 1.

CONCLUSIONS

An on-line fermentation monitoring system has been developed for the analysis of alcohols present in fermentation broths. The system utilizes membrane-covered enzyme-based biosensors as a postcolumn detection system in combination with a microdialysis sampling unit and a polymer-based chromatographic separation system. The improved selectivity of the biosensor with different membranes deposited on the electrode surface can easily be applied not only in this process but in others as well. Analytical information on alcohols and also aldehydes and carboxylic acids can be obtained very rapidly by using the set-up. On-line separations of ethanol can be analysed every 5th minute.

The whole system is totally automated and is therefore simple to operate, providing highly reproducible process data. Although only short fermentations were run with the described system, the same performance has been observed for more extended fermentations. In current

work, new types of microdialysis probes are being investigated in terms of operational stability over 24-h fermentations. Applications with similar waste waters for the analysis of phenolic compounds are under study and show similar high recoveries as for the carbohydrates [64].

New types of alcohol sensors with broader selectivity have been developed and are also being studied in combination with these fermentations, and carbohydrate sensors are being developed for the simultaneous monitoring of sugars in these processes.

ACKNOWLEDGEMENTS

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