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review

Enzyme Electrodes for Food Analysis

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Summary

Enzyme electrodes are the most extensively studied type of biosensor and their application in food sensing is an area with enormous potential for growth. This review introduces the principles of enzyme electrodes, focusing on amperometric transduction. The requirements for sensing in the food industry are discussed along with the potential for enzyme electrodes to fulfil these needs and the challenges presented by technology transfer into food applications. Approaches to construction of enzyme electrodes with respect to enzyme immobilisation and choice of electrode material are briefly described along with the most commonly employed analysis format, flow injection analysis. Examples of enzyme electrodes described in the literature, for analysis of carbohydrates, organic acids, alcohols, additives, pesticides and fish/meat freshness are presented.

Keywords: food analysis, enzyme electrodes, biosensors, flow injection analysis

Introduction

A biosensor has been defined as an analytical device composed of a biological recognition element (e. g. enzyme, antibody, receptor, microbe) coupled to a chemical or physical transducer *(1)*. Four main types of transducer have been described, electrochemical, optical, mass and thermometric, of which electrochemical transducers are by far the most important ones. Electrochemical measurements need a minimum of instrumentation, can be carried out in opaque samples, do not need strict temperature control, and can be made with a high degree of sensitivity.

The first biosensor concept to be proposed, by Clark and Lyons in 1962 *(2)*, was an enzyme electrode system for blood glucose based on oxidation of glucose to gluconic acid, catalysed by the oxidoreductase enzyme glucose oxidase (GOD):

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} \text{D-gluconic acid} + H_2O_2 \qquad /1/$$

In one embodiment the enzyme was entrapped between two polymeric membranes and retained over an oxygen electrode which could then measure the consumption of the oxygen co-substrate due to the enzymic reaction:

$$O_2 + 2H_2O + 4e^- \xrightarrow{-600\ \text{mV vs. Ag/AgCl}} 4OH^- \qquad /2/$$

Updike and Hicks *(3)* demonstrated the application of this principle using glucose oxidase entrapped in an acrylamide gel over an amperometric oxygen electrode, but employed a second non-enzymic control electrode to compensate for background oxygen fluctuations.

Alternatively hydrogen peroxide generated in the enzymic reaction (eq. 1) can be measured amperometrically by oxidation and directly related to glucose concentration *(4–6)*:

$$H_2O_2 \xrightarrow{-650\ \text{mV vs. Ag/AgCl}} O_2 + 2H^+ + 2e^- \qquad /3/$$

This has the advantage of requiring no oxygen background current correction.

The range of oxidase enzymes available, of which glucose oxidase is by far the most commonly studied, has provided a convenient basis for constructing enzyme electrodes *(7)* based on either O_2 or H_2O_2 detection.

Where H_2O_2 is detected problems of electrochemical interference result due to the high overpotentials employed for noble electrodes *(8)*. These result from a range of electrooxidisable species found in biological matrices, especially ascorbate and urate *(9–11)*. Furthermore, low ambient oxygen concentrations may prove rate-limiting for the oxidase enzyme *(5)*.

Second generation enzyme electrodes were developed to avoid both interference and oxygen effects on response. These involve the use of electron mediators *(12)* which achieve direct electron transfer between the active site of the enzyme and the working electrode *(7)*. The mediator should ideally operate at a low redox po-

tential, allowing monitoring of the enzymic reaction at a low electrode potential and thus minimising interference. Cass *et al. (13)* described a mediated glucose enzyme electrode, using electrochemically regenerated ferrocene to re-oxidise the reduced flavine adenine dinucleotide prosthetic group of reduced glucose oxidase. Ferrocene typically allows electron transfer from glucose oxidase at +240 mV *vs.* Ag/AgCl *(7)*, and as the mediator replaces oxygen as normal electron acceptor in the oxidase system, the electrode becomes relatively insensitive to changes in ambient oxygen concentration. The most successful application of a mediated glucose enzyme electrode has been the Exac Tech disposable glucose sensor *(14,15)* for whole blood glucose monitoring in diabetics *(16)*. This may be regarded as a model for the development of commerical enzyme electrodes for food.

The ferrocenes remain the most extensively studied of all mediators *(17–19)*, but others include dichlorophenolindophenol *(20,21)*, ferricyanide *(22–24)*, pyrroloquinolinequinone (PQQ) *(25)*, Meldola's Blue *(26)*, cobalt phtalocyanine *(27)* and poly(methylene blue) *(28)*.

Mediators can also permit the use of other oxidoreductases such as dehydrogenases, which require the cofactors nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) as electron acceptors. The reduced forms of these cofactors, NADH and NADPH may be detected by direct electrochemical oxidation, but the high overpotential required and electrode fouling due to side reactions on the electrode make this unattractive *(29)*. With suitable mediators, electron transfer from reduced cofactor to the electrode can be faciliated at low potentials *(30,31)*. Thus, a range of dehydrogenase enzyme electrodes become feasible, including electrodes for common food analytes such as malate *(22)*, lactate *(26)*, and fructose *(23)*.

The desired electron transfer characteristics of the mediator may, however, cause problems due to the parallel mediation of interferent oxidation. Daurtartus and Evans *(32)* showed that ferrocene could catalyse the electrooxidation of ascorbate, so partially negating the specificity advantage of low operating voltages.

The successful application of a mediator requires intimate contact between the mediator molecule and the enzyme/cofactor and free passage to the electrode surface. This may be achieved by supplying the mediator in solution or by entrapping within the bioreactive layer. Entrapment has been variously achieved by the use of pre-cast films *(26,33,34)*, covalent attachment of mediator to electrode surface *(35)*, direct attachment of the mediator to the enzyme *(36)* and *in situ* entrapment in electropolymerised films *(37–39)*.

»Third generation« enzyme electrodes have been developed in order to remove the need for mediators entirely and are the subject of much ongoing research. Here the electrode material comprises an organic conducting salt, most commonly TTF^+TCNQ^- (tetrathiafulvalenium tetracyanoquinodimethanide) *(40–42)*, where it has been suggested that the reduced glucose oxidase is oxidised directly at the electrode surface *(43)*. This form of electrode could reduce problems associated with mediator leakage in second generation devices. The mechanism of direct electron transfer from the enzyme active site to the adjacent electron mediator on the conducting surface is, however, controversial *(41)* and it is likely that mediation is via the solubilised conducting salt. There are also interference *(44)* and toxicity *(45)* problems.

Despite extensive research into mediated/modified electrodes, the non-mediated peroxide detecting system remains the most common operational form of amperometric enzyme electrode.

Requirements and Problems in Food Sensing

The major driving force behind enzyme electrode development remains the need for clinical monitoring *(46)*, and since the development of the first commercial medical glucose analyzer, the Yellow Springs Instrument, in 1975, several laboratory based and portable devices have reached the medical market. The technology has, however, been slow to penetrate the food industry *(47)* despite the considerable opportunities in food analysis.

Current analytical practices in the food industry are time-consuming and may require highly skilled labour and expensive equipment. The food industry needs rapid, affordable and simple techniques to both replace existing methods and for measurement of previously analytically inaccessible compounds *(48)*. Pocket-sized devices capable of simplified in-field measurement on unmodified samples are very attractive, as is the possibility of on-line monitoring of biochemical parameters in complex matrices during the production or processing of foods. Basic characteristics of enzyme electrodes such as the exquisite selectivity of the enzyme and sensitivity extension to analytes difficult to access using synthetic reagents, appear to make them suited to at least some of the needs of the industry. However, to gain the confidence of the market, enzyme electrode-based sensors need also to be inexpensive, reliable and robust, and demonstrably superior to existing methods.

The enormous scale of food production and quality control should provide a natural home for technology advances from the medical field. The increasing demands of legislation relating to labelling of food constituents and additives and the need for effective detection of contaminants including toxins, microorganisms, pesticides and insecticides in a wide variety of products help further drive this technology transfer. Also, enzyme electrode generated information may confer competitive advantage resulting from improvements in raw material quality and harvesting efficiency, process optimisation and manufactured product quality *(47–49)*.

Operational issues relating to the food industry which lead to problems in food sensor development include a high material diversity, diverse processing and wide variations in physical conditions. Problems referable to a given enzyme electrode will depend on the specifics on analyte, enzyme and food matrix including analyte concentration, electrochemical interferents and pH. The transfer of the intensively studied glucose oxidase enzyme electrode from clinical to food applications provides an illustrative model for the various problems.

For clinical glucose measurement the concentration range is relatively restricted (≤40 mM *(50)*), and this therefore demands a relatively small extension of the apparent enzyme K_m from that of free glucose oxidase (4.2 mM *(51)*) to obtain a linear or nearlinear calibration. This

has mostly been achieved by the use of diffusion restricting membranes to limit glucose flux so avoiding enzyme saturation *(52)* and substrate linearity has been extended to a maximum of 150 mM *(53)*. In foodstuffs such as fruits, glucose concentration may be up to 500 mM *(54)*. Amine *et al. (55)* reported the measurement of glucose up to 1000 mM using a buffered FIA system, but in this instance inevitably the device was not in equilibrium with the analyte. Other workers have measured high concentrations of glucose in banana *(56)* and molasses *(57)* but only after dilution. Such systems may provide the basis for laboratory-based methods but are not suitable for direct in-field use on unmodified food samples. By the use of specialised cellulose acetate-based outer membranes, our group has extended response linearity to at least 400 mM (Fig. 1) *(52)*.

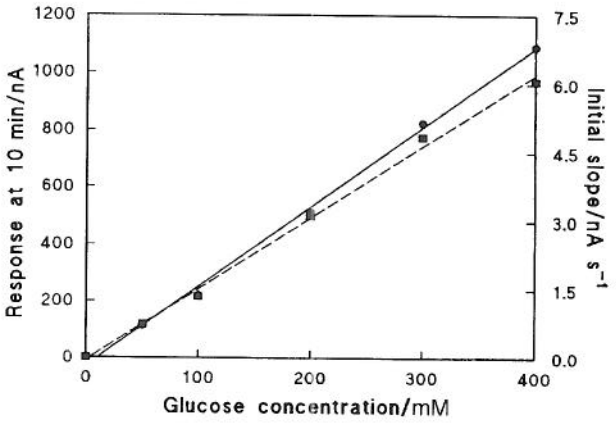

Fig. 1. Glucose calibration using Cuprophan glucose oxidase laminate with outer Tween-80 modified cellulose acetate membrane. ● response at 10 min; ■ initial slope response. [From ref. *(52)*].

In food samples, pH values may differ significantly from the enzyme optimum (5.6 for glucose oxidase) *(51)*, for example in citrus fruits the pH can be 2–3. Amine *et al. (55)* found immobilised glucose oxidase activity to decrease significantly below pH = 4 and Maines *et al. (52)* demonstrated rapid deactivation of a glucose oxidase enzyme electrode at pH = 2.4. However, by the use of specialised outer membranes it is possible to protect the enzyme from pH deactivation for sufficient time for pH independent measurement an re-use (Fig. 2) *(52)*.

In first generation enzyme electrodes, the high polarising voltage for amperometric H_2O_2 detection, typically +650 mV *vs.* Ag/AgCl, leads to serious interference from electrooxidisable species found in food matrices, the most important being ascorbate. Ascorbate can also be oxidised by homogeneous chemical reaction with hydrogen peroxide thus reducing the signal *(58)*. In citrus fruits especially, ascorbate up to 3 mM *(54)*, causes a serious interference problem. In order to reduce such interference several techniques have been employed, including preliminary oxidation on separate electrodes *(59)* and elimination by an ascorbate oxidase layer *(60)*. Selective restriction by the use of membranes and polymer coatings has also been widely used *(61,62)*. Since ascor-

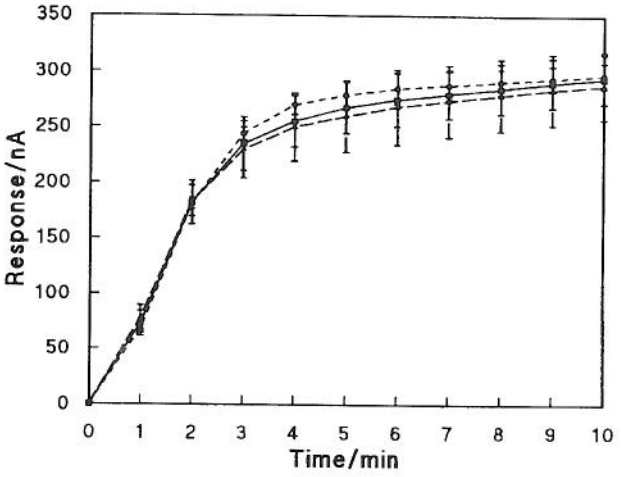

Fig. 2. Effect of step exposure of Cuprophan glucose oxidase laminate plus outer cellulose acetate/Tween-80 membrane to varying pH (100 mM glucose) plotted as response during initial 10 min. Data plotted as mean of 3 runs, standard deviation as y error bar. ■ pH = 7.4; ▲ pH = 3.4; ● pH = 2.4. [From ref. *(52)*].

bate is anionic at neutral pH research has focused on the use of anion rejecting membranes such as cellulose acetate *(63,64)*, poly(ester-sulphonic acid) *(61)* and negatively charged Nafion *(65,66)*. Plasticized PVC has also been shown to demonstrate high selectivity for hydrogen peroxide over ascorbate *(67)*.

The problem of direct ascorbate oxidation is less in mediated second generation enzyme electrodes where lower polarising voltages are employed, but reaction with the mediator itself may compromise this effect. A combination of the membrane and mediator approach appropriate to the particular sample may prove most effective.

Electrode Materials

A wide variety of electrode materials exists for the determination of oxidizable or reducible analytes or products of enzyme-catalysed reactions, and the majority of these have been employed in food analysis. The transfer of electrons between analyte and electrode is a necessary step in the electrochemical process, and materials are selected which maximise the rate of electron transfer between the electrode and the particular electroactive species being determined. As the enzymes used in food analysis are predominantly oxidases *(68–72)* and dehydrogenases *(73,74)* the species determined at the electrode is usually O_2, H_2O_2, NADH or mediators (which shuttle electrons between the enzyme and electrode).

It is generally acknowledged that the determination of oxygen and of H_2O_2 is best carried out with gold and platinum working electrodes, respectively. Noble metals capable of catalytic decompositions of H_2O_2 (because of facile redox chemistry of their surface oxides) are in general good anodes for H_2O_2 oxidation. Various forms of carbon are usually used to determine NADH and me-

diators. These have taken the form of solid carbon, based on graphite or glassy carbon, or carbon paste (graphite admixed with a low volatility oil e.g. paraffin oil or silicone oil *(75,76)* usually mixed in the ratio 2 g oil to 3–5 g carbon). The oil acts principally as a binding medium, enabling the carbon, enzymes, cofactors, etc. to be maintained in close proximity to a conducting strip.

An inert conducting material can be used as a pseudoreference, since precise voltages are not as critical in amperometry as in potentiometry. Pt, Ag, Au have all been used, but Ag/AgCl is commonly employed as it offers the desired level of voltage stability. It is important that the reaction of analyte with the working electrode is the rate-determining step. If the pseudoreference is too small, then electron transfer at this electrode will be limiting, and the current produced will not reflect the working electrode reaction. The reference electrode should be ≈ 10 times the area of the working electrode. Pontentials applied to the electrodes are 600–800 mV *vs.* Ag/AgCl for H_2O_2 and NADH oxidation, and –600 to –800 mV for O_2 reduction.

Enzyme Immobilization

The high cost of enzymes has led to a whole technology for enzyme immobilization *(4,17,77,78)* which has contributed to the creation of robust sensors. Enzymes can be immobilized by simple physical means, or by chemical attachment, or combinations of these.

Physical Binding

The most straightforward way of immobilizing enzymes is to confine them behind a mass-discriminating barrier membrane *(79–81)*. The (small) enzyme substrates and, as necessary, cofactors, can then diffuse from solution through the membrane and reach the enzyme, where the electroactive product will be formed, some of which will diffuse to the electrode surface. Membranes for this purpose would normally be made from a hydrophilic polymer e.g. cellulose acetate, cellulose nitrate, poly(vinyl acetate) or poly(urethane). Preformed commercial membranes can be employed, mechanically held over the electrode e.g. by an »O« ring *(82)*. Recently, reagentless sensors have been constructed by retention of dehydrogenase enzyme, cofactor and mediator by an *in situ* solvent cast cellulose acetate membrane *(26)*. This method does not, however, give close control over membrane characteristics.

The enzyme can also be entrapped within a gel *(83)* resulting in an easily handled sensor layer. Here, the enzyme is introduced into a warm solution of water gellable polymer e.g. gelatin or polyacrylamide. On cooling a semi-rigid gel is formed which, when protected by a hydrophilic membrane, efficiently entraps enzyme. A modified form of gel entrapment involves the enzyme being mixed into a carbon paste electrode, together with any cofactors and mediators *(76)*.

A more sophisticated form of enzyme immobilization, allowing a degree of time control, is by entrapment in a growing polymer film of electropolymerisable small molecules. Examples of these are phenol *(84)*, pyrrole *(85)*, indole *(86)* and aniline *(87)*. An advantage of this approach is that the enzyme is deposited only over the electrode surface, important where microelectrodes are concerned. If many electrodes are present on one support a different enzyme can thus be deposited at individual electrode surfaces.

Where there are strong hydrophilic or hydrophobic interactions between enzyme and support materials a successful immobilization can sometimes be obtained by direct adsorption, achieved simply by allowing the enzyme and support to come into contact *(82)*.

A stronger type of binding is obtained where ionic forces are involved. If a support material contains charged sites (e.g. ion exchange membrane) or chelating groups, enzymes, having charged sites themselves, may be held firmly. Nafion, an anion exchanger, has been used extensively as a membrane material *(88,89)* but has the drawback that it cannot be cast directly on to an electrode surface. An alternative, water-soluble, anion exchanger *(90,91)* is available which overcomes some of the deficiencies of Nafion *(92–94)*.

Covalent Binding

If a long operational lifetime is required, covalent binding of the enzyme to a support material or to the electrode is the method of choice. The binding has to be accomplished by involving active groups on the enzyme e.g. -NH_2, -COOH, -SH, -OH, which are remote from the site of catalytic activity.

The functionalisation of the support material or the electrode surface is usually the most problematic. Typical materials for working electrodes are the noble metals, and various forms of carbon. Anodic oxidation of the electrode surface *(95)* results in oxygen-containing functional groups (-OH or -COOH). The oxidized surface of metals can be treated further with a silanizing reagent, e.g. aminopropyltriethoxy silane to give, in this case, a covalently bound amino group *(96–98)* which can be coupled to an amine group of the enzyme by a bridging bifunctional reagent e.g. glutaraldehyde. Enzyme activity may well be affected by this process if the amino groups involved are too close to the enzyme active site, and it is usual to admix the enzyme with an inert protein e.g. bovine serum albumin (BSA), prior to coupling. By this procedure the majority of covalently bound protein is BSA, with the active enzyme enmeshed with the BSA polymer chains.

Oxidised carbon surfaces can be treated with carbodiimide to give a modified surface capable of reacting with enzyme amino groups. The resulting linkage of enzyme to electrode is by an amide group, itself not a particularly stable linkage.

The previously mentioned technique of immobilization in an electropolymerising film has been adapted to allow covalent binding of enzyme *(99)*. The polymerization of *N*-(2-carboxyethyl)pyrrole, to which glucose oxidase had been covalently attached, resulted in an electrogenerated film containing covalently immobilized enzyme *(100)*.

Immobilization on to inert solid supports, rather than directly on a working electrode, has been more commonly reported. Such supports may themselves be close to/remote from an electrode surface. Commercial

membranes e.g. Biodyne Immuno Affinity membranes (Pal Industries S.A., France) are available, which are pre-activated for facile enzyme immobilization. Alternatively Nylon membranes can be used, which have to be activated prior to use using a sequential treatment with dimethyl sulphate, lysine and glutaraldehyde *(101)*.

The most widely used remote support is controlled pore glass (CPG). Activation of this material involves silanization of CPG with one of a variety of silanization reagents e.g. 3-aminopropyltriethoxysilane, 3-glycidoxypropyltrimethoxysilane, 3-mercaptopropyltrimethoxysilane or 3-chloropropyltrimethoxysilane. The most successful method is to use the aminopropyl reagent *(102)*, resulting in a covalently bound terminal amino group. Alternatively commercially available aminopropyl CPG may be employed *(103)*. In either event treatment of the surface-bound amino groups with glutaraldehyde, followed by enzyme, establishes a covalent bond (imine type) between enzyme and glass *(104)*. The imine linkage, in common with the amide link, is not particularly stable, being prone to cleavage by hydrolysis. Reduction of the imine to the amine improves stability, accomplished by treatment with sodium cyanoborohydride *(105–106)*.

All the above immobilization methodologies can be adapted in principle to enzyme electrode use. However, in all instances the enzyme layer would constitute a physical barrier, the porosity, tortuosity, depth and partitioning behaviour of which would be important considerations in the choice of a particular immobilization phase.

Flow Injection Analysis

A need exists for automated inexpensive and fast analyses of materials in foodstuffs, and the requirements have created opportunities for measuring analytes in flowing liquids. If the enzyme-catalysed reaction produces a soluble electroactive product or utilises a soluble co-substrate, the enzyme and electrode need not be in close proximity. The reaction occurring within a matrix of immobilized enzyme can be monitored downstream at a remote electrode.

A commonly used flow technology is flow injection analysis (FIA). This was introduced in 1975 *(107)* as a means of automated liquid handling and analysis. A defined sample volume, typically 20–200 μL, is injected into a flowing carrier stream (flow rate 0.5–3.5 mL min^{-1}), which in most instances is an aqueous solution, maintained at a suitable pH by buffer, containing any necessary co–factors, co-substrates, etc. The sample passes through the immobilized enzyme phase (enzyme reactor) and the product is subsequently monitored.

After injection the sample concentration profile progressively changes from a rectangular shape to (approximately) that of a triangle as a result of mixing with the carrier stream due to diffusion. Provided the electrode response is linear with respect to analyte concentration (achieved by judicious choice of electrode and membrane materials and electrode geometry) the analyte concentration is proportional to the area under the curve. Signal integration techniques can readily provide this figure, but when these are absent, and provided the curve is reasonably symmetrical, the peak height can be taken, rather than peak area.

Constructionally the enzyme reactor is a tube of inner diameter 1–3 mm, and length 1–10 cm, containing a particulate support material onto which the enzyme is immobilized. This enzyme phase is held in the tube by a mesh made from inert material, e.g. Nylon or stainless steel, at the tube entrance and exit. An alternative form has the enzyme immobilized on the inner surface of a glass or Nylon tube.

There are several advantages to FIA. Automation is facile, consisting essentially of sequentially injecting samples into a flowing carrier stream, allowing sample frequencies of up to 60 h^{-1}. The enzyme can be immobilized on the most suitable support, without regard for electrode materials. The enzyme loading may be increased many-fold over that on an electrode by simply increasing the quantity of enzyme-loaded support. Thus enzyme saturation is improbable and a quantitative conversion of substrate to product results. This, in turn, means that undiluted samples may be injected with virtually 100% of substrate converted to product, which extends the lower detection limit at the downstream sensor compared to an enzyme electrode.

Examples of Enzyme Electrodes for Food Analysis

Carbohydrates

Glucose

Of the very large number of published reports on glucose enzyme electrodes few deal specifically with food applications. However, glucose remains the most thoroughly studied analyte for food enzyme electrodes. This section reviews recent strategies for amperometric detection.

The majority of laboratory analyses are based on FIA, which has been used to overcome some of the problems associated with non-flow batch measurement in unmodified samples and to facilitate a degree of automation. Amine *et al. (55)* immobilised glucose oxidase by glutaraldehyde crosslinkage onto a cellulose acetate membrane overlying a platinum electrode for hydrogen peroxide detection. By the use of silanised polycarbonate outer membranes and incorporation into a flow-injection system, the determination of glucose up to 1 M in soft drinks without dilution, at 60 samples per hour was achieved with a correlation coefficient of 0.989 against a Beckman glucose analyzer. Glucose oxidase has also been immobilised in graphite paste and the resulting electrode incorporated into a wall-jet flow-through cell of a flow injection system *(108)*, giving a linear range of 30 mM, a sampling rate of 120 samples per hour and an average lifetime of 3 weeks. Matsumoto *et al. (109)* immobilised glucose oxidase on aminocellulofine packed in a glass tube (2 mm × 10 cm) with a multichannel flow-through cell for hydrogen peroxide detection. A correlation with a photometric enzymic assay of 0.97 was obtained for various beverages, but dilutions of 100–200 were involved. Recently, Wei *et al. (110)* used both flow

injection and batch steady state measurements for glucose determination in a range of foods including honey, syrup and fruit juice, and obtained correlation coefficients with a spectrophotometric method of 0.997 and 0.998, respectively. In order to prevent ascorbate interference a hydrophobic inner membrane was used, and similarly, Mitzutani *et al. (65)* used an anion-rejecting Nafion coating in their glucose enzyme electrode for use in fruit juices.

Mediated enzyme electrodes have allowed decreased oxidising potentials to be used. Bradley *et al. (57)* determined glucose in molasses using a ferrocene-mediated batch system with an electrode potential of 220 mV (*vs.* Ag/AgCl), achieving a correlation coefficient of 0.98 with a GLC method. A similar system was employed by D'Costa *et al. (56)* for determination of glucose in banana as an indicator of fruit maturation, with a correlation coefficient of 0.992 against an HPLC method.

Progress towards the development of portable devices for in-field use has been made with the fabrication of multi-channel screen printed electrodes *(111)*. The enzyme was immobilised here by glutaraldehyde crosslinking or adsorption in graphite paste modified with the mediator tetrathiafulvalene or finely divided platinum, with polyvinylpyrrolidone or hydroxyethylcellulose as the binder. This paste could be screen printed to produce enzyme electrodes for fruit juice analysis with coefficient of variation of 5–10%. The ability to manufacture such reproducible devices facilitates mass production of disposable »one-shot« probes. Alternatively, Haginoya *et al. (112)* fabricated sensors in needle format, showing a correlation coefficient with HPLC of 0.989.

Fructose

Fructose is widely distributed in many fruits and vegetables, has greater sweetness than glucose or sucrose and is frequently used in diabetic sweeteners *(24)*. Enzyme electrodes for fructose determination are based on D-fructose-5-dehydrogenase which catalyses the oxidation of fructose to 5-keto-D-fructose in the presence of a mediator.

$$\text{D–fructose} + \text{mediator}_{(ox.)} \xrightarrow[\text{dehydrogenase}]{\text{fructose}} \text{5–keto–D–fructose} + \text{mediator}_{(red.)} \quad /4/$$

The enzyme requires no additional cofactor as it contains PQQ and heme *c* as redox active sites *(81)*. Mediators such as hexacynoferrate(III) can be used *(24,113)*. Xie *et al. (113)* adsorbed the enzyme onto a carbon paste electrode and covered it with a dialysis membrane. The soluble hexacynoferrate (III) mediator needed to be added to each sample. Ascorbate was a strong interferent at the polarising voltage used (500 mV *vs.* Ag/AgCl) but could be removed by co-immobilising ascorbate oxidase. The sensor was used for fructose determination in fruit and showed good correlation with a conventional enzymatic assay. Matsumoto *et al. (24)* developed a flow-injection system with the mediator in the carrier stream, an ascorbate oxidase pre-column. More recently, a biosensor comprising a rotating bioreactor and a stationary phase platinum ring amperometric detector was used for the simultaneous determination of fructose and ascorbate *(23)*. The determination takes advantage of the fast chemical oxidation of ascorbate by hexacyanoferrate (III) ions and the subsequent slower production of hexacyanoferrate (II) by the enzyme reaction which allows time resolution of the two signals.

Sucrose

Sucrose determination requires a multi-enzyme system. Sucrose is hydrolysed enzymatically by the enzyme invertase:

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \text{fructose} + \alpha\text{–D–glucose} \quad /5/$$

An invertase, glucose oxidase combination may thus be used to produce a sucrose enzyme electrode. Glucose oxidase and invertase have been co-immobilised in poly(vinylacetate) *(114,115)* and gelatin *(116)* and used to determine sucrose in fruits, vegetables and soft drinks. However, glucose oxidase is specific for β–D-glucose, so conversion from the α to β forms can be rate limiting; conversion is therefore accelerated by mutarotase. Bilitewski *et al. (111)* found the addition of mutarotase to co-immobilised invertase and glucose oxidase to increase sensitivity by 100 times. The three enzymes may be co-immobilised over the electrode *(111,117)* or in a column *(118)*, or immobilised as separate layers *(119)*. Matsumoto *et al. (118)* developed a multichannel flow injection system (Fig. 3) to simultaneously determine sucrose plus glucose and fructose in cola and fruit juices showing good comparison with the enzymatic/spectrophotometric F-kit method. Mitzutani and Asai *(119)* immobilised the three enzymes over an oxygen electrode and measured oxygen consumption in a stirred cell, obtaining a correlation coefficient for sucrose of 0.994 with the F-kit method, coefficient of variation of 3.7% and sampling rate of >20 h^{-1}.

Inevitably free glucose is a potential interferent in the above sucrose sensors. A separate glucose electrode may be employed and the measured glucose concentration subtracted from the total sucrose plus glucose *(114)*. Other strategies include the use of glucose eliminating layers comprising glucose oxidase and catalase *(116,118)*, and time resolution at a single electrode by exploiting differences in sucrose and glucose response rates *(119)*.

Lactose

Lactose is a disaccharide present in milk at concentrations of 4–6% *(111)*. It is hydrolysed by the enzyme β-galactosidase to galactose and glucose:

$$\text{lactose} + H_2O \xrightarrow{\beta\text{–galactosidase}} \text{D–galactose} + \alpha\text{–D–glucose} \quad /6/$$

A combination of β-galactosidase with glucose oxidase can thus be used to make a lactose electrode. The enzymes have been immobilised in a gel *(83)*, on a membrane *(101,120)*, crosslinked with glutaraldehyde *(111)*, and also deposited on glassy carbon electrodes *(96)*. Hamid *et al. (101)* added mutarotase to enhance lactose signals by acceleration of conversion from α to β–D-glucose.

Galactose oxidase catalyses oxidation of D-galactose, producing hydrogen peroxide and has been used in tandem with glucose oxidase to amplify the lactose response *(101)*. Galactose oxidase also catalyses the oxidation of lactose to produce hydrogen peroxide, therefore a sin-

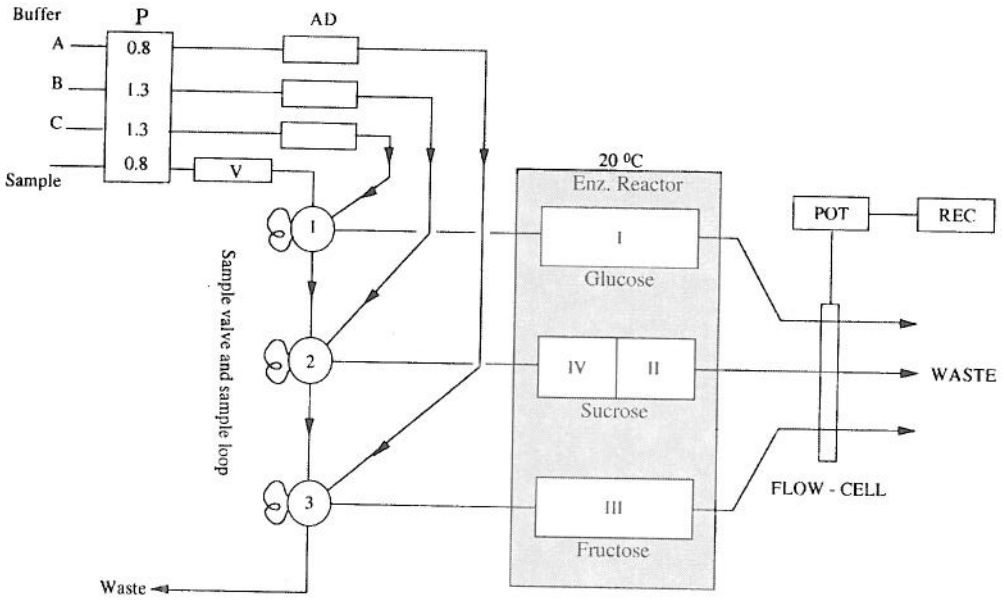

Fig. 3. Schematic diagram of multichannel flow injection analytical system; P, microtube pump; AD, air dampener; POT, multichannel potentiostat; REC, multipen recorder; I, glucose reactor; II, sucrose reactor; III, fructose reactor; IV, glucose-eliminating reactor; V, ascorbate-eliminating reactor; A, McIlvaine buffer (containing 6 mM $K_3[Fe(CN)_6]$, pH = 5.0); B, phosphate buffer (pH = 7.0); C, phosphate buffer (pH = 6.0). [From ref. *(118)*].

gle enzyme lactose sensor is possible *(121,122)*, though sensitivity and galactose interference will depend on the relative lactose : galactose activity ratio.

Organic Acids

Lactate

The importance of lactate measurement in medicine has lead to numerous studies for such applications, employing for example, lactate oxidase *(123)* and lactate dehydrogenase *(124)*. Flow injection *(125)*, continuous flow *(124)*, stirred cells *(124)* and disposable strips *(26)* have all been proposed. However, relatively few lactate enzyme electrodes have been applied to food analysis.

Lactate oxidase catalyses the oxidation of lactate to pyruvate and hydrogen peroxide. Palleschi *et al. (126)* chemically immobilised the enzyme on a Nylon net secured over an electrode and variously monitored hydrogen peroxide and oxygen consumption at the lactate probe in a flow-through cell. Wine samples were assayed following 100–200 times dilution; correlations with a spectrophotometric method showed 5–10% relative error and reproducibility of triplicate sample measurements was better than spectrophotometry. FIA systems for wine analysis have also shown good correlations with standard assays while allowing rapid sampling *(109,127)*.

Lactate dehydrogenase catalyses oxidation of lactate to pyruvate with NAD^+ as cofactor. The resulting NADH can be reoxidised by a soluble mediator such as ferricyanide using diaphorase *(22)*, or alternatively non-enzymic conversion is possible *(26)*. Reduced mediator can then be oxidised at an anode to generate current. Gilis and Comtat *(22)* retained lactate dehydrogenase, diaphorase and NAD^+ as a solution film over the electrode using an unspecified semi-permeable membrane, and supplied ferricyanide mediator in the external sample. A response time of 3 min and a lifetime of 40 days was achieved. A significant step towards de-skilled portable devices has been made by the development of a reagentless, disposable lactate probe in which lactate dehydrogenase and NAD^+ were retained in a dry state over a screen printed carbon electrode containing Medola's Blue mediator, and covered by a drop-coated cellulose acetate membrane *(26)*. A single batch of sensor strips was reproducible to 8.7% (relative standard deviation).

Malate

Malate determination is of special value in the wine industry. The quality of wines and their organoleptic characteristics are defined considering the effects of malo-lactic fermentation. The natural transformation of malic to lactic acid produces a cascade of collateral phenomena such as the decrease of fixed acidity in wine and the formation of volatile acids and esters responsible for wine »bouquet«. Control of these phenomena before bottling allows control of final quality and requires accurate malate and lactate determination *(126)*. NAD^+-dependent malate dehydrogenase or $NAD(P)^+$-dependent malic enzyme may be used with a mediator and an optional diaphorase as for lactate. Gilis and Comtat *(26)* employed a membrane-retained malate dehydrogenase, diaphorase and NAD^+ combination, with ferricyanide in solution. Willner and Riklin *(25)* immobilised the mediator PQQ by covalent attachment to a self-assembled cysteamine monolayer on a gold electrode, with subsequent covalent attachment of malic enzyme to PQQ (Fig. 4), but $NAD(P)^+$ needed to be supplied in solution. The need for mediator has been removed altogether by the use of the malate dehydrogenase plus NADH oxidase with detection of oxygen consumption at an amperometric oxygen electrode *(128)*.

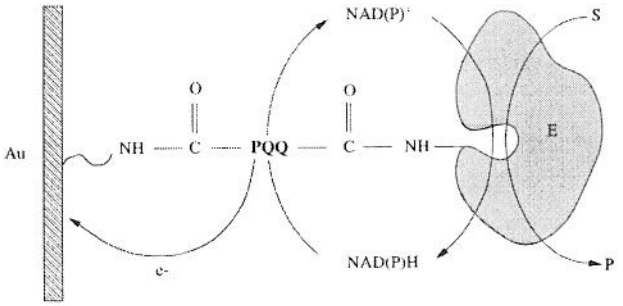

Fig. 4. Electron-transfer communication of self-assembled monolayer of cysteamine, PQQ and malic enzyme by a diffusional $NAD(P)^+$ cofactor; Au, gold electrode; E, malic enzyme; S, substrate; P, product. [From ref. *(25)*].

Ascorbate

Ascorbate is readily oxidised at an anode, but detection methods based on this direct eletrochemistry, lack specificity, being prone to interference from other reducing agents in a sample. Ascorbate oxidase catalyses the oxidation of ascorbate to the non-electroactive dehydroascorbate and water, hence hydrogen peroxide detection cannot be used. However, enzymic detection of ascorbate can be achieved using a combination of ascorbate oxidase and an oxygen electrode *(129)*. In an alternative approach. Greenway and Ongomo *(130)* used FIA with the enzyme immobilised in a Sepharose column. Sample passage through the enzyme column led to fractional conversion of ascorbate to dehydroascorbate and the selective decrease in a direct electrochemical signal was compared with a blank column and correlated with ascorbate concentration.

Glutamate

Glutamate is a major taste component in food, added as a seasoning or flavour enhancer. Several enzyme electrodes have been reported based on the enzyme glutamate oxidase:

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glutamate}} \alpha\text{-ketoglutarate} + NH_3 + H_2O_2 \quad /7/$$

Either oxygen or hydrogen peroxide electrodes have been used previously as detectors in FIA *(131,132)* and in disposable thick-film format *(98)*. Measurements have been made in soy sauce *(131,133)*, seasonings *(97)* and other foodstuffs *(134,135)*. A mediated glutamate oxidase electrode has also been described using carbon paste modified with tetrathiafulvalene, allowing operation at +150 mV (*vs.* Ag/AgCl) *(136)*.

Citrate

Citrate is widespread in plants, and the predominant organic acid in citrus fruits, contributing significantly to taste and quality of the fruits and products. It is also used as an antibacterial agent and as an additive to control pH *(137)*. Due to the multi-enzyme pathway required for measurement and the stability and activation problems with the candidate enzymes involved, relatively few publications report citrate enzyme electrodes. The obvious scheme is where citrate lyase (CL) splits citrate into oxaloacetate and acetate, and oxaloacetate decarboxylase (OAD) generates pyruvate from oxaloacetate. The pyruvate may be directly detected by, for example, differential pulse polarography after flowing the sample through a CL/OAD reactor *(138)*, or by reduction at a mercury film electrode at a constant cathodic potential of –1.4 V (*vs.* saturated calomel electrode) *(139)*. A third enzyme, pyruvate oxidase (POD) may be used in combination with a hydrogen peroxide or oxygen electrode. Matsumoto *et al. (140)* co-immobilised OAD and POD on controlled pore glass in a packed column, with CL in solution in the flow system. They achieved a detection limit of 0.02 mM, linearity to 1 mM, sampling rate of 15 h^{-1} and good correlation with the F-kit method when applied to citrus fruit samples. The requirement for additional cofactors (thiamine pyrophosphate and flavine adenine dinucleotide) for POD and divalent cation activators for all three enzymes makes the development of a reagentless citrate sensor for a portable device very difficult. Gajovic *et al. (141)* immobilised CL, OAD and POD in a gelatin membrane over an oxygen or hydrogen peroxide electrode and achieved a reponse time of 2.5 min with a lower detection limit for citrate of 0.5 μM, but phosphate, magnesium and thiamine pyrophosphate had to be added in solution with the sample. Furthermore CL undergoes a reversible inactivation upon reaction *(142,143)*, and therefore for re-use requires either constant replenishment, a large enzyme reservoir or limitation of citrate exposure of the enzyme by means of a membrane barrier.

Ethanol

Determination and control of ethanol is important in the brewing, winemaking and distilling industries, and tax regulations require exact determination of ethanol content, especially in spirits. A number of ethanol enzyme electrodes have been proposed, based respectively on alcohol oxidase, or alcohol dehydrogenase:

$$RCH_2OH + O_2 \xrightarrow[\text{oxidase}]{\text{alcohol}} RCHO + H_2O_2 \quad /8/$$

$$RCH_2OH + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{alcohol}} RCHO + NADH + H^+ \quad /9/$$

Sensors using alcohol oxidase employ the immobilised enzyme in combination with an oxygen *(82)* or hydrogen peroxide *(144–146)* electrode. Váradi and Adányi *(147)* immobilised alcohol oxidase on a protein membrane for use in a flow injection system with hydrogen peroxide detection at a platinum electrode (+600 mV *vs.* Ag/AgCl). They achieved a linear range of $\varphi = 1$–8% (volume fraction), a detection limit of $\varphi = 0.1\%$ and a correlation of 0.992 with a calorimetric reference method in beer samples. A disposable sensor with alcohol oxidase based on screen-printing technology has alo been reported *(146)*.

Unfortunately alcohol oxidase lacks specificity and its main substrate is actually methanol. Alcohol dehydrogenase is specific to ethanol, but the challenges related to the use of cofactors and mediators in dehydrogenase systems then apply. Sim *(79)* entrapped the NAD^+ and enzyme behind a dialysis membrane, but found a 30% loss in activity over 5 h. Stability was improved using a solid state sensor formed by mixing NAD^+, en-

zyme and NMP^+TCNQ^- (conducting salt as mediator and electrode material) with PVC *(80)*, with only 10% of activity lost in the first hour of use. Both sensors were used for wine analysis. The stability problem can apparently be avoided in FIA systems by adding NAD^+ to the carrier solution *(148)*.

Food Additives

Aspartame

Aspartame (N-L-α-aspartyl-L-phenylalanine methyl ester) is an artificial sweetener known by the brand name NutraSweet. Enzyme electrodes have been developed for aspartame based on different enzyme pathways. The peptide bond in aspartame can be cleaved enzymatically (aspartate peptide bond cleaving enzyme – APBC) to yield aspartate and phenylalanine/phenylalanine methyl ester. The aspartate can then be converted to glutamate by aspartate aminotransferase and determined by a glutamate oxidase enzyme electrode *(149)*, or the phenylalanine can be monitored using L-amino acid oxidase. Male *et al. (150)* immobilised APBC on activated glass beads in a column and L-amino oxidase on an activated Nylon membrane in series in a flow injection system, with a platinum hydrogen peroxide electrode (+700 mV *vs.* Ag/AgCl). The sensor displayed linearity to 1 mM aspartame with a detection limit of 25 μM, reproducibility of 0.3% (relative standard deviation) and a sampling rate of 15 h^{-1}, and was used for determinations in dietary food products.

Alternatively, the methyl ester group of aspartame can be cleaved by the enzyme α-chymotrypsin and the resulting methanol determined with alcohol oxidase. Chou *et al. (151)* entrapped the two enzymes behind a dialysis membrane over an oxygen electrode, and achieved linearity to 2 mM, sampling rate of 6 h^{-1} and results that agreed with the manufacturers labelled value within 5%.

Sulphite

Sulphite (SO_3^{2-}) is one of the most important inorganic acids found in food, and is added as a preservative, inhibiting bacterial growth and preventing oxidation. Enzyme electrodes for sulphite are generally based on sulphite oxidase, the enzyme being immobilised on Sepharose *(152)*, in a gel between membranes *(153,154)*, on an activated Nylon membrane *(155)* or in an electropolymerised poly(pyrrole) film *(156)*, and used in combination with an oxygen or hydrogen peroxide electrode.

Lecithin

Lecithin is an emulsifying compound added to food. It can be hydrolysed by phospholipidase D, producing choline which can be detected *via* choline oxidase. Campanella *et al. (157)* immobilised choline oxidase on Nylon net overlying an oxygen electrode with phospholipase in solution, and measured lecithin in chocolate, soya flour and egg yolk.

Pesticides

The enzyme acetylcholinesterase (AChE) can be used for the detection of insecticides. Effective enzyme electrodes can be made due to the selectivity and specificity of the inhibition of AChE by organophosphorus and carbamate insecticides. However, these pesticides are highly soluble only in organic solvents. Mionetto *et al. (158)* constructed an AChE electrode capable of detecting pesticides in a range of organic solvents. The sensor could be re-used by reactivation of the enzyme with 2-pyridine aldoxime methiodide.

Fish and Meat Freshness

The advantages of sensors for rapid evaluation of meat and fish freshness are obvious. Fish quality can be related to the naturally occurring chemical sequence of ATP decomposition *(159)*:

$$ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow HxR \rightarrow Hx \rightarrow X \rightarrow U \qquad /10/$$

where ADP is adenosine diphosphate, AMP is adenosine-5-phosphate, IMP is inosine-5-phosphate, HxR is inosine, Hx is hypoxanthine, X is xanthine, and U is uric acid. Following death of the fish the relative concentrations of these compounds change and sensors based on enzymes specific for these compounds thereby provide a route to freshness assessment. Enzyme electrodes can determine hypoxanthine and inosine using the enzymes xanthine oxidase and nucleotide phosphorylase co-immobilised with an oxygen electrode *(160–162)*:

$$HxR + \text{phosphate} \xrightarrow[\text{phosphorylase}]{\text{nucleoside}} Hx + \text{ribose-1-phosphate}$$

$$Hx + 2O_2 \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{uric acid} + 2H_2O_2 \qquad /11/$$

The difference between the electrode response with and without phosphate addition represents HxR concentration in fish extract. More precise information has been obtained by using multi-enzyme electrodes to determine the ratios of several compounds used as indices of freshness *(163,164)*.

Fish quality can also be related to the degradation of proteins and amino acids to biogenic amines. Increased concentrations of these amines, particularly putrescine and cadaverine can be used as indicators of freshness. Functional electrodes using putrescine oxidase with hydrogen peroxide *(165)* or oxygen *(166)* detection have been reported.

In fresh meat, microbial activity leads to development of amines which may be used as freshness indicators in similar systems to those developed for fish. However, before significant amounts of amines are formed, the meat glucose is depleted by surface microbial activity, and a gradient of glucose concentration from surface to bulk develops. This gradient has been shown to correlate well with microbial surface counts *(167)*. Kress-Rogers and D'Costa *(49)* described a planar »knife« glucose sensor array exploiting a mediated glucose oxidase system which could be inserted into meat joints.

Conclusions

Amperometric enzyme electrodes have been the most extensively studied form of biosensor and their application in clinical monitoring remains the major driving force behind biosensor research. However, the potential for food sensing applications is enormous. The appeal to the food industry of pocket-sized devices ca-

pable of easy in-field measurements and on-line monitoring of specific parameters in complex matrices is obvious. The selectivity and sensitivity characteristics, along with the potential for electronic integration, miniaturisation, and mass-production, of enzyme electrodes can meet these needs.

Enzyme electrodes have been developed for a wide range of food analytes, employing single and multi-enzyme systems in various formats. The majority of researchers describe flow systems which are more suited to centralised laboratory analysis. However, there is a recent trend towards the development of miniaturised, reagentless flat-form and probe type sensors relying on »packaging technology« such as specialised permselective membranes to create »solid state« systems. Such devices appear ideally suited to industry requirements for in-field and on-line sensing.

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Enzimske elektrode za analizu namirnica

Sažetak

Enzimske su elektrode najsvestranije proučavani tip biosenzora, a njihova primjena u kontroli kakvoće namirnica ima velike mogućnosti daljnjeg razvoja. U ovom su pregledu prikazana načela enzimskih elektroda, usredotočujući se na amperometrijsku transdukciju. Razmatrane su sve mogućnosti primjene enzimskih elektroda u biosenzorskim postupcima pri kontroli kakvoće namirnica u prehrambenoj industriji. Ukratko su opisani načini konstrukcije enzimskih elektroda imobilizacijom enzima i izborom materijala za elektrode, te najčešće primijenjivani automatizirani analitički postupak, tzv. analiza s injektiranjem (engl. flow injection analysis, FIA). Navedeni su u literaturi opisani primjerci enzimskih elektroda za analizu ugljikohidrata, organskih kiselina, alkohola, aditiva, pesticida, te za određivanje svježine mesa i ribe.