Review

Enzyme Based Amperometric Biosensors for Food Analysis

Mamas I. Prodromidis and *Miltiades I. Karayannis**

Department of Chemistry, University of Ioannina, 45 110 Ioannina, Greece; e-mail: mkaragia@cc.uoi.gr

Received: June 4, 2001
Final version: July 25, 2001

Abstract
This review introduces the principles of amperometric detection e.g. oxygen electrodes, hydrogen peroxide electrodes, NADH detection, mediators-aid detection, conductive organic salts and wiring electrodes. A short categorization and description of the materials commonly used for the construction of electrodes, e.g., platinum, glassy carbon, different types of graphite, screen-printed electrodes, rigid carbon-polymer biocomposites, zeolites, clays, and polymeric membranes is given. Approaches to construction of biosensors with respect to various strategies of enzyme immobilization, e.g., physical binding, covalent binding, gel entrapment, electropolymerization, sol-gel techniques and self-assembled architectures are also presented. The requirements and problems for sensing in food industry, examples of enzyme electrodes, published in the literature during the last half-decade, commercial biosensors released into the market along with the current and modern instrumentation, are also presented.

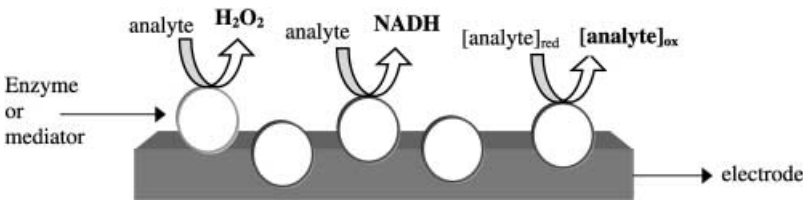
Keywords: Amperometric biosensors, Enzyme electrodes, Food analysis, Commercial biosonsors, Review

1. Introduction

This review focuses on enzyme-based transducers with amperometric transduction, therefore all examples and elucidations originated from these types of biosensors. Table 1 lists some examples of review articles of the topics presented here along with the number of the references cited in each review and the year of publication.

A biosensor is a system of two transducers, biochemical and physical, in intimate contact or in close proximity with each other, that relates the concentration of an analyte to a measurable signal. The action of the biochemical transducer over the applied system (enzyme-catalyzed reaction) results in the change of a physical property or to the commencement of a process (electrons flux originated by a redox reaction), which is sensed and converted into an electrical signal by the physical transducer (electrode, under constant potential), as shown schematically in Scheme 1.

Amperometric biosensors are based on enzymes that, either consume oxygen e.g. all the oxidases, or produce



Scheme. 1. Schematic representation of various types of amperometric biosensors.

Table 1. Review articles citied in this article.

Topic	*Nr. of citations*	*Reference*	*Year*
Catalytic electroxidation of NADH; mediators	148	[4]	1997
Chemically modified electrodes; mediators	220	[5]	1995
Modified electrodes in flowing streams	54	[8]	1990
Principles of wiring electrodes	82	[15]	1990
Polymeric membranes in biosensors	77	[33]	1995
Ion-exchange polymeric membranes in (bio)sensors	100	[34]	1997
Electropolymerized polymeric membranes	155	[37]	2000
Sol-gel based biosensors	43	[50]	1999
Rigid conducting carbon-polymer composites	30	[57]	2000
Screen-printed enzymatic biosensors	42	[60]	2000
Alkanethiol SAMs to enzyme electrodes	46	[61]	1999
Enzyme electrodes in food analysis	167	[62]	1996
Applications of biosensors in food analysis	37	[80]	1997

  1040-0397/02/0402-0241 $ 17.50+.50/0

hydrogen peroxide (excluding oxidases which produce water), or produce (indirectly) the reduced form of β-nicotinamide adenine dinucleotide (phosphate), NAD(P)H, e.g., dehydrogenases, during the course of the catalytic reaction on the substrate of interest. The general equations of the forementioned types of amperometric biosensors are:

$$\text{Substrate} + \mathbf{O_2} \xrightarrow{Enzyme} \text{Product} + H_2O \quad (1)$$

$$\text{Substrate} + \mathbf{O_2} \xrightarrow{Enzyme} \text{Product} + \mathbf{H_2O_2} \quad (2)$$

$$\text{Substrate} + NAD^+ \xrightarrow{Enzyme} \text{Product} + \mathbf{NADH} + H^+ \quad (3)$$

The depletion of oxygen according to the Equations 1 and 2 can be measured by a Clark-type electrode at a platinum cathode at 0.6 – 0.9 V versus a Ag/AgCl reference electrode, according to the following equation.

$$O_2 + 2H_2O + 4e^- \xrightarrow{-0.6\ V\ (vs.\ Ag/AgCl)} 4OH^- \quad (4)$$

An electrical bridge between the two electrodes is established with a saturated KCl and the whole system is firmly covered by a thin polyethylene or Teflon membrane (0.03 – 0.13 mm). This membrane prevents premature poisoning of the electrode from several materials of the tested matrix, is impermeable to potential interfering solutes, but is nevertheless permeable to oxygen. Oxygen electrodes are quite sensitive and provide an interference free biosensor; however, care for the oxygen back-diffusion from the atmosphere should be taken, especially in the case of concentrated samples. Some inaccuracy in direct measurements could be overcome using derivative signals [1]. The employed enzyme(s) can be entrapped either between the oxygen permeable and the outer membranes or can be immobilized onto a third one, which is being placed in the middle of the others (Scheme 2)[2].

Hydrogen peroxide generated according to Equation 2 can be measured amperometrically by oxidation at the anode of a solid (platinum, glassy carbon) electrode, polarized at +0.65 V, according to the Equation 5.

$$H_2O_2 \xrightarrow{0.65\ V\ (vs.\ Ag/AgCl)} O_2 + 2H^+ + 2e^- \quad (5)$$

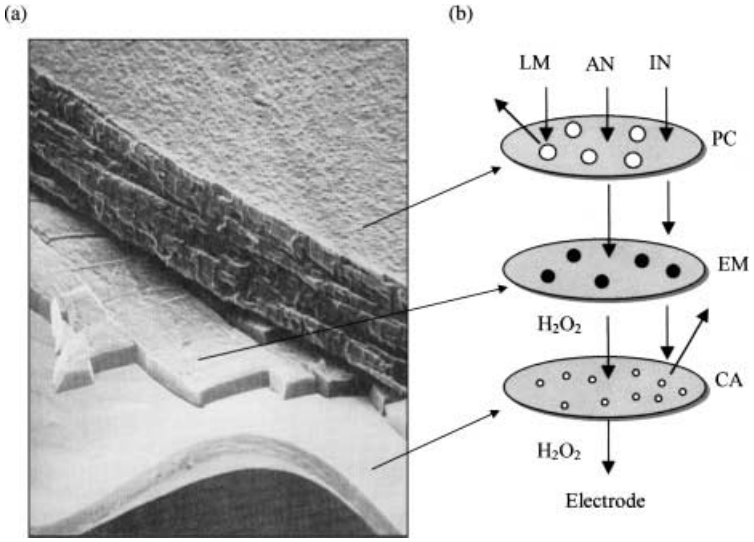
At the potential where the anodic oxidation of hydrogen peroxide is taken place, various organic compounds, e.g., ascorbic acid and uric acid are co-oxidized resulting thus in a poor selectivity sensor. This is a severe problem in electrochemistry as specificity is inversely related to the magnitude of the applied potential. Moreover, prolonged use of electrochemical detectors, especially in biological matrix and undiluted samples, results in fouling of the surface of the solid electrodes commonly used as sensing probes [3].

Similar problems should be faced in the case of dehydrogenase-based biosensors where the measurable species is NADH, the concentration of which is directly proportional to the concentration of the analyte of interest.

Although there is still considerable uncertainty about the detailed nature of the electrochemical oxidation of NADH the following (tentative) reaction scheme is generally accepted [4]:

$$NADH \xrightarrow[slow]{e^-} NADH^{\bullet+} \underset{+H^+}{\overset{-H^+}{\longleftrightarrow}} \underset{\text{intermediate}}{NAD^\bullet} \underset{+e^-}{\overset{-e^-}{\longleftrightarrow}} \underset{\text{fast}}{NAD^+} \quad (6)$$

The use of mediators has been proven as an effective approach to restrict or to eliminate these problems. A lot of work has been done in this direction and the advantages from the use of mediators have been documented in comprehensive reviews [4, 5]. However, some drawbacks are introduced since the mediators may facilitate charge transfer between possible interferants and the electrode, increasing the interference problem and leaching from the system, producing thus a progressively diminishing response to the analyte. A detailed review dealing with the criteria that should be satisfied by a mediator is reported by Turner [6]. Mediators that can be used for electron transfer include: ferrocene, ferricyanide, meldola blue, nile blue, methylene



Scheme. 2. a) SEM picture for a three-membrane configuration, b) schematic representation of the diffusion of the compounds present in a sample through the multi-membrane configuration. LM, large molecules, e.g., proteins; AN, analyte; IN, interferants; PC, polycarbonate membrane; EM, enzyme membrane; CA, cellulose acetate membrane.

blue, dichlorophenolindophenol, phenazine methosulfate, quinone etc. [4, 5]. Mediators have been extensively used for the indirect monitoring of H_2O_2 or NADH according to the following equations.

$$H_2O_2 + [Med]red \xrightarrow{Peroxidase} [Med]ox + H_2O \quad (7)$$

$$NADH + [Med]ox + H^+ \xrightarrow[or\ non\text{-}enzymatic]{Diaphorase} [Med]red + NAD^+ \quad (8)$$

$$Substrate + [Med]ox \xrightarrow{Enzyme} Product + [Med]red \quad (9)$$

A lot of work has been focused on the combination of the advantages of working at low redox potential of the mediator and/or the permselective properties of a polymeric membrane employed in the probe. Different transport mechanisms based on size exclusion (e.g., cellulose acetate), charge exclusion (e.g., Nafion), polarity (e.g., phosphatidylcholine), mixed control (e.g., cellulose acetate/Nafion) or permselectivity/electrocatalysis (e.g., cellulose acetate/dichlorophenoloindophenol [7], Nafion/cobalt phthalocyanine) have been extensively reviewed [8]. Chemical modifications of several polymeric membranes, such as cellulose acetate with non-anionic surfactants [9], polyvinylchloride with surfactants [10] or different plasticizers [10] have been reported to provide promising approaches for increasing selectivity of electrochemical sensors.

For membrane-based electrochemical devices, various characteristics of great analytical interest such as, the retention of the mediators, co-factors, permeability of the membrane to the analyte, extension of the linearity over the environmental concentration range, and protection of fragile biomaterial from low pH inactivation, have been reported [11]. However, under the diffusion limitations of these configurations sensitivity of probes is decreasing.

An alternative approach for facile charge transfer at low applied potential was introduced by Kulys [12]. In this configuration, conductive organic salts, commonly TTF^+ $TCNQ^-$, are used as electrode material on which the reduce coenzyme (e.g., $FADH_2$ or NADH) can be directly oxidized at low applied potentials. This type of biosensors although subject of ongoing research is not widespread, probably because of some difficulties in their synthesis. [13].

Another class of amperometric biosensors is that of "wiring" electrodes. A biosensor based on the concept on electrical "wiring" of the used biomolecule consists of three parts: the biorecognition element (enzyme), the electron abstraction and propagation chemistry (redox polymer) and the retaining surface (electrode) [14]. An important parameter for the improvement of their performance is the increase of the diffusion coefficient of electrons through the molecular wire, which is two orders of magnitude lower than the diffusion coefficient of a freely diffusing mediator. A comprehensive article concerning the principles of wiring electrodes has been reported by Heller [15].

2. Electrode Materials

Working electrodes used in amperometric biosensors are normally solid. The mercury electrode is the only liquid electrode at room temperature and its application to amperometric biosensor configuration is not common [16]. The most common types of working electrodes in amperometric biosensors are platinum, glassy carbon and gold for the anodic oxidation of hydrogen peroxide, whereas all types of carbon electrodes, e.g., graphite, carbon pastes and glassy carbon are used for the anodic oxidation of NADH and mediators. These types of electrodes fulfill the requirements demanded for good performance of a working electrode as they are relative inert [17], highly conductive (metal solids mainly) resulting thus in low background currents, their surfaces can be modified by electrodeposition or chemical modification, the latter being more common with carbon electrodes. Other solid electrode materials used are semiconductors, e.g., metal oxides [18] and conductive organic salts (see Sec. 1).

The introduction of metallized graphite powder, containing distinct metals including palladium, platinum, ruthenium and cobalt provides larger electrocatalytic efficiency such as shorter response times and lower applied potentials compared to conventional graphite pastes. Carbon pastes doped with different metals Pt, Pd and Ru have also been used as anodes of NADH, vitamin C and hydrogen peroxide [19]. Recently, a versatile way for construction of gold band nanoelectrodes with areas as small as $10^{-6}\,cm^2$ using recordable CDs as the gold source was proposed by Angnes et al. [20].

A new class of materials with promising application to many areas, among them (multi)sensors development, is that of hierarchically ordered oxides which exhibit structural ordering at multiple discrete length scales [21].

3. Immobilization Supports

The benefits that can be obtained from an efficient protocol of immobilization are very important namely the prolonged use of the sensor and an anticipated extended storage and working stability. We do not aim to provide a comprehensive coverage of the vast range of methods available, but to provide a snapshot of immobilization techniques for potential users. The reader is referred to several books for a wider and more detailed description of immobilization techniques [2, 3, 22 – 25].

3.1. Controlled Pore Glass (CPG), Zeolites, Clays

CPG has been widely used in many applications in enzymic analysis, mostly in combination with flow injection analysis (FIA). CPG provides high efficiency of immobilization, has very good flow characteristics and is commercially available in a wide variety. Detailed protocols for several activation and coupling techniques are given by Weetall [24]. Glycerol

dehydrogenase [26], malate dehydrogenase and diaphorase [27] have been immobilized into aminopropyl-CPG using glutaraldehyde as crosslinking agent, isothiocyanato-CPG and into carboxy-CPG using *N*-hydroxysuccinimide ester.

Zeolites are a class of aluminosilicates with a high ion-exchange capacity that has been used as molecular sieves based on shape or size exclusion properties. A reagentless amperometric biosensor for hydrogen peroxide [28], and a glucose biosensor on a dealuminized Y zeolite has been reported [29].

Another class of inorganic materials that have been used as matrices for the immobilization of enzymes is clays. Immobilization of polyphenol oxidase into a laponite clay matrix [30], a glucose oxidase clay-modified electrode for aerobic glucose monitoring [31] and a composite clay glucose biosensor based on an electrically connected HRP [32] have been published.

3.2. Polymeric Membranes

Polymeric membranes are the most commonly reported matrix for the immobilization of enzymes. Different types of polymeric films were implemented in various capacities in amperometric biosensor designs. They are ordered as conductive, nonconductive and composite (see above) and they are fabricated by solvent casting, spin coating and electropolymerization. Their use in amperometric biosensors is extensively reviewed by Erm and Yacynych [33] and Bontempelli et al. [34]. Here, some selected examples from the recent literature will be presented.

Glucose, lactate and glutamate dehydrogenases were immobilized into nonconducting films by controlled electropolymerization of the corresponding monomers (1,2-, 1,3-, 1,4-diaminobenzene or 4,4-dihydroxybenzophenone) [35]. A distinctive feature of enzyme entrapment within electropolymerized films is the possibility to fabricate controlled architectures based on spatially segregated multilayers that allow particular detection schemes (e.g., enzymic elimination of interferants, integration of a redox mediator, functionalization of the electrode surface for covalent attachment of biomolecules) and/or tailoring of some sensor properties (e.g., sensitivity, stability, selectivity) [36]. A detailed review of amperometric biosensors based on electrosynthesized polymeric films is given by Palmisano et. al [37]. Nafion, an anion exchanger, has been extensively used as a membrane material for the immobilization of biomolecules through ionic forces [38].

A big variety of commercially available membranes have also been used in multienzyme biosensors (Scheme 2). Among the most widely used are MF-Millipore membranes (Millipore corp., MA) and Biodyne, Immunodyne, Ultrabind by Pall Biosupport, UK. A biologically inert mixture of cellulose acetate and cellulose nitrate composes MF-membranes [39]. Nylon 66 membranes have been applied as supports for the immobilization of various enzymes for use in both batch and flow conditions. Due to the diverse functionalization of their surface enzymes can be immobilized either through ionic force [39], covalent binding [39], or they can further modified with glutaraldehyde [40] or carbodiimide [41]. Other commercial membranes such as polycarbonate by Nucleopore (Cambridge, MA) and Immobilon AV affinity by Millipore (Bedford, MA) [42] have been used as supports for enzyme immobilization.

Physical entrapment into a glycerol-BSA gel was reported by Kim [43] for the preparation of a multienzyme sensor for glucose, ascorbic and citric acid. Co-immobilization of oxaloacetate decarboxylase (OACD) and pyruvate oxidase (POD) by adsorption into a wet poly(vinyl chloride) membrane was reported by Kihara and co-workers [44]. Malic enzyme and and salicylate hydroxylase were physically co-immobilized into a gelatin gel [45]. An octapine biosensor based on the immobilized POD and octapine dehydrogenase into a cellulose triacetate membrane [46]. Enzyme laminates using Cuprophan dialysis membranes, were recently reported for the construction of reagentless biosensors for malic and citric acid [47, 48].

3.3. Sol-Gel Matrices

Sol-gel derived glasses have emerged as a new class of materials well suited for the immobilization of proteins. Sol-gel chemistry has shown to provide a means of immobilizing enzymes in a manner that preserves their selectivity and activity and allows their use as platforms for (bio)sensors. The sol-gel network is prepared by a generalized synthesis involving an acid or base catalyst, an aqueous or non-aqueous solvent and an organic precursor (or a mixture of them) $(RO)_4Si$ or $[(RO)_4Si]X$, where R is an alkyl group and X represents an organic moiety. Three steps namely hydrolysis of the silicate, condensation and polycondensation are involved during the formation of the sol-gel network. Synthesis protocols and characterization of the products are detailed covered by the book of Brinker and Scherer [49], whereas the use of sol-gel in biosensors is reviewed by Wang [50]. Diffusion effects of redox probes in hydrated sol-gel derived glass [51], a recent study of three oxidases for glucose, hydrogen peroxide and phenols assay in natural samples [52] and a stability study of oxidase immobilized in silica gels [53] have been published.

Incorporation of a grafting copolymer of poly(vinyl alcohol) with 4-vinylpyridine into the silica sol was found to prevent the cracking of conventional sol-gel-derived glasses and to eliminate the swelling of the hydrogel [54]. Silica-carbon and silica-gold composite materials have found great applicability to screen-printed sensors as reported by Wang and co-workers [55, 56]. Such coupling of sol-gel and thick-film technologies offers a one-step fabrication of disposable enzyme electrodes, as it obviates the need for thermal curing.

3.4. Carbon Based Biosensors

One of the most commonly used designs of amperometric biosensors involves the incorporation of a biocatalyst within a carbon matrix. The resulting bulk modified electrodes offer several advantages such as close proximity of the biocatalytic and sensing sites, the possibility to produce reagentless biosensors, the renewing of the surface, the economy of fabrication and the stability. This type of biosensors can be shorted into three main categories namely carbon paste electrodes (CPE), screen-printed electrodes and rigid carbon-polymer (or solid carbon) composites.

Biosensors based on CPEs, while an important function, are too vast for review here. Readers are referred to several reviews dealing with the construction, the properties and the analytical applications of CPE [4, 5, 18].

A recent, and growing, trend in the field of carbon-based electrodes is that of rigid conducting carbon-polymer composites. In these materials, carbon and the polymer are mixed together in order to prepare a rigid, conductive matrix where the enzyme(s) and/or other components (cofactors, mediators) can be immobilized. The sensing surface can be renewed by a simple polishing procedure. Alegret and co-workers have reviewed the most important electrochemical and mechanical features of these materials as well as their applications to enzyme electrodes [57]. Miertuš and co-workers have been published a lot of work on solid binding matrices-based enzyme electrodes that have been applied for food quality monitoring [58, 59].

In screen-printed electrodes, a paste with the appropriate viscosity and thixotropy is pressed onto the substrate with a squeegee through openings in the emulsion on a stainless steel screen. A recent review dealing with the configurations that have been used in the design of screen-printed enzymatic biosensors was recently published by Alegret and co-workers [60].

3.5. Self-Assembly Monolayers

The use of self-assembled monolayers (SAMs) in various fields of research is rapidly growing. In particular, biosensors apply SAMs as an interface-layer between a metal surface and a solution or vapor. The most common compounds that are able to undergo the process of SAMs are alkanothiols, dialkyl disulfides or dialkyl sulfides on gold. The use of SAMs in the construction of enzyme electrodes is not widespread to a large variety of enzymes, however, a growing trend is observed. A comprehensive review dealing with the application of alkanethiol self-assembled monolayers to enzyme electrodes has been published by Gooding and Hibbert [61].

4. Requirements and Problems in Food Sensing

The major driving force behind enzyme electrode development remains the need for clinical monitoring. The technology is, however, slowly penetrating in the food industry [62] despite the considerable opportunities in food analysis (see also Sec. 6). It has been estimated that the food industry spends, on average, 1.5 to 2% of its turnover on quality control and appraisal.

Demands of sensitivity, specificity, speed and accuracy of analytical measurements have stimulated considerable interest in developing biochemical sensors as diagnostic tools in food industry. It is characteristic that over 10 million penicillin assays are performed on the milk for diagnostic reasons. The measurement of alcohol content is used as a guide to the quality of the final product in the wine and beverage industries; glucose in fermentation of liquors and syrups; biogenic amines or inosine and hypoxanthine are used as indicators for food freshness and aging; hydrogen peroxide and sulfites as preservatives in frozen products; malic and citric acid in fruits industries as indicators of maturity; ascorbic acid in fruit juices and acetic acid in vinegar. Determination of peracetic acid or/and hydrogen peroxide is needed in disinfection processes in breweries; of ammonia in bakery and meat products; of lecithin as a measure of egg content of foods. Nicotinic acid is determined in meat products; of lactate in yogurt, beer, fruit juices and wine; of glutamate as flavor enhancer; of lysine as a marker of nutritional damage due to heat; of polyphenols in olive oils and of catehols as quality indicator in tea processing. Many other examples as well advocate the growth of biosensors in the field of food analysis. Most of these assays are routinely performed by various regulatory agencies or is of concern of health authorities.

Current analytical practices in food industry are time-consuming and require skilled labor. They need lengthy separations, expensive instrumentation and high purity chemicals as mobile-phase modifiers. Most of these drawbacks can be overcome by applying enzymatic analysis. Excellent books [22, 63] and a powerful manual by Boehringer Mannheim provide all the background and detailed instructions for the determination of more than 40 compounds in foods. However, modern food industry needs pocket-sized devices capable of simplified in-field measurement on undiluted samples and to provide on-line monitoring of one or more parameters simultaneously during the production or processing of foods. Most of these requirements can be met admirable by enzyme electrodes. However to gain the confidence of the market they need also to be inexpensive, reliable, robust and demonstrable superior to existing methods. Moreover, enzyme-electrodes generated information may confer competitive advantage resulting from improvements in raw material quality and harvesting efficiency, process optimization and manufactured product quality [62].

Problems referable to a given enzyme electrode will depend on the specifics of the analyte, enzyme and food matrix including analyte concentration, electrochemical interferents and pH. Interference by various electroactive species, commonly ascorbates, has been faced either by appropriate manipulation of the signals recorded by a dual measurement system consisting of a plain and an enzymic

electrode [64], or by using ascorbate oxidase in order to eliminate ascorbic acid present in the sample [47, 65] or by using polymeric membranes with certain properties of size exclusion or electrostatic repulsion [38 – 42, 47, 48]. The use of diffusion restricting membranes has also been used as an effective tool towards a major challenge of biosensor that of in-field measurements. In-field measurements presuppose a portable device (sensor and metric arrangement) and this is attainable with the current technology in electronics and the existing know-how in sensor fabrication. Another requirement is the ability of the sensor to operate over the environmental concentration range of the analyte of interest in order to eliminate steps as sample dilution. Glucose determination up to 2000 mM [9], malic acid up to 40 mM [47] and citric acid up to 100 mM [48] have been reported. These approaches however are not such as yet able to be used directly in natural products, e.g., fruits, since their pH values differ significantly from the enzyme optimum. Vadgama and co-workers [11] demonstrated rapid deactivation of a glucose oxidase enzyme electrode at pH 2.4. However, by the use of specialized outer membranes it is possible to protect the enzyme from pH deactivation for sufficient time for pH independent measurement and reuse. Similar strategies have been employed for the construction of a calibration plot (4 – 20 mM) for malate at pH 3.3 [47].

Sterilization or disinfection of biosensors is important in two respects. On the one hand, the biocatalytic sensing part without protection will become subject to bacterial attack or to degradation by enzymes. On the other hand, sterilization is demanded for in-line process control to protect foods or fermentations from leaking biosensor material. Hamid et al. [66] devised a method to sterilize biosensor by radiation while Bradley et al. [67] reported the construction of an in situ fermentation probe, which is stable enough to be used in fermentations for over a month. The sterilizable probe was introduced commercially in late 1990 by Biotrace (Braunschweig, Germany) but it was withdrawn. In order to overcome this handicap several approaches have been proposed for sampling under sterile conditions. Microdialysis (passive diffusion of analytes across a semipermeable membrane), filtration probes and bypass module are currently employed by TraceBiotech in the ProcessTRACE analyzer (see Table 3).

An enhancement of the stability of the proposed biosensors models will certainly increase their commercialization, since a stable product satisfies some of the market demands namely reusesability, stock ability, no need for recalibration within runs, low cost of storage and distribution, and easy handling. Except the reuseability, all the above demands are also valid in the case of disposable sensors, since the profits of the low cost mass production are not attainable with short-lived sensors. Since most biosensors are not optimized with respect to stability, progress in this field is expected. Some enzyme stabilization protocols that use polyelectrolytes and sugar derivatives have been reported by Gibson and co-workers [68]. Improved performance of carbon paste amperometric biosensors through the incorporation of fumed silica have been reported by Wang and Naser [69] (see also Sec. 6).

5. Instrumentation and Cell Design

Amperometric electrodes can be designed to work using a two-electrode (working/auxiliary) system or a three-electrode (working/auxiliary/reference) system. Two-electrode system is simpler and requires little instrumentation, however, we should ensure that the auxiliary electrode does not limit the current. An auxiliary electrode of large area helps in this regard. If a reference electrode is employed we can arrange to control the potential of the working electrode with respect to this reference electrode while measuring the current between the working and the auxiliary electrode [23, 70].

A need exists for inexpensive and fast analyses of materials in foodstuffs, and the requirements have created opportunities for measuring analytes in flowing liquids. Flow injection analysis (FIA) is the most commonly used technique.

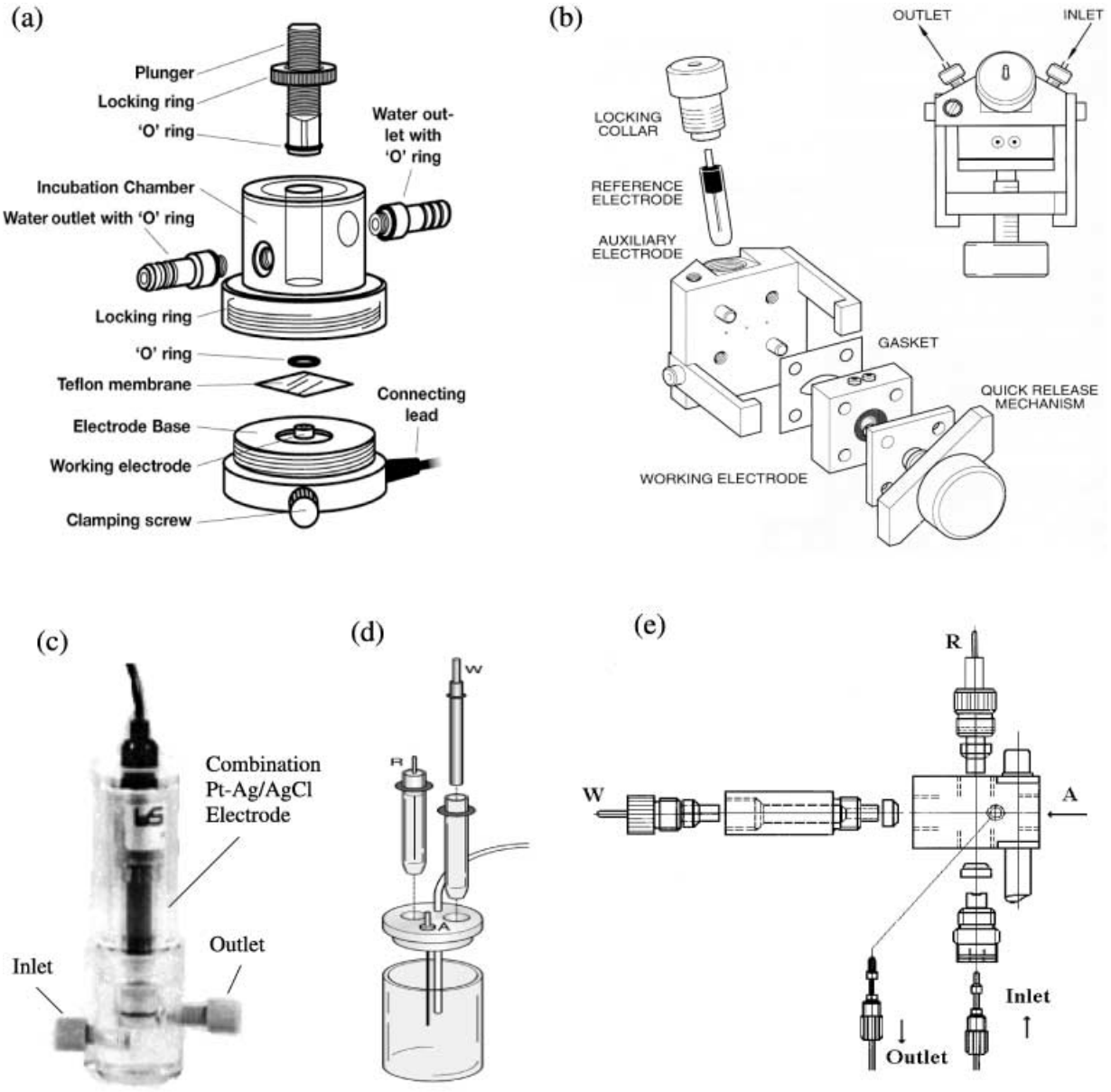
Schematic diagrams of some commercially available flow cells that have been extensively used in flow applications are shown in Scheme 3. Recently a new electrochemical cell is provided by TraceBiotech AG (Braunschweig, Germany) under the brandname TRACE Flowcell. This cell is available in different geometries and has been equipped with dual-channel sensor-chips, thus allowing multiple and difference measurements (see also Table 3).

Prodromidis et al. [71] proposed the upgrading of a semi-automatic flow injection manifold to a fully-automatic one by means of a resident software. Economou et al. [72] described a virtual instrument for amperometry and voltammetry in stationary and flowing solutions, based on LabView software and a potentiostat. Remote monitoring and control of a FIA system via the Internet was proposed by Frederik et al., holding promise of being a mean for long-distance collaborations with feasibility of real-time data acquisition and control [73]. Gooding and Hall described a new fill-and-flow biosensor design, with the reagents located upstream from the transducer [74].

Recently a microseparation chip for performing multi-enzymatic dehydrogenase/oxidase assays in connection with amperometric detection was published by Wang and co-workers [75] (see below).

6. Commercial Biosensors and Future Prospects

According to recent strategic research by Frost & Sullivan, the biosensor market is expected to continue growing and have revenues of $560 million by 2002. Since 1993, this market has increased 21% a year, and in 1996 the market was worth $413.1 million [76]. Biosensors market by sectors is illustrated in Table 2. Results presented in Table 2 were retrieved by the website of Cranfield Biotechnology Center (*www.cranfield.ac.uk*) and according to the copyright holders, these results are their best estimate based on limited available information. However, these results are indicative



Scheme. 3. Different commercial flow and batch cells: a) oxygen electrode assembly by Rank Brothers Ltd (Cambridge, UK), b) thin layer flow-through cell by BAS (IN, USA), c) flow-through cell by Universal Sensors Ltd. (Metairie, LA), d) VC-2 voltammetry cell by BAS, and e) electrochemical detector cell 656 (flow-through) by Metrohm Ltd. (Herisau, Switzerland).

Table 2. Biosensor market by sectors.

Sectors	*US$ (million)*	*%*	*Comments*
Medical: glucose	456	90	Diabetics control
Medical: other	11	2	Lactate, urea, etc.
Environmental	10	2	Mainly BOD indicators
Other	31	6	Fermentation monitoring, food analysis, alcohol
Total	**508**		

Source: Cranfield Biotechnology Center (1996)

for the status of biosensors towards the currently human and industrial analytical demands.

The market share of biosensors would be probably larger if the considerable academic research on biosensors would be reoriented from the concept demonstration to the improvement of crucial parameters. These include the test of the developed biosensors to real samples for which they are supposed to be operated and the extension of storage and operating stability of the enzyme membranes or enzyme cartridge that accompany them. In many cases, biosensors

Table 3. A selection of commercial available analyzers for use in food analysis based on biosensor technology.

Manufacturer/instrument/contact data	*Enzymic pathway*	*Analyte/applied matrix*		*General comments*
Yellow Springs Instruments (OH, USA) **YSI 2700 Select Food Analyser** Tel: (+1) 937-767-7241 ext. 2 Fax: (+1) 937-767-8058 *http://www.ysi.com*	Glucose oxidase Invertase/Mutarotase/Glucose oxidase Galactose oxidase L-Lactate oxidase L-Glutamate oxidase Choline oxidase Glutaminase/L-Glutamate oxidase Alcohol oxidase Amyloglucosidase/Glucose oxidase	Glucose (Dextrose) Sucrose Lactose L-Lactate L-Glutamate Choline L-Glutamine Ethanol Starch	Molasses, Potatoes, Milk Cheese, Whey Lunch meat Chicken broth, Spice mix, Tomatoes Infant formula, Pet foods Cell culture Beer, wine Starch in foods	Automatically up to 24 samples 10–25 μL sample size Immobilization on polymeric membranes Typical membrane working life: 5–21 days H_2O_2 anode oxidation Response time: 1 min Precision CV (n = 10) ~ 2%
Universal Sensors Inc., (Metairie, LA) **ABD 3000 Biosensor Assay System** Tel: (+1) 504-885-8443 Fax: (+1) 504-885-8443 *http://intel.ucc.ie/sensors/universal/*	Lysine oxidase Alcohol oxidase L-Amino acid oxidase Ascorbate oxidase Glucose oxidase L-Glutamate L-Lactate oxidase Galactose oxidase Oxalate oxidase Invertase/Mutarotase/Glucose oxidase	L-LysineAlcohol L-amino acids Ascorbate Glucose Lactate Lactose/Galactose Oxalate Sucrose	Determination of the analytes in foods samples	Enzyme Immobilization on polymeric membranes Lysine oxidase is immobilized on metallized (Ru/Pd) carbon electrode using electropolymerized poly(*o*-phenylenediamine) H_2O_2 anode oxidation or O_2 cathode reduction Reproducibility < 3% Staedy-state response: 1–2 min Storage Lifetime: 1 to 12 months Analog or RS-232 outputs
Nova Biomedical (Waltham, MA) **Bio Profile Chemistry Analyzer** Tel: (+1) 781-894-0800 Fax: (+1) 781-899-0417 *http//Uwww.novabiomedical.com*	No details available	Glucose L-Lactate L-Glutamate L-Glutamine	Determination of the analytes in cell culture and fermentation processes	Auto sampling, fully automated O_2 cathode reduction Enzyme-membranes 0.5 mL sample size Imprecision/Resolution 5%
TRACE Biotech AG (Braunschweig, Germany) **Process TRACE 1.2** Tel: (+49) 531-261-33-0 Fax: (+49) 531-261-33-38 *http://www.trace-ag.de*	No details available	Glucose L-Glutamate L-Glutamine	Determination of the analytes in pilot plants and fermentation processes	Flow analyzing system with injection Amperometric with sensor chips as biosensors Temperature stabilization, R.S.D. < 1.5% Lifetime: > 14 days or 5000 measurements Bioreactors or screen-printed electrodes for several analytes are available on request.
SensAlyse Ltd. (Manchester, UK) **Alcohol Sensor** Tel: (+44) 161-232-1922 Fax: (+44) 161-226-2263 *http://www.sensalyse.com*	No details available	Alcohol Sugars Ascorbic acid Malic acid	Beer and wine Under development	Amperometric disposable, screen printed electrodes H_2O_2 anode oxidation Selectivity: a polmyeric membrane screens out possible interferants Response time: 3 minutes
Gwent Sensors Ltd (Pontypool, UK) **The Answer 8000** Tel.: (+44) 1495-750-505 Fax: (+44) 1495-752-121 *http://www.g-s-l.co.uk*	Horse Radish Peroxidase/Glucose oxidase/Ferrocene No details available	Glucose Sucrose	Potatoes and other industrial applications Industrial applications	Flow analysis system Graphite enzyme-cartridge Cartridge lifetime: 50 runs Chronoamperometric measurements at −0.1 V Build-in dilutor (for high concentrations)
Applied Enzyme Technoloy Ltd. (Leeds, UK) Tel: (+44) 113-233-31940 Fax: (+44) 113-233-2593 *http://www.leeds.ac.uk/ulis/*	L-Glutamate oxidase Alcohol oxidase	L-Glutamate Alcohol		Biosensor stabilization protocols ensure retention up to 80% of the original enzyme activity after a period of: 12 weeks L-Glutamate oxidase) 25 weeks (Alcohol oxidase)

Table 3. (cont.)

Manufacturer/instrument/contact data	*Enzymic pathway*	*Analyte/applied matrix*		*General comments*
Bioanalytical Systems, Inc. (IN, USA) **Peroxidase Redox Polymer Kit** Tel.: (+1) 765-463-4527 Fax: (+1) 765-497-1102 *http://www.bioanalytical.com*	Glucose oxidase Acetylcholine esterase/ Choline oxidase	Glucose Acetylcholine/Choline	General applications General applications	Enzymes are covalently bounded within a post-column reactor (IMER); shipped in cold containers H_2O_2 oxidation at Pt anode at 0.1 V; mediated by Osmiun(III) Polyvinylpyridine redox polymer
Biotech Products (IN, USA), Distributor **Micro Dialysis Biosensor by Sycopel** Tel: (+1) 317-577-4194 Fax: (+1) 317-842-9935 *http://www.biotechproducts.com*	Glucose oxidase Lactate oxidase Glycerol kinase/glycerol-3-P oxidase Acetylcholine esterase/ Choline oxidaseNon-enzymatic L-Glutamate oxidase D/L-Amino acid oxidase Adenosine oxidase Xanthine oxidase	Glucose Lactate Glycerol Acetylcholine Ascorbate L-Glutamate (D/L-Amino acids) Adenosine Xanthine	General Applications	Electropolymerized poly(*o*-phenylenediamine) is used either as a molecular barrier to electroactive species or to immobilized the enzyme onto the Pt-working electrode. Ascorbate oxidase is used for the elimination of ascorbates interference. Enables real time measurements of analytes in vivo *Specifications for glutamate analysis* Linear range: 0.5 μM to 1 mM glutamate Response time: 20 s for 90% steady state response
Flownamics Analytical Instruments, Inc. (USA) **FAIZA 110-P** Tel: (+1) 608-240-1604 Fax: (+1) 608-240-1606 *http://www.flownamics.com*	No details available	Glucose Lactose Sucrose Galactose Lactate Ethanol/Methanol L-Glutamate L-amino acids	General Applications Fermentation Process	Flow injection analyzer equipped with the appropriate enzyme cartridge. Response time: 3 min Reproducibility: < 3% Simultaneously analysis of 6 samples Bioreactor lifetime: 1500 runs or 6–12 months depending upon the analyte
Analox Instruments Ltd. (London, UK) **LM5 Lactate Analyzer** **AM2 Industrial Alcohol Analyser** **GM10 Industrial Glucose Analyser** **GM7 Micro-Stat Multiassay Analyser** Tel: (+44) 181-749-7635 Fax: (+44) 181-740-6608 http://www.analox.com	Lactate oxidase Alcohol oxidase Glucose oxidase Invertase/Glucose oxidase β-Galactosidase/Glucose oxidase Glutaminase/Glutamate dehydrogenase /NADH/Horse radish peoxidase Glutamate dehydrogenase/ NADH/ Horse radish peroxidase Alcohol oxidase	Lactate Alcohol Glucose Sucrose Glutamine Ammonia Methanol	Dairy products (directly on milk) Beers, Wines, Consumer products Soft drinks, Distilleries, Industrial plants Aqueous solutions and extracts Aqueous solutions and extracts Aqueous solutions culture fluid, Fermentation Process Aqueous solutions culture fluid, etc. Fermentation Process Soft drinks, colas and other aqueous products, still or carbonated.	Enzyme-based biosensor; O_2 cathode reduction using a Clark-type amperometric electrode Fluid pumps: peristaltic Sample size: 3.5–25 μL depending on the assay Response time: 20 seconds Reagent stability: 6–12 months unopened at 0–5 °C. Lactate: up to 20 mM Alcohol: 0–40% v/v Glucose: 0–20% w/v Methanol: 0–500 ppm Sucrose/Lactose: up to 50 mM total glucose Glutamine/NH_3: 0.5–20 mM
Toyo Jozo (Japan [a] **PM-1000, PM-1000DC analyzers** **M-100, AS-200 ANALYZERS**	No details available	Glucose, Sucrose, Lactose, Lactate, Alcohol, Glycerin, Ascorbic acid, L-amino acids, Glutamate, Lysine	Food Analysis	Enzymic membranes onto a Clark oxygen electrode Two-point calibration is necessary Sample size: 1 to 50 μL; CV 1%
Oriental Electric Co., Ltd. (Japan) [b] **Freshness Meter KV-101**	No details available	ATP	Indicator of fish freshness And meat aging	Soluble enzymes and a Clark oxygen electrode Sample preparation and extraction is necessary Response time: 5–6 min

Table 3. (cont.)

Manufacturer/instrument/contact data	*Enzymic pathway*	*Analyte/applied matrix*		*General comments*
IBA GmbH (Göttingen, Germany) **OLGA, On-Line General Analyser** Tel: (+49) 551-506-72-0 Fax: (+49) 551-506-72-181 *http://www.iba-go.de*	No details available	Sucrose Glucose Alcohol	Quality control of foods Juice and Beer	Automatic calibration, on-line analysis system, based on sequential injection mode Exchangeable biosensors
		Acetate, Nitrate, Nitrite and phosphate sensors are Under development		
BioSensor Technology GmbH (Berlin, Germany) **Enzyme membranes, Thick Film Biosensor** Tel: (+49) 30-476-4594/2934 Fax: (+49) 30-476/4594 *http://www.bst-biosensor.de*	Glucose oxidase Lactate oxidase Ascorbic acid oxidase β-Galactosidase/Glucose oxidase	Glucose Lactate Ascorbic acid Lactose	General Applications Provider of some Analyzers	Amperometric enzyme sensor; H_2O_2 anode oxidation or O_2 cathode reduction in the case of ascorbic acid Storage stability: > 6 months (GOD > 9 months) Operational stability: > 10 days or 2000 analysis (GOD > 28 days or 3000 analysis; GOD screen printed > 15 days or 3000 analysis) Linear range: Glucose, 0.2 – 24 mM; Lactate, 0.2 – 20 mM; Ascorbic acid, 0.6 – 40 mM; Lactose, 0.1 – 100 mM.
EKF diagnostic GmbH (Magdeburg, Germany) **Biosen 5040** Tel: (+49) 39-203-785-0 Fax: (+49) 39-203-785-14 *http://www.ekf-diagnostic.de*	Glucose oxidase Lactate oxidase	Glucose Lactate	General applications General applications	Amperometric; H_2O_2 anode oxidation Enzymic membranes Sample volume: 20 μL Lifetime: 15 days or 3000 analysis Response time: 10 – 15 seconds Linear range: 0.5 – 50 mM glucose; 0.5 – 40 mM lactate
BioFutura S.r.l (Gorizia, Italy) **PerBaco 2000** **PeBaco 2002** Tel: (+39) 481-527-723 Fax: (+39) 481-527-770 *http://www.biofutura.com*	Glucose oxidase Fructose dehydrogenase Lactate dehydrogenase/ Diaphorase /Ferricyanide Malate dehydrogenase/ Diaphorase /Ferricyanide	Glucose Fructose Lactate Malate	Wine Must	Enzymic; Amperometric Solid binding matrices bearing the biocatalysts. Dialysis membranes for preventing interferences Dilution and decolorazation of the samples is recommended In red wines or in a high content of polyphenols.
Ismatec S.A. (Glattbrugg, Zurich) **ASIA Flow Injection Analyzer** Tel: (+41) 1-810-30-40 Fax: (+41) 1-810-52-92 *http://www.ismatec.com*	No details available	Glucose Alcohol Xanthine Galactose Choline	Quality control of foods and drinks	Automate sampling Wall-jet flow through cell Reproducibility: < 1% Sampling throughput: 30 – 120 h^{-1} Stability: 1000 runs per cartridge

[a] Data are taken from [79]. [b] Data are taken from [80].

were never challenged with real samples, and the gap between academic biosensor developments and practical applications remains wide.

Moreover, the food industry commonly tests a variety of analytes in a large diversity of materials. The major difference between clinical tests and food tests is that the former ones are carried out on the same materials (blood and urine), while the latter ones should be carried out on a wide range of diverse materials, each one of these bearing particular difficulties in terms of matrix effects and environmental concentration range. Once a clinical test is developed for an analyte and is adapted for use with blood and urine, it can be made available to all hospitals and general practitioners. Once a food test is developed, it needs to be adapted for use within a large range of foodstuffs before it can be made available to diverse users (industry, farmers, processors and retailers).

As it has been stated above, the most important parameter for an immobilized enzyme is its stability during the solid form. To make the enzyme element of the biosensor a practicable commercial entity, it must be stored and shipped dry. Unfortunately, many enzymes are relatively unstable and they lose activity during drying or during storage over extended periods. The stabilization of the enzymes has been attempted using a large number of chemicals, including mono-. di-, and trisaccharides, polysaccharides and other polymers, such as polyethylene glycol [68, 69, 77, 78].

Glucose oxidase is a very stable glycoprotein, however the majority of the commercial available analyzers dealing with the assay of glucose in various real samples, is restricted mostly in blood for diabetes control. However, these analyzers are also applicable to other natural matrices such as foods. Except glucose, other analytes such as sucrose, lactate, lactose, ethanol, methanol, glutamate, glutamine, acetylcholine, L-aminoacids, ascorbate, oxalate, glycerin, lysine, etc. can be analyzed by using commercially available analyzers (Table 3).

More information concerning the specifications, the principles of operation, the applications and the capabilities of the proposed biosensor-based analyzers can be obtained through the web sites of the companies as well as in literature from several comprehensive books or reviews [22, 79–81].

Searching the various web databases in order to retrieve the data presented in Table 3, we found out that only a limited number of biosensor-based products have been exclusively dedicated to the food industry. Most of them are applied to clinical analysis, although the analytes studied may also be found in foods. Many promising biosensors fail miserably during their first contact with food samples, due largely to the high complexity of the samples involved [80]. Another interesting point that occurred from the evaluation of these data is the almost total absence of the dehydrogenase-based biosensors. This can be attributed to the need of cofactors for their operation (e.g., NAD^+), which cannot easily be immobilized in a successful manner, and therefore a continuous supply of them through the carrier stream, is necessary. However, the majority of the presented analyzers is not based on a reagentless mode of biosensors and therefore more attention must be paid to the development of dehydrogenase based biosensors, since their variety can increase the number of the analytes reported in the manuals of the analyzers.

Table 4. Various analytes presented in this review.

Analyte	*References*
Glucose	[9, 10, 11, 29, 31, 35, 38, 43, 52, 55, 56, *59*, 68, *75*, 78, *82, 83, 84, 85, 86, 91*]
Fructose	[*59, 87, 88, 89, 90*]
Sucrose	[*66, 83, 91, 92a, b*]
Lactulose	[*42, 93*]
Lactose	[*94*]
Lactate	[35, *59*, 74, *85, 86, 95, 96, 97*]
Malate	[*27, 45, 47, 59, 95, 98*]
Citrate	[16, *39, 43, 48, 64, 65*]
Glutamate	[35, *84, 99, 100*]
Ascorbic acid	[19, 43, *101, 102*]
Glycerol	[*26*, *40*, 43, 71, *103*]
Ethanol	[*59*, *75*, *104, 105, 106, 107*]
Lysine	[*108*, 109]
Freshness indices	[*110*] (inosine), [*111, 112*] (amines), [*46*] (octapine)
Cholestrerol	[*113*]
Essential fatty acids	[*114*]
Lecithin	[*115*]
Sulfite	[*59, 116*]
Reducing compounds	[*117*]
Choline	[*118*]
Nicotinic acid	[*1*]
Pyruvic acid	[14, 41, 44]
Hydrogen peroxide	[19, 28, 32, 52]
Polyphenols	[*119, 120*]
Carbamates	[*121*]

New legislation and regulations may open new markets for biosensors in food industry, but biosensors will still have to compete with other methods such as gas chromatography, and HPLC with certain good analytical characteristics such as reliability and their ability for simultaneous analysis of several compounds. The major advantages of biosensors over these techniques are their ability to operate in natural samples with little or no pretreatment as well as their ability for on-line performance, in order to facilitate the quality control of products during processing, which is of great importance in the food industry [80].

7. Examples of Enzyme Electrodes for Food Analysis

In this section some selected examples of the literature are briefly presented. Selection was made giving a priority to these articles dealing with applications in food analysis and published after 1995; however some interesting articles may not be included because of space constrains. Similar presentations can be found in various reviews [62, 80] and books [22, 81] dedicated to biosensor food analysis. Table 4 summarizes the analytes considered along with the corresponding references. Citations referring to food analysis are in italics.

7.1. Glucose

The catalytic action of glucose oxidase is described by the following equation

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\textit{Glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \quad (10)$$

Mutlu and co-workers [82] developed a single-layer glucose enzyme electrode with extended linearity up to 340 mM glucose with a response time 500 seconds at pH 4, which operates at +650 mV (vs. Ag/AgCl). The method was applied to orange juice, several beverages and tonic with good agreement to a conventional spectrophotometric method.

Glucose and sucrose were simultaneously determined by the use of an enzyme sensor system consisting of a glucose-sensing electrode based on a lipid-modified glucose oxidase and a measuring cell which contains an invertase/mutarotase-coimmobilized layer. After a period of 2–6 s and 8–20 s from the injection of the analytes, steady state currents were recorded and linear calibration plots over the concentration ranges of 0.2 μM–3 mM glucose and of 10 μM–6 mM sucrose, were, respectively, constructed. The method was applied to jams and juices for the rapid determination of glucose and sucrose and correlated well with a reference spectrophotometric method [83].

An amperometric FIA with an enzyme electrode bearing glucose oxidase behind a polyanion layer consisting of poly-L-lysine and poly(4-styrenesulfonate) was effectively used for the determination of glucose in beverages. Polyanion membrane was useful for preventing electrochemical interferences from reaching the electrode surface. A dynamic linear range from 10 μM to 3 mM glucose with a sampling rate of 180 h^{-1} and a storage stability of two months were reported [84].

An interference free amperometric biosensor integrated with a microdialysis sampling for the flow determination of glucose in untreated tomato juice samples was reported by Palmisano et al. [85]. Each electrode was modified by a composite bilayer consisting of an electrosynthesized over-oxidized polypyrrole anti-interference membrane covered by an enzyme entrapping gel, obtained by glutaraldehyde co-crosslinking of glucose oxidase with bovine serum albumin.

Recently a FIA system for the simultaneously amperometric determination of glucose and lactate was described by Marzouk et al. [86]. Oxidases were co-immobilized into two-parallel enzyme reactors. The produced H_2O_2 was amperometrically monitored by a Pt electrode coated with an electropolymeric-permselective layer of *m*-phenylenediamine. The method correlates well with a reference photometric method when tested in dairy products.

Towards the development of miniaturized sensors a lot of work has been made by Wang and co-workers [38, 55, 56, 75]. An enzyme nanosensor, based on a carbon fiber cone microelectrode modified by co-deposition of Prussian blue and glucose oxidase offers great promise for measurements of glucose in extremely small volumes [38]. The development of a sol-gel derived enzyme-containing carbon inks display compatibility with the screen-printing process [55]. The operation of a new oxidase/dehydrogenase reaction/separation microchip was developed for the simultaneous measurement of glucose and ethanol in connection with the corresponding glucose oxidase and ethanol dehydrogenase reactions. Enzymatically produced H_2O_2 and NADH are separated on the basis of their different charges and detected amperometrically at an end-column thick-film detector. The method was applied to wine samples without any sample preparation, with the exception of a 100-fold dilution [75].

Vadgama and co-workers developed a glucose oxidase electrode covered by a cellulose acetate membrane modified with Tween-80, which ensures extended linearity over the environmental concentration range of glucose in fresh fruits and also provides protection for the immobilized glucose oxidase from low pH deactivation in low-pH samples (pH < 3) such as citrus fruit [11].

Appleton et al. [78] presented a novel system for the immobilization of glucose oxidase onto alkylamine-CPG beads through a glutaraldehyde activation step using an enzyme-polyelectrolyte complex in order to increase the thermal and operational stability of the biocatalyst. The immobilized enzyme displayed high operational stability when tested at elevated temperatures up to 100 °C for a 15-min time interval.

7.2. Fructose

Fructose is widely distributed in many fruits and vegetables, has greater sweetness than glucose or sucrose and is frequently used in diabetic sweeteners [63]. Enzyme electrodes for fructose determination are based on D-fructose-5-dehydrogenase (FDH) in the presence of a mediator. The enzyme requires no additional cofactor as it contains PQQ and heme c as redox active sites.

$$\text{D-Fructose} + \text{mediator}_{(ox)} \xrightarrow{FDH} \text{5-keto-D-fructose} + \text{mediator}_{(red)} \quad (11)$$

An amperometric fructose biosensor, based on FDH and the coenzyme ubiquinone-6 immobilized in a membrane mimetic layer on gold, was proposed by Kinnear and Monbouquette [87]. The sensor exhibits a response time of less than 20 s, a sensitivity of 15 $\mu A/cm^2$ mM and a detection limit of less than 10 μM. The membrane mimetic layer blocked effectively access of ascorbates to electrode surface; a 4% positive error was recorded in its presence. Biosensor measurements of fructose in apple and orange juices agreed to within a few percent with those made with an enzymic spectrophotometric assay.

An unmediated enzyme carbon paste electrode bearing FDH and addition of polyethyleneimine was proposed by Parellada et al. [88]. An operation potential of +400 mV was applied and a linear calibration plot up to 10 mM fructose was constructed. The operational stability of the sensor was 10 h. Using a flow injection manifold the sensor was applied for the quantitative determination of fructose in honey samples. Results were validated by liquid chromatography-refractive index and a good agreement was achieved.

Garcia et al. [89] utilized a carbon paste electrode modified with silica gel coated with FDH and Meldola's blue for the determination of fructose in sweets and fruit jellies. A recovery higher than 96% was obtained. The working plot was linear over the concentration range 0.1 to 0.8 mM, with a correlation coefficient of $r = 0.999$ and an RSD of 0.68% for $n = 7$. A storage stability of 2 months and an operational stability of 300 successive runs were observed.

Bassi et al. [90] immobilized FDH behind a thin non-conducting electropolymerized film of 1,3-plenylene-diamine-resorcinol for the construction of a fructose biosensor. Ferricyanide and tertacyanoquinodimethane were tested as redox mediators for the amperometric measurements of fructose. Using the latter the proposed sensor was applied for the determination of fructose in diluted (1:1000) honey samples. The method correlates well with a chemical assay.

7.3. Sucrose

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

$$\text{Sucrose} + H_2O \xrightarrow{Invertase} \text{fructose} + \alpha\text{-D-glucose} \quad (12)$$

Ivertase in combination with glucose oxidase may thus be used to produce a sucrose enzyme electrode, however, a third enzyme, mutarotase, is usually utilized in order to convert α-D-glucose to its β-isomer on which glucose oxidase is specific.

A multi-enzyme electrode obtained by a two-step immobilization of the enzymes glucose oxidase, mutarotase and invertase was developed for the determination of sucrose. Glucose oxidase was entrapped in a poly-1,3-diaminobenzene film on a platinum electrode by electrochemical polymerization and a combination of mutarotase and invertase was cross-linked over the electrode via bovine serum albumin and glutaraldehyde. A second electrode, for glucose only, was constructed containing inactive invertase, thus being used for signal subtraction. Application to a number of different soft drinks gave a good agreement with a standard liquid chromatography (LC) method [91].

Maestre et al. [92a] based on an enzymatic cascade involving sucrose phosphorylase, phosphoglucomutase and glucose-6-phosphate 1-dehydrogenase that have been proposed by Kogure et al. [92b] for the spectrophotometric determination of sucrose, developed an amperometric electrode for its assay without interference from glucose or fructose. From the last enzymatic reaction NAD(P)H is produced and recycled into $NAD(P)^+$ through its electrocatalytic oxidation by $Os(4,4'\text{-dimethyl-2,2-bypyridine})_2$ (1,10-phenanthroline-5,6-dione) at 0.15 V (vs. Ag/AgCl). The proposed electrode was applied to the flow analysis of sucrose in fruit juices and showed good agreement with the reference method.

7.4. Lactulose

Lactulose, the epimerized lactose (see Sec. 7.5), is a synthetic disaccharide consisting of galactose and fructose and is absent in raw milk. It is formed in alkaline lactose solutions or by heating of milk due the epimerization of lactose. Therefore, it can be used as an indicator for the severity of heat treatment of milk and to distinguish between pasteurized, ultra-heat treated and sterilized milk. Lactulose is enzymatically hydrolyzed to D-galactose and fructose according to the following reaction scheme

$$\text{Lactulose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{D-galactose} + \text{fructose} \quad (13)$$

β-galactosidase in combination with fructose dehydrogenase may thus be used to produce a lactulose enzyme electrode.

An enzymatic procedure for the determination of lactulose was developed by Bilitewski and co-workers [93]. Lactulose was hydrolysed to fructose and galactose by using soluble β-galactosidase, and the amount of fructose was determined by using immobilized FDH and $K_3[Fe(CN)_6]$ as mediator. The reduced mediator was re-oxidized at a screen-printed Pt-electrode at +385 mV (vs. a screen-

printed Pt-pseudo reference electrode). An automated flow method was applied to milk samples utilizing a dialysis unit for separating the analyte from the sample matrix. Data correlates well to the official spectrophotometric method, except in the case of condensed milk where a deviation of up to 20% was observed. The effect of interferences was manipulated by subtracting the signal taken after the addition of β-galactosidase with that recorded before its addition.

Based on the same enzyme cascade Moscone et al. [42] proposed a flow method for the determination of lactulose in milk by combining a FDH enzyme electrode and a D-galactosidase enzyme reactor. Ferricyanide was used as a mediator. A dialysis unit was also used for sampling and interference problem was circumvented by the use of a flow path by-passing the β-galactosidace reactor (blank subtraction). Recovery studies in milk samples gave values between 95 and 105%.

7.5. Lactose

Lactose is the main disaccharide present in milk and dairy products at concentrations of 4 – 6%. It is hydrolyzed by the enzyme β-galactosidase to galactose and glucose:

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{D-galactose} + \text{glucose} \qquad (14)$$

β-galactosidase in combination with glucose oxidase or galactose oxidase may thus be used to produce a lactose enzyme electrode. Two types of amperometric biosensors for lactose detection based either on co-immobilization of two enzymes (galactose oxidase with peroxidase) or co-immobilization of three enzymes (β-galactosidase, galactose oxidase and peroxidase) were constructed by Tkác and co-workers [94]. A graphite rod with pre-adsorbed ferrocene was used as a working electrode. The use of galactose oxidase instead of the frequently used glucose oxidase resulted in the construction of a glucose-non-interfering lactose sensor. The presence of β-galactosidase greatly enhances the sensor's sensitivity, but its linear range is narrower than that of the sensor without β-galactosidase. Addition of DEAE-dextran and inositol to the enzyme layer improved the half-life more than 16-fold compared with the sensor without stabilizers. Analyses of real samples showed good correlation with HPLC analysis.

7.6. Lactic Acid

Lactic acid is an extensively studied organic acid due to its importance in many fields of food industry such as the cheese manufacturing as the main product of lactose fermentation or in wine industry during the malo-lactic fermentation. Generally during microbial cultivations, lactate can function as a carbon source, as a fermentation product, or as an end product of anaerobic carbohydrate metabolism.

A multisensor composed of two enzyme electrodes allowing a simultaneous determination of malate and lactate was constructed by Katrlik et al. [95] utilizing transducers based on a solid binding matrix where NAD^+ have been incorporated. Lactate dehydrogenase (or malate dehydrogenase) and diaphorase, were placed onto the transducer surface and covered by a dialysis membrane, which substantially reduced interferences derived from easily oxidable compounds, e.g., polyphenols, of wine. Hexacyanoferrate(III) was used as a mediator. The biosensors showed an excellent long-term stability, after five months storage at room temperature and were applied for the simultaneously determination of malate and lactate in diluted (up to 1:200) wine samples. The results obtained were in a good agreement with those obtained by liquid chromatography.

Esti et al. [96] developed an amperometric method for the determination of lactate during the production of mozzarella cheese. A platinum electrode covered with immobilized lactate oxidase was mounted onto a wall-jet flow-cell. Real time analysis of lactate in raw milk and during the manufacturing of cow and mozzarella cheese allowed a control of the curd-ripening evolution at different pasteurization temperatures of the milk. The method proved to be more sensitive than the pH measurement procedures.

Bioprocess applications of screen-printed lactate sensors fabricated with UV-polymerizable enzyme pastes have been applied by Bilitewski and co-workers [97]. The sensor is based on immobilization of lactate oxidase by printing and subsequent UV irradiation within a polymerizable paste containing different supplements. The sensor was applied in a flow injection system based on dialysis to off-line and on-line bioprocess monitoring of *Geotrichum candidum* cultivations in complex media. The correlation factor between amperometric results and a homogenous photometric assay using lactate dehydrogenase was $r = 0.985$.

7.7. Malic Acid

Malic acid is predominant acid in many fruits and vegetables and the second highest in citrus fruits [62]. Except fruits industry, malic acid determination is of great value in the wine industry. Its determination by using biosensors has been proposed utilizing various enzymic pathways involving malate dehydrogenase and diaphorase [27, 47], malic enzyme in combination with pyruvic oxidase [77] and malic enzyme in combination with salicylate hydroxylase [45].

Prodromidis et al. [27] immobilized malate dehydrogenase and diaphorase in isothiocynate-CPG and have proposed the flow determination of malate in fruits and vegetables by monitoring the oxidation of NADH at +0.3 V (vs. Ag/AgCl) in the presence of ferricyanide as mediator. A relative error of 4% and 2% was, respectively, calculated, compared with a reference photometric method. Enzyme reactors have shown an extended operational and storage stability reducing thus the cost per analysis.

Using the same enzymic pathway in addition to ascorbate oxidase in order to eliminate ascorbates interference, a

reagentless enzyme electrode for malate was proposed by Maines et al. [47] based on an unplasticized spin coated PVC/polycarbonate resin. The proposed electrode is able to determine malate in undiluted neutral or acidic media as it responds linear up to 20 (pH 3.3) or 40 (pH 7.8) mM malate.

The co-immobilization of a $NAD(P)^+$-dependent malic enzyme with salicylate hydroxylase in front of a Clark-electrode was proposed by Sheller and co-workers [45] as a malate sensor. It ensures an effective re-oxidation of NADPH due to the presense of salicylate hydroxylase, yielded a linear range from 0.01 to 1.2 mM malate and strongly reduced $NADP^+$-requirement (< 0.025 mM), while the working stability was increased to more than 30 days. The results obtained from wine, fruit and juice samples showed a close correlation with the standard enzymatic method.

Palleschi and co-workers proposed an amperometric malate enzyme electrode using a hydrogen peroxide-based sensor coupled with malic and pyruvate oxidase enzymes [98]. The first enzyme catalyzes the oxidation of malic acid, which in the presence of $NADP^+$ yields pyruvate as product. The oxidation of pyruvate is catalyzed by pyruvate oxidase, which yields H_2O_2 as product in the presence of phosphate, thiamine pyrophosphate and Mg^{2+} as co-factors. Recovery studies of malate in a wine matrix have been carried out. Malic acid has been determined in grape musts during grape maturation. Results correlated well when compared with those from a spectrophotometric procedure.

7.8. Citric Acid

Citric acid is present in numerous natural products. Several fresh fruits such as lemons and limes owe their sharp taste to the presence of the citrate anion. Citric acid is also an additive in the industry, mainly as a preservative and an acidulant. Due to the instability of the citrate lyase (CL) a few papers have been published with immobilized CL. Proposed methods are based on a sequence of the enzymes, CL, oxaloacetate decarboxylase (OACD) and pyruvate oxidase (POD) in the presense of its co-factor, thiamine pyrophosphate (TPP), according to the following scheme:

$$\text{Citrate} \xrightarrow{CL} \text{Oxaloacetate} + CH_3COOH \quad (15)$$

$$\text{Oxaloacetate} \xrightarrow{OACD} \text{Pyruvate} + CO_2 \quad (16)$$

$$\text{Pyruvate} + HPO_4^{2-} + O_2 \xrightarrow[TPP,\ Mg^{2+}]{POD} \text{acetyl-phosphate} + CO_2 + H_2O_2 \quad (17)$$

Prodromidis at al. [39] proposed an enzymic method for the determination of citric acid in fruits, juices and sport drinks. The method is based on the action of the enzymes citrate lyase in soluble form and oxaloacetate decarboxylase and pyruvate oxidase in immobilized form. A multi-membrane system, consisting of a cellulose acetate membrane for the elimination of interferants, an enzymic membrane and a protective polycarbonate membrane were placed on a Pt electrode and used with a fully automated flow injection manifold. The mean relative error was 2.4% compared with a standard enzymatic method (Boehriger F-kit). An 8 – 10% loss of the initial activity of the sensor was observed after 100 – 120 injections.

Using the same enzyme cascade, a novel amperometric biosensor for the determination of citric acid in food samples and fermentation broths has been developed by Scheller and co-workers [64]. All the enzymes were immobilized in gelatin and a reactivation method involving incubation with ATP and acetic acid is suggested. Measurements using a non-enzymic membrane were a proposed route to the elimination of interferent species. Sensor response remained constant for 8 days and decreased to 25% after 18 days at 20 – 23 °C. The results obtained from citrate determinations in orange juice, tomatoes, jam, soy sauce samples and fermentation broths agree well with those determined by enzymatic sample analysis.

Karayannis and colleagues developed an enzyme electrode for extended linearity of citrate measurements based on modified polymeric membranes [48]. Co-immobilization of POD/OACD and CL as well as of the co-factors FAD and TPP onto a high protein binding membrane of mixed cellulose ester was proposed. pH independent rejection of ascorbate was achieved by the use of a novel cellulose acetate membrane incorporating isopropyl myristate. Extended linearity up to 20 or 100 mM citrate was achieved utilizing outer membranes of unplasticized PVC/polycarbonate resin or PVC/Pluronic F-68, respectively.

A flow injection analytical system for the determination of citrate in mandarin, grapefruit, *citrus iyo* and lemon was developed by Matsumoto et al. [65] using soluble CL and OACD and POD immobilized into a CPG reactor. Ascorbate oxidase was also immobilized into a CPG reactor in order to eliminate the ascorbate interference. The proposed method has a sampling rate 15 h^{-1} and a relative standard deviation of 1.2% for 10 successive runs. The accuracy was tested comparing the results with those obtained by a reference photometric assay and a good correlation was achieved.

7.9. Glutamic Acid

Glutamic acid is a major component in a variety of vegetables, meats, and sea products and is used to enhance flavor in foods prepared at home, in restaurants, and by the food processing industry. Several enzyme electrodes have been reported based on the enzymes glutamate oxidase and glutamate dehydrogenase.

An enzymatic method for the determination of glutamate in food products has been developed by Mottola and Janarthanan [99], by using rotating bioreactors. Two enzymatic approaches have been implemented. One of the methods uses glutamate dehydrogenase in the main enzymatic reaction and diaphorase in the indicator reaction, which involves NADH and hexacyanoferrate(III). The

second method utilizes a single enzyme, glutamate oxidase and amperometric monitoring of H_2O_2. Samples used to illustrate the approaches included: beef and chicken bouillon cubes, soy sauce, chicken broth, seasoning salt, fruit and vegetable juices, and skim milk. Interference by ascorbate present in some samples is eliminated by in-line use of a packed reactor containing ascorbate oxidase.

Kwong et al. reported the fabrication of thick-film biosensors for the determination of L-glutamate in foodstuffs [100]. The sensors were prepared by immobilization of glutamate oxidase by using polycarbamylsulfonate-hydrogel on a thick-film sensor. Glutamate oxidases obtained from Streptomyces sp. with different degree of purification were compared with their characteristic response to L-glutamate at different conditions and for their specificity, inhibition, and storage properties. These sensors were applied to determine monosodium glutamate in soy sauce samples and show good correlation with colorimetric method.

7.10. Ascorbic Acid (Vitamin C)

The determination of ascorbic acid is of great value in food industry since it is an important nutrition compound due to its redox properties it is widely used as a preservative. The majority of the proposed methods are based on voltammetric chemical sensors rather than biosensors, since various selective approaches, based on permselective membranes and specific compounds, have been successfully tested in natural samples.

Guilbault and co-workers [101] developed a sensor that selectively catalyzes the oxidation of ascorbic acid at low potentials (+100 mV). The low applied potential minimizes the effects of many common electrochemical interferents. The interferents tested are those commonly found in juices and pharmaceutical preparations, including 4-acetamidophenol (paracetamol), uric acid and citric acid. Polyaniline was electrochemically grown on both glassy carbon and screen-printed electrodes mounted in a flow cell. The proposed method was tested in various fresh and concentrated juices achieving a good correlation with the official titrimetric method.

Karayannis and co-workers [102] developed an ascorbate sensor based on a glassy carbon electrode modified with a cellulose acetate polymeric film-bearing 2,6-dichlorophenolindophenol. The modified electrodes were mounted in a flow injection (FI) manifold, poised at +100 mV (vs. Ag/AgCl) at pH 6.5 and utilized for the determination of ascorbic acid in beverages and juices. Good correlation with a reference method was attained. The recovery was 92–110%. The sensors showed very good repeatability and operational stability.

7.11. Glycerol

Glycerol is the most important secondary product of alcoholic fermentation contributing to the smoothness and viscosity of a wine with a favorable effect on the taste [63]. Biosensors based on glycerol dehydrogenase [26, 103] or on hexokinase/glycerol-3-phosphate oxidase enzyme combination [40] have been published.

A FIA manifold incorporating amperometric detection and enzyme reactor for glycerol determination in alcoholic beverages has been proposed by Prodromidis et al. [26]. The reactor is based on the glycerol dehydrogenase system, and the enzyme was immobilized through chemical modification onto aminopropyl and isothiocyanate CPGs, aminopolystyrene resin and *m*-aminobenzyloxymethyl cellulose. NADH, the product of the enzymatic reaction, was monitored at +0.5 V (vs. Ag/AgCl). Reactors are stable for a period over 3 months and after about 2500 injections. The successive application of the method was confirmed by comparison with a reference method. The mean relative error is 2.2% and the recovery 95–102%.

A biosensor for the measurement of glycerol in FIA was constructed by Compagnone et al. [40]. The most effective configuration for measurement in FIA was the immobilization of glycerokinase in a glass beads reactor coupled with glycerol-3-phosphate oxidase on a preactivated Immobilon AV membrane kept at the electrode surface. A recovery study to ascertain the effect of the matrix (wine) on the biosensor performance was carried out at different dilutions of the sample; recovery values between 90 and 103% were reported. The biosensor was also used to monitor off-line glycerol production during alcoholic fermentations carried out at different pHs and temperatures.

Tuñón-Blanco and co-workers [103] published a reangentless glycerol dehydrogenase (GlDH) based biosensor for the determination of glycerol in a plant-extract syrup, with results being in good agreement with those of a standard spectrophotometric method. CPEs modified with the oxidation products of NAD^+ show excellent electrocatalytic activity toward NADH oxidation at +50 mV (Ag/AgCl). GlDH and its cofactor NAD^+ were co-immobilized in a carbon paste electrode using an electropolymerized layer of nonconducting poly(*o*-phenylenediamine). After partial oxidation of the immobilized NAD^+, the modified electrode allows the amperometric detection of the NADH enzymatically obtained at applied potential above 0 V (Ag/AgCl) thus eliminating the interference effect by various electroactive species present in tested samples.

7.12. Ethanol

The determination and control of ethanol is important in brewing, winemaking and distilling industries. Tax regulation requires also exact determination of ethanol content, especially in spirits. A plethora of ethanol biosensors have been proposed in the literature based on alcohol oxidase or alcohol dehydrogenase.

Amplified biosensing of ethanol based on biocatalytic accumulation of the Meldola blue mediator was published by Wang and Naser [104]. By performing the biochemical/chemical step under open-circuit conditions, accumulation of the reduced form of the mediator was achieved, prior to the chronoamperometric quantitation of the enriched layer. Such temporal separation of the recognition and transduction events offered a 35-fold sensitivity enhancement, following a 5-min accumulation. The method was applied to various wine samples diluted in a $1:10^3$ or $1:4\times10^3$ ratio and good agreement with the labeled values was achieved.

Garcia Mullor et al. [105] developed a yeast alcohol dehydrogenase amperometric carbon paste-based biosensor, with Meldola Blue as a mediator and a dialysis membrane with a very small molecular weight cut-off for protection. The influence of membrane pore size on the stability and the overall kinetics of the biosensor is established by using cyclic voltammetry and stationary potential measurements. Application of this device to the determination of ethanol in diluted ($1:5\times10^3$) whisky, gin, anis and ($1:2\times10^3$) wine samples was achieved successfully. Recovery was ranging between 95–108%. During the analysis of samples and at this working potential (+50 mV vs. Ag/AgCl) no interferences were observed.

Karube and co-workers [106] developed a reagentless alcohol dehydrogenase-based sensor using a gas-permeable membrane, which was successfully applied to the determination of ethanol in diluted whisky and sake. Results obtained with the sensor and by gas chromatography agreed within 3.4%. The sensor consists of an anode mounted with immobilized alcohol dehydrogenase, a cathode and an electrolyte containing hexacyanoferrate(III). Its surface was covered with a gas-permeable membrane. Measurements were made without consumption of reagent by pumping a sample solution through the flow cell for 1 or 2 min. Penetration of interfering compounds was prevented by the gas-permeable membrane.

Hart and co-workers [107] developed a disposable amperometric biosensor for the measurement of ethanol. It was comprised of a screen-printed carbon electrode doped with 5% cobalt phthalocyanine, and coated with alcohol oxidase; a permselective membrane on the surface acts as a barrier to interferents. The measurement of ethanol is based on the signal produced by H_2O_2, the product of the enzymatic reaction. Amperometric measurements in stirred solution and chronoamperometric measurements for in-field applications were made in beer samples. The precision and recovery data indicated that both configurations of the biosensor should give reliable results.

7.13. Lysine

Lysine is easily damaged during heat treatment and storage conditions of food, therefore it is used in the assessment of food processing techniques as well as an index of the nutritional quality of foods.

Guilbault and co-workers [108] developed an amperometric biosensor based on lysine oxidase for the determination of lysine in food. A ruthenium/rhodium coated glassy carbon electrode covered with 1,2-diaminobenzene polymer was used. Nil response to ascorbates was observed. Interference by other amino acids like ornithine, arginine and phenylalanine were reduced to 3.4, 1.1 and 0.7% of the response to lysine (taken as 100%). No other amino acids interfere. The sensor was successfully applied to the determination of lysine (protein content) in milk and pasta samples, following rapid microwave digestion of the product.

The construction of a lysine biosensor on a Si-gold strip electrode was developed by Papastathopoulos and co-workers [109]. The construction comprised of (a) the formation of poly(*o*-phenylenediamine), membrane onto the electrode surface via electropolymerization and (b) the immobilization of lysine oxidase over the membrane-modified electrode with glutaraldehyde. The biosensor responded mainly against tyrosine and cysteine, while the response to phenylalanine, arginine, histidine and ornithine was very low, thus indicating its suitability for application in food analysis.

7.14. Freshness Indices

Biosensors have been also widely used as analytical devices for the evaluation of fish freshness, shellfish freshness, meat aging, and quality control of fruits during storage. Some of these biosensors have already been released into the market (see Table 3).

7.14.1. Inosine

Fish quality can be related to the naturally occurring chemical sequence of ATP decomposition:

$$\mathrm{ATP}\rightarrow\mathrm{ADP}\rightarrow\mathrm{AMP}\rightarrow\mathrm{IMP}\rightarrow\mathrm{HxR}\rightarrow\mathrm{Hx}\rightarrow\mathrm{X}\rightarrow\mathrm{U} \quad (18)$$

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 thereby corresponding biosensors provide a route to freshness assessment.

Carsol and Mascini [110] developed a FIA system incorporating a 3-electrode screen-printed element for the determination of fish freshness with double enzyme reactors for the determination of the freshness indicator $K = 100(\mathrm{HxR}+\mathrm{Hx})/(\mathrm{IMP}+\mathrm{HxR}+\mathrm{Hx})$. The determination of the total amount of HxR and Hx is achieved by passing the sample through two reactors in series: one reactor was packed with nucleoside phosphorylase and the other with xanthine oxidase immobilized on CPG. Similarly, the other term of the equation was evaluated by flowing the sample through the two reactors and treating by alkaline phosphatase for 5–10 min at 45 °C. One assay could be completed

within 5 min. More than 200 – 300 samples could be analyzed in about one month by using these enzyme reactors provided that the disposable screen-printed electrode are replaced after 30 – 40 determinations. *K* values were compared with those obtained by the XO-reactor. A correlation factor of 0.992 was achieved. Authors are finally suggesting the FIA system with xanthine oxidase and nucleoside phosphorylase reactors as simpler and more easily handled.

7.14.2. Biogenic Amines

Biogenic amines, mainly putrescine, cadaverine, spermidine, histamine and tyramine, are not only biosynthesized in animal and plant cells but also produced by microbial decarboxylation of amino acids. The amount and type of amine formed is strongly dependent on the food composition, microbial flora and other parameters, which allow bacterial growth during food storage, food additives, temperature, moisture, ripening and packaging.

Chemnitius and Bilitewski [111] developed screen-printed electrodes for the determination of biogenic amines using monoamine oxidase and putrescine oxidase. The electrodes using monoamine oxidase as the biochemical component, respond to several amines including histamine, an important amine for the determination of fish freshness. The putrescine oxidase electrodes show a significant response not only to putrescine and its homologue cadaverine but also to tyramine, an electrochemically active amine. The sensors were used to monitor the freshness of mackerel and codfish in storage. Sensor signals increase with storage time of the fish, indicating the production of biogenic amines.

Esti et al. [112] developed a method for the determination of amines in fresh or modified atmosphere packaged fruits using electrochemical biosensors based on immobilized diamine oxidase and polyamine oxidase onto polymeric membranes. The method was applied for the investigation of postharvest life of apricots and sweet cherries in modified atmosphere storage at 0 °C, by measuring the concentration of the amines at harvest and after a period of 20 days under the specified storage conditions.

7.14.3. Octapine

Formation of octopine from arginine and pyruvic acid is one of the major biochemical processes occurring in scallop muscle postmortem. The concentration of octopine in scallop muscle held on ice for 4 – 5 days is about 1%.

An octopine sensor was developed by Shin et al. [46], which was based on the immobilized enzymes pyruvate oxidase and octopine dehydrogenase (ODH):

$$\text{Octapine} \xrightarrow{ODH} \text{Pyruvate} \quad (19)$$

$$\text{Pyruvate} + HPO_4^{2-} + O_2 \xrightarrow[TPP,\ Mg^{2+}]{POD} \text{acetyl-phosphate} + CO_2 + H_2O_2 \quad (17)$$

The sensor consisted of a reactor, an oxygen electrode, a flow cell, a peristaltic pump, a recorder, and a buffer tank. A good correlation was obtained between the octopine contents in scallop adductor muscle determined with the sensor and those determined with high performance liquid chromatography.

7.15. Miscellaneous Biosensors

7.15.1. Cholesterol

Pena et al. [113] reported a bienzyme amperometric composite biosensor for the determination of free and total cholesterol in butter, lard and egg yolk. Cholesterol oxidase and horseradish peroxidase, together with ferrocyanide as a mediator, are incorporated into a graphite-70% Teflon matrix. The compatibility of the sensor with predominantly nonaqueous media allows the use of reversed micelles (formed with ethyl acetate and AOT as emulsifying agent) as working medium. The validity of the sensor was testified by comparing the results obtained with those taken by using a commercial Boehringer F- kit.

7.15.2. Essential Fatty Acids

Schoemaker et al. [114] used both an enzyme electrode (in a batch system) and an enzyme column (in a FIA manifold) with immobilized lipoxygenase for the determination of essential fatty acids, e.g., linoleic and linolenic acids. The oxygen depletion due to the action of the enzyme was monitored amperometrically. The enzyme electrode showed different sensitivities for each acid due to the different dialytic behavior of the substrates. Only the FIA system was finally used for the determination of essential fatty acids in real matrices such as vegetable oils and margarines. The results correlate well with the standard gas chromatographic method. In the presence of detergent the triglycerides of the hydrophobic food samples were converted into water-soluble glycerol and free fatty acids after a 15 min incubation with a ready to use lipase/esterase-mix, avoiding thus the use of organic solvents.

7.15.3. Lecithin

Campanella and co-workers [115] developed an organic phase bienzyme electrode for the determination of lecithin in food products such as egg yolk, soya flour, oil as well as in some diet integrators, operating in various mixtures of organic solvents such as chloroform, hexane and methanol. The organic phase electrode was constructed by using an oxygen electrode as electrochemical transducer. Enzymes phospholipase D and choline oxidase were both immobilized either on a dialysis membrane or in a kappa-carrageenan gel.

7.15.4. Sulfite

Gooding and co-workers [116] developed a sulfite sensor based on the immobilization of sulfite oxidase in electro-

deposited polytyramine over a glassy carbon electrode for the determination of sulfite in various types of wines. The sensor exhibits a linear response over the concentration range 0.002 – 0.3 mM sulfite. Despite its reasonable good correlation between the proposed method and the AOAC method ($r = 0.967$), the biosensor showed an apparent trend to give analytical results above the AOAC method for red wines (recovery 114 – 123%) and below the AOAC method for white wines (recovery 81 – 96%).

7.15.5. Reducing Compounds

Wabner and co-workers [117] reported an automated voltammetric determination of reducing compounds in beer. Different types of reductones have a considerable influence on the stability of beer. Authors used the redox compound 2,6-dichlorophenol-indophenol to determine indirectly the reducing power of beer thus providing a potential application for quality control. Its reduced form is electrochemically reoxidized, followed by internal calibration with a standard of ascorbic acid.

7.15.6. Choline

Panfili and co-workers [118] reported a rapid assay of choline in foods using microwave hydrolysis and a choline biosensor. The proposed method was set out against methods involving hydrolysis at 70 °C for a period of 180 min or using a bienzyme system of phospholipase and choline oxidase. The proposed biosensor is based on a Clark-type oxygen electrode, which monitors the depletion of the oxygen during the action of the enzyme to the substrate. Comparative studies between standard (heating-enzymic-colorimetric assay) and the proposed methods in milk (pasteurized, powder, enriched for children), soy lecithin, pasta, pasta with eggs, dietetic meal, yoke, egg powder gave a correlation coefficient $r = 0.998$.

7.15.7. Polyphenols

Capannesi et al. [119] compared different techniques, in evaluating the phenolic content of an extra-virgin olive oil with varying storage time and storage conditions. A disposable screen-printed sensor was coupled with differential pulse voltammetry to determine the phenolic fractions after extraction with a glycine buffer. Moreover a tyrosinase-based biosensor operating in hexane was also assembled, using an amperometric oxygen probe as transducer. A good agreement with a classical spectrophotometric assay using Folin-Ciocalteau reagent and HPLC analysis was achieved.

Campanella and co-workers [120] evaluated the progressive rancidification of olive oil by simultaneously using two different indicators: the peroxide number, and an innovative one consisting of the progressive decrease in the content of polyphenols, the main natural antioxidants contained in the oil, as determined rapidly by means of a new organic phase enzyme electrode based on tyrosinase. A comparison test regarding the phenol content in extra virgin, virgin and a commercial olive oil samples was made. A good correlation between the data obtained with the reference spectrophotometric method and the tyrosinase enzyme sensor was achieved.

7.15.8. Carbamates

An amperometric biosensor for the determination of carbamate pesticides directly in water, fruit and vegetable samples was proposed by Nunes et al. [121], utilizing a screen-printed biosensor strip modified by a layer of carbon paste mixed with cobalt(II) phthalocyanine and acetylcellulose. Cholinesterase was immobilized on this layer. The biosensor was applied and for the direct determination of some *N*-methylcarbamates in fruit and vegetable samples at ppb concentration levels without any sample pretreatment. A comparison of the obtained results for the total carbamate concentration was done against those obtained using HPLC measurements.

8. References

[1] T. Hamano, Y. Mitsuhashi, N. Kojima, N. Aoki, M. Semma, Y. Ito. Y. Oji, *Analyst* **1995**, *120*, 135.
[2] F. Scheller, F.Schubert, *Biosensors*, Elsevier, Amsterdam **1992.**
[3] A. P. F. Turner, I. Karube, G. S. Wilson., *Biosensors, Fundamentals and Applications*, Oxford University Press, Oxford **1987.**
[4] I. Katakis, E. Dominquez, *Mikrochim. Acta* **1997**, *126*, 11.
[5] L. Gorton, *Electroanalysis* **1995**, *7*, 23.
[6] A. P. F. Turner, in *Analytical Uses of Immobilized Biological Compounds for Detection, Medical and Industrial Uses*, Vol. 226 (Eds.: G. G. Guilbault, M. Mascini), NATO ASI Series, D. Reidel, Dordrecht **1988**, pp. 131 – 141.
[7] A. B. Florou, M. I. Prodromidis, M. I. Karayannis, S. M. Tzouwara-Karayanni, *Electroanalysis* **2000**, *12*, 361.
[8] J. Wang, *Anal. Chim. Acta* **1990**, *234*, 41.
[9] A. Maines, D. Ashworth, P.Vadgama, *Anal. Chim. Acta* **1996**, *333*, 223.
[10] S. Reddy, P.Vadgama, *Anal. Chim. Acta* **1997**, *350*, 67.
[11] A. Maines, A. Cambiaso, L. Delfino, G. Verreschi, I. Christie, P. Vadgama, *Anal. Commun.* **1996**, *33*, 27.
[12] J. J. Kulys, *Biosens. Bioelectron.* **1986**, *2*, 3.
[13] J. P. Lowry, R. D. O'Neill, *J. Electroanal. Chem.* **1991**, *300*, 175.
[14] N. Gajovic, G. Binyamin, A. Warsinke, F. Scheller, A. Heller, *Anal. Chem.* **2000**, *72*, 2963.
[15] A. Heller, *Accounts Chem. Res.* **1990**, *23*, 128.
[16] S. Hikima, S. Hasebe, M. Taga., *Electroanalysis* **1992**, *4*, 801.
[17] R. N. Adams, *Electrochemistry at Solid Electrodes*, Marcel Dekker, New York **1969.**
[18] R. C. Murray, in *Electroanalytical Chemistry*, Vol. 13 (Ed.: A. J. Bard), Marcel Dekker Inc., New York **1984**, p. 191.
[19] J. Wang, N. Naser, L. Angnes, H. Wu, L. Chen. *Anal. Chem.* **1992**, *64*, 1285.
[20] L. Angnes, E. M. Richter, M. A. Augelli, G. H. Kume, *Anal. Chem.* **2000**, *72*, 5503.
[21] P. Yang, T. Deng, D. Zhao, P. Feng, D. Pine, B. F. Chmelka, G. M. Whitesides, G. D. Stucky, *Science* **1998**, *282*, 2244.

[22] *Food Biosensors Analysis* (Eds.: G. Wagner, G. G. Guilbault), Marcel Dekker, New York **1994.**
[23] *Biosensors. A practical Approach* (Ed.: A. E. G. Cass), Oxford University Press, Oxford **1990.**
[24] *Methods in Enzymology* (Ed.: K. Mosbach), Academic Press, New York **1976**, Vol. XLIV; a) H. H. Weetall, in this volume ch. 10; b) J. Porath, R. Axen, in this volume, ch. 3.
[25] *Methods in Enzymology* (Ed.: K. Mosbach), Academic Press, New York **1988**, Vol. 137.
[26] M. I. Prodromidis, C. D. Stalikas, S. M. Tzouwara-Karayanni, M. I. Karayannis, *Talanta* **1996**, *43*, 27.
[27] M. I. Prodromidis, S. M. Tzouwara-Karayanni, M. I. Karayannis, P. Vadgama, A. Maines, *Analyst* **1996**, *121*, 435.
[28] B. Liu, F. Yan, J. Kong, J. Deng, *Anal. Chim. Acta* **1999**, *386*, 31.
[29] B. Liu, R. Hu, J. Deng, *Anal. Chem*. **1997**, *69*, 2343.
[30] L. Coche-Guerente, V. Desprez, P. Labbe, S. Therias, *J. Electroanal. Chem*. **1999**, *470*, 61.
[31] J-M. Zen, C-W. Lo, P-J. Chen, *Anal. Chem*. **1997**, *69*, 1669.
[32] S. Cosnier, F. Lambert, M. Stoytcheva, *Electroanalysis* **2000**, *12*, 356.
[33] S. A. Erm, A. M. Yacynych, *Electroanalysis* **1995**, *7*, 913.
[34] G. Bontempelli, N. Comisso, R. Toniolo, G. Schiavon, *Electroanalysis* **1997**, *9*, 433.
[35] A. Curulli, I. Carelli, O. Trischitta, G. Palleschi, *Talanta* **1997**, *44*, 1659.
[36] Z. Sun, H. Tachikawa, *Anal. Chem*. **1992**, *64*, 1112.
[37] F. Palmisano, P. G. Zambonin, D. Centonze, *Fresenius J. Anal. Chem*. **2000**, *366*, 586.
[38] X. Zhang, J. Wang, B. Ogorevc, U. Spichiger, *Electroanalysis* **1999**, *11*, 945.
[39] M. I. Prodromidis, S. M. Tzouwara-Karayanni, M. I. Karayannis, P. Vadgama, *Analyst* **1997**, *122*, 1101.
[40] D. Compagnone, M. Esti, M. C. Messia, E. Peluso, G. Palleschi, *Biosens. Bioelectron.* **1998**, *13,* 875.
[41] M. Mascini, F. Mazzei, *Anal. Chim. Acta* **1987**, *192*, 9.
[42] D. Moscone, R. A. Bernardo, E. Marconi, A. Amine, G. Palleschi, *Analyst* **1999**, *124*, 325.
[43] V. Volotovsky, N. Kim, *Sens. Actuators* **1998**, *49*, 253.
[44] L.Kihara, E. Yasukawa, S. Hirose, *Anal. Chem*. **1984**, *56*, 1876.
[45] N. Gajovic, A. Warsinke F. W. Scheller, *J. Biotechnol*. **1998**, *61*, 129.
[46] S. J. Shin, H. Yamanaka, H. Endo, E. Watanabe, *Enz. Microb. Technol*. **1998**, *23*, 10.
[47] A. Maines, M. I. Prodromidis, S. M. Tzouwara-Karayanni, M. I. Karayannis, D. Ashworth, P. Vadgama, *Anal. Chim. Acta* **2000**, *408*, 217.
[48] A. Maines, M. I. Prodromidis, S. M. Tzouwara-Karayanni, M. I. Karayannis, D. Ashworth, P. Vadgama, *Electroanalysis* **2000**, *12*, 1118.
[49] C. J. Brinker, G. W.Scherer, *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*, Academic Press, San Diego **1990.**
[50] J. Wang, *Anal. Chim Acta* **1999**, *399*, 21.
[51] A. R. Howells, P. J. Zambrano, M. M. Collinson, *Anal. Chem*. **2000**, *72*, 5265.
[52] J. Li, S. N. Tan, *Anal. Lett*. **2000**, *33*, 1467.
[53] Q. Chen, G. L. Kenausis, A. Heller, *J. Am. Chem. Soc*. **1998**, *120*, 4582.
[54] B. Wang, J. Zhang, S. Dong, *Biosen. Bioelectron*. **2000**, *15*, 397.
[55] J. Wang, P. V. A. Pamidi, D. S. Park, *Anal. Chem*. **1996**, *68*, 2705.
[56] J. Wang, P. V. A. Pamidi, *Anal. Chem*. **1997**, *69*, 4490.
[57] F. Cespedes, S. Alegret, *Trends Anal. Chem*. **2000** *19*, 276.
[58] M. Stred'anský, A. Pizzariello, S. Stred'anská, S. Miertuš, *Anal. Chim. Acta* **2000**, *415*, 151.
[59] S. Miertuš, J. Katrlík, A. Pizzariello, M. Stred'anský, J. Švitel, J. Švorc, *Biosen. Bioelectron.* **1998**, *13*, 911.
[60] M. Albareda-Sirvent, A. Merkoci, S. Alegret, *Sens. Actuators* **2000**, *69*, 153.
[61] J. J. Gooding, D. B. Hibbert, *Trends Anal. Chem*. **1999**, *18*, 525.
[62] A. Maines, D. Ashworth, P. Vadgama, *Food. Technol. Biotechnol.* **1996**, *34*, 31.
[63] Y. Pomeranz, C. E. Meloan, *Food Analysis. Theory and Practice*, 3rd ed., Chapman and Hall, New York **1994.**
[64] N. Gajovic, A. Warsinke, F. W. Scheller, *J. Chem. Biotechnol*. **1995**, *63*, 337.
[65] K. Matsumoto, T. Tsukatani, Y. Okajima, *Electroanalysis* **1995**, *7*, 527.
[66] J. A. Hamid, G. J. Moody, J. D. R. Thomas, *Analyst* **1988**, *113*, 81.
[67] J. Brandley, A. P. F. Turner, R. D. Schimd, in *Biosensors: Applications in Medicine, Environmental Protection, and Process Controls* (Eds.: R. D. Schmid, F. Scheller), Vol. 13, GBF Monographs, VCH, New York **1989**, p. 85.
[68] V. G. Gavalas, N. A. Chaniotakis, T. D. Gibson, *Biosen. Bioelectron*. **1998**, *13*, 1205.
[69] J. Wang, N. Naser, *Electroanalysis* **1994**, *6*, 571.
[70] C. M. A. Brett, A. M. O. Brett, *Electrochemistry. Principles, Methods and Applications.* Oxford University Press, New York **1993.**
[71] M. I. Prodromidis, A. B. Tsibiris, M. I. Karayannis, *J. Autom. Methods Manage. Chem*. (former: *J. Autom. Chem.*) **1995**, *17*, 187.
[72] A. S. Economou, G. J. Volikakis, C. E. Efstathiou, *J. Autom. Methods Manage. Chem*., **1999**, *21*, 33.
[73] O. Frederik, J. R. Danaraj, B. Fleet, H. Gunasingham, S. Jaenicke, V. E. Hodgkinson, *Electroanalysis* **1999**, *11*, 1027.
[74] J. J. Gooding, E. A. H. Hall, *Anal. Chem*. **1998**, *70*, 3131.
[75] J. Wang, M. P. Chatrathi, B. Tian, *Anal. Chem*. **2001**, *73*, 1296.
[76] Frost & Sullivan, *Expansion of Biosensor Market Evident in Everyday Applications*, March 31, **1997.**
[77] T. D. Gibson, J. R. Woodward, in *Biosensors and Chemical Sensors* (Eds.: P. G. Eldeman, J. Wang), ACS, Washington, DC **1992**, p. 40.
[78] B. Appleton, T. D. Gibson, J. R. Woodward, *Sens. Actuators* **1997**, *43*, 65.
[79] *Commercial Biosensors. Applications to clinical, Bioprocess, and Environmental Samples* (Ed.: G. Ramsey), Wiley, New York **1998.**
[80] J. H. T. Luong, P. Bouvrette, K. B. Male, *Trends Biotechnol.* **1997**, *15*, 369.
[81] *Biosensors for Food Analysis* (Ed.: A.O. Scott), The Royal Society of Chemistry, Cambridge **1998.**
[82] B. Alp, S. Mutlu, M. Multu, *Food Research International* **2000**, *33*, 107.
[83] F. Mizutani, S. Yabuki, *Biosen. Bioelectron*. **1997**, *12*, 1013.
[84] F. Mizutani, Y. Sato, Y. Hirata, S. Yabuki, *Biosen. Bioelectron*. **1998**, *13*, 809.
[85] E. Palmisano, R. R. D. Centonze, P. G. Zambonin, *Biosen. Bioelectron*. **2000**, *15*, 531.
[86] S. A. M. Marzouk, H. E. M. Sayour, A. M. Ragab, W. E. Cascio, S. S. M. Hassan, *Electroanalysis* **2000**, *12*, 1304.
[87] K. T. Kinnear, H. G. Monbouquette, *Anal. Chem*. **1997**, *69*, 1771.
[88] J. Parellada, E. Dominquez, V. M. Fernandez, *Anal. Chim. Acta* **1996**, *330*, 71.
[89] C. A. B. Garcia, N. Graciliano de Oliveira, L. T. Kubota, L. A. Grandin, *J. Electroanal. Chem*. **1996**, *418*, 147.

[90] A. S. Bassi, E. Lee, J. X. Zhu, *Food Research International* **1998**, *31*, 119.
[91] W. Surareungchai, S. Worasing, P. Sritongkum, M. Tanticharoen, K. Kirtikara, *Anal. Chim. Acta* **1999**, *380*, 7.
[92] a) E. Maestre, I. Katakis, E. Dominquez, *Biosen. Bioelectron.* **2001**, *16*, 21; b) M. Kogure, H. Mori, H. Ariki, C. Kojima, H. Yamamoto, *Anal. Chim. Acta* **1997**, *337*, 107.
[93] M. Mayer, M. Genrich, W. Kunnecke, U. Bilitewski, *Anal. Chim. Acta* **1996**, *324*, 37.
[94] J. Tkác, E. Šturdík, P. Gemeiner, *Analyst* **2000**, *125*, 1285.
[95] J. Katrlík, A. Pizzariello, V. Mastihuba, J. Švorc, M. Stred'anský, S. Miertuš, *Anal. Chim. Acta* **1999**, *379*, 193.
[96] M. Esti, M. C. Messia, E. LaNotte, P. Lembo, D. Compagnone, G. Palleschi, *J. Agric. Food Chem.* **1996**, *44*, 3102.
[97] I. Rohm, M. Genrich, W. Collier, U. Bilitewski, *Analyst* **1996**, *121*, 877.
[98] M. C. Messia, D. Compagnone, M. Esti, G. Palleschi, *Anal. Chem.* **1996**, *68*, 360.
[99] C. Janarthanan, H. A. Mottola, *Anal. Chim. Acta* **1998**, *369*, 147.
[100] A. W. K. Kwong, B. Grundig, J. Hu, R. Renneberg, *Biotechnol. Lett.* **2000**, *22*, 267.
[101] P. J. O'Connell, C. Gormally, M. Pravda, G. G. Guilbault, *Anal.Chim. Acta* **2001**, *431*, 239.
[102] A. B. Florou, M. I. Prodromidis, M. I. Karayannis, S. M. Tzouwara-Karayanni, *Anal. Chim. Acta* **2000**, *409*, 113.
[103] M. I. Álvarez-González, S. B. Saidman, M. J. Lobo-Castañón, A. J. Miranda-Ordieres, P. Tuñón-Blanco, *Anal. Chem.* **2000**, *72*, 520.
[104] J. Wang, N. Naser, *Electroanalysis* **1995**, *7*, 362.
[105] S. Garcia Mullor, M. Sanchez-Cabezudo, A. J. Miranda Ordieres, B. Lopez Ruiz, *Talanta* **1996**, *43*, 779.
[106] M. Hikuma, M. Takeda, H. Matsuoka, I. Karube, *Anal. Chim. Acta* **1995**, *306*, 209.
[107] M. Boujtita, J. P. Hart, R. Pittson, *Biosen. Bioelectron.* **2000**, *15*, 257.
[108] S. C. Kelly, P. J. O'Connell, C. K. O'Sullivan, G. G. Guilbault, *Anal. Chim. Acta* **2000**, *412*, 111.
[109] I. D. Karalemas, C. A. Georgiou, D. M. Papastathopoulos, *Talanta* **2000**, *53*, 391.
[110] M.-A.Carsol, M. Mascini, *Talanta* **1998**, *47*, 335.
[111] G. C. Chemnitius, U. Bilitewski, *Sens. Actuators* **1996**, *32*, 107.
[112] M. Esti, G. Volpe, L. Massignan, D. Compagnone, E. LaNotte, G. Palleschi, *J. Agric. Food Chem.* **1998**, *46*, 4233.
[113] N. Pena, G. Ruiz, A. J. Reviejo, J. M. Pingarron, *Anal. Chem.* **2001**, *73*, 1190.
[114] M. Schoemaker, R. Feldbrugge, B. Grundig, F. Spener, *Biosen. Bioelectron.* **1997**, *12*, 1089.
[115] L. Campanella, F. Pacifici, M. P. Sammartino, M. Tomassetti, *Bioelectrochem. Bioenerg.* **1998**, *47*, 25.
[116] M. Situmorang, D. B. Hibbert, J. J. Gooding, D. Barnett, *Analyst* **1999**, *124*, 1775.
[117] R. M. Sobiech, R. Neumann, D. Wabner, *Electroanalysis* **1998**, *10*, 969.
[118] G. Panfili, P. Manzi, D. Compagnone, L. Scarciglia, G. Palleschi, *J. Agric. Food Chem.* **2000**, *48*, 3403.
[119] C. Capannesi, I. Palchetti, M. Mascini, A. Parenti, *Food Chem.* **2000**, *71*, 553.
[120] L.Campanella, G. Favero, M. Pastorino, M. Tomassetti, *Biosen Bioelectron.* **1999**, *14*, 179.
[121] G. S. Nunes, D. Barcelo, B. S. Grabaric, J. M. Diaz-Cruz, M. L. Ribeiro, *Anal. Chim. Acta* **1999**, *399*, 37.