

Biosensors for Food Analysis in Aqueous and Non-aqueous Media

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Summary

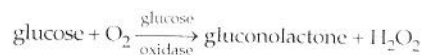
The increasing interest in biosensors as analytical devices is surely related to their simplicity, inexpensiveness, adaptability to automation, continuous monitoring and control. Food samples are commonly analysed by these new tools. The most common biosensors developed, used to analyse food components, to assay their freshness and to detect preservatives or pesticides contained, are briefly described. Biosensors capable to act in non-aqueous solvents and having a real or potential interest for food analysis are also briefly reviewed. Finally, we describe some of our recent research in this field.

Keywords: biosensors, food analysis, aqueous media, non-aqueous media

There is a strong need in the field of chemical analysis of foodstuffs, to develop methods that are rapid and cheap, but in the same time reliable, precise and accurate, which rely as little as possible on separation techniques or extensive pretreatment. Some of the known instrumental analytical techniques only partially satisfy all these requirements. On the other hand, most of the mentioned demands can be successfully tackled using a number of modern devices known as biosensors. Biosensors are different devices consisting of a biological system (one or more enzymes, specific animal or plant tissues, colonies of bacteria or cells, subcellular organelles, antibodies, etc.) which combined with a transducing system (electrochemical, optical, calorimetric, electromagnetic or piezoelectric, etc.) produce an analytical signal. The interaction between the biological system and the corresponding substrate i.e. a neutral or charged species that usually is of high chemical complexity and thus difficult to determine, produces a highly specific reaction (chemical, heat exchange, electromagnetic radiation effects, etc.) that used together with corresponding transducer enables selective analyte determination. Biosensors using electrochemical transducers were among the first developed and are certainly those that have been most extensively investigated, diffused (1) and used also in the agro-food sector, where they found numerous useful analytical applications.

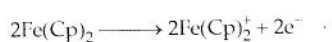
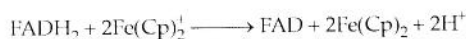
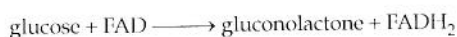
Current interest in biosensor research is not limited only to extend their range of application but also their engineering is undergoing rapid development. In addition

to the classical potentiometric and amperometric sensors, modified with enzymes and mediators, microbial sensors, cell or tissue sensors and immunosensors (2), considerable attention is now being focused on the combination of biological systems with field effect transducers (FET) (3). Nevertheless, the most common biosensors, which are also commercially available, are of the enzymatic type. However, even the latter ones are currently undergoing numerous, often substantial, innovations. Many enzymatic sensors actually consist of modified potentiometric or amperometric electrodes. For instance, the most extensively investigated enzymatic sensor is the one used for glucose determination. The utility of this sensor in the analysis of foodstuffs, at the industrial level has been known for some time. Its on-line use was recently confirmed (4) in processes of milk serum enhancement and the disposal of the relative processing waste. The enzyme used to catalyse the reaction involved in its functioning is glucose oxidase (GOD) and the reaction can be written in concise form as:



The natural electron acceptor during glucose oxidation is oxygen, which is reduced to hydrogen peroxide. The majority of glucose sensors developed consist of gels or membranes, containing immobilized glucose oxidase, superimposed on amperometric electrodes for H_2O_2 or O_2 determination. This type of biosensor, also known as the first generation type, is without any doubt

the most common and widely used in practice, also in the food analysis sector (5). However, these sensors have a number of limitations and in certain cases, the range of linearity of the sensor is limited by the value of the partial pressure of the O₂ in solution. In specific applications, the oxidation of glucose should take place under anaerobic, or in any case, oxygen deprived conditions. The idea thus arose of seeking electron carriers other than oxygen which would allow the enzymatic reaction to be uncoupled from the need to use O₂ as an electron acceptor. Electron carriers other than oxygen have been used such as ferrocene/ferricenium [Fe(Cp)₂/Fe(Cp)₂⁺], etc. A second generation of amperometric sensors has thus been developed to measure various substrates (6), including glucose, based on enzymatic-electrochemical reactions of the type described below for the glucose oxidase sensor:



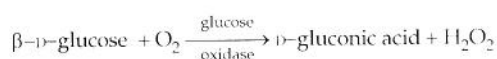
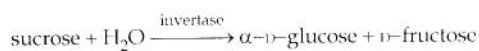
In this case problems are encountered, due to the need to maintain the electron carrier in solution at a constant concentration. One further step forward was the introduction of insoluble organic redox salts that could act as electron carriers between the reduced enzyme and the electrode. These salts are also known as charge transfer complexes (7). The enzymatic-amperometric sensors based on these systems for charge transfer purposes are also known as third generation sensors. A new type of enzymatic-amperometric system currently being developed consists of an amperometric electrode of platinum, gold, or graphite which is «activated» by depositing on it (usually electrochemically) a film of conducting polymer which is supposed to have the dual task of immobilizing the enzyme and acting as electron carrier between the reduced enzyme and the metal electrode. The conducting polymers most frequently used for this purpose include polypyrrole and derivatives (8). Research has also been carried out to find ways and means of introducing the carrier into the enzyme and chemically binding it so that the reduced enzyme will be capable of reoxidising itself directly on the metal electrode, maintained at the fixed potential, on which the enzyme itself is suitably immobilized (9).

Basically, in the field of the chemical analysis of foodstuffs electrochemical biosensors may be used for the following purposes:

- 1) to analyze certain food components that have to be determined fast and cheap with great sensitivity and specificity;
- 2) to determine chemical compounds used as indicators, for instance, of microbial pollution of a food or the deterioration of the latter due to, for example, oxidative processes. They can therefore be used to make a reasonable assessment of the probability of the satisfactory preservation of certain food products under certain conditions and for variable time intervals;
- 3) to detect traces of chemical compounds, pollutants, additives, preservatives, toxins, pesticides, etc.;

- 4) to act as detectors in FIA and HPLC chromatographic systems used to determine components in various foodstuffs such as alcohols, organic acids, amino acids, etc. (10).

Most biosensors built and used for food analysis were developed for the first type of purpose. For example, a large number of enzymatic sensors have been proposed for the analysis of (mono- and disaccharide) carbohydrates. The glucose electrode is without any doubt the most exhaustively studied enzymatic sensor and, as we have seen, has been constructed in a large number of different versions and types. Excellent glucose biosensors, among the most classical ones, have been obtained by covalently immobilizing glucose oxidase on Nylon membranes and fixing the latter to amperometric oxygen (11) or hydrogen peroxide (12) sensors. By using this technique, but with two or three enzymes immobilized, such as amyloglucosidase and glucose oxidase on a Clark or hydrogen peroxide electrode, it has proved possible to determine maltose or starch. It is also possible to assay lactose by using the same technique and the same type of electrode to immobilize β-galactosidase and glucose oxidase. Lastly, the same indicator electrode and the same immobilization technique has been used to develop a sucrose sensor (13). In this case three enzymes (invertase, mutarotase and glucose oxidase) catalysing the following three enzymatic reactions were immobilized simultaneously:

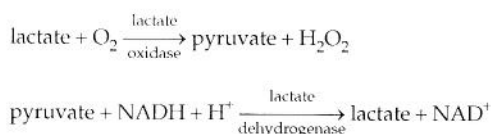


This type of sensor has been used to make glucose determinations in honey and jams. Also the authors of the present paper have built enzymatic sensors for glucose and sucrose by immobilizing the above enzymes in cellulose triacetate membranes (14). This type of glucose and sucrose sensor has been used to determine these analytes in sweets, soft drinks, some drugs, or glucose in biological fluids. The multiple immobilization of several enzymes has often been achieved in various cases obtaining different geometries, for instance by immobilizing the enzymes in a single membrane or in different membranes, which are then suitably superimposed (15). A very interesting sensor which can be used for sucrose determination in samples containing also glucose has been developed by superimposing on an H₂O₂ sensor first a membrane containing glucose oxidase and invertase, followed by a second, or «anti-interference» membrane, containing glucose oxidase and catalase, and finally a dialysis membrane (16). Recently, one problem involving considerable research carried out on the glucose electrode and which may have also a practical spin off in the direct analysis of foodstuffs, has been how to develop a sensor capable of providing a linear response at high glucose concentrations. Under these conditions the dissolved oxygen deficit leads to a loss of linearity of the response when common enzymatic amperometric electrodes are used. The problem has been approached

by various authors in different ways. In this respect the type of enzymatic amperometric sensors tested did not differ substantially from the classical types described earlier, but equipped also with special membranes (17) to limit the diffusion rate of glucose towards the enzymatic membrane. A different approach to the problem was recently proposed by the authors of the present paper (18). It consists in the immobilization inside a membrane sandwich of two different enzymes that operate in parallel and compete for the same substrate (glucose), but consume oxygen in only one of the two enzymatic reactions that they catalyse, i.e. the species determined by the indicator electrode associated with the membrane sandwich. Basically, one of the two enzymes »steals« the substrate from the other, but does not consume oxygen. This allows a substantial extension of the range of linearity of the biosensor.

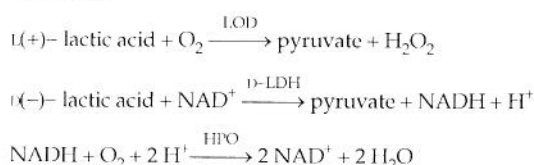
Lactate analysis in wine is of great importance because of the malo-lactic fermentation. This process results in the production and proper control of lactic acid from malic acid to avoid collateral reactions that can modify the wine composition of volatile acids and esters, which are responsible of the wine »bouquet«. An electrochemical procedure for the analysis of L-lactate, based on the lactate dehydrogenase plus lactate oxidase enzyme reactions cycle, has been developed by Compagnone *et al.* (19) and recently applied in wine analysis. The reaction of lactate with lactate oxidase produces pyruvate, which is the substrate, at physiological pH, of the enzyme lactate dehydrogenase.

This enzyme, in the presence of NADH, converts pyruvate to lactate consuming H^+ :



An automated software-controlled differential pH-meter was used for H^+ detection and the ΔpH measured was correlated to the lactate concentration present in the sample. Analysis of the lactate in several wine samples has been carried out. Comparison with a reference spectrophotometric procedure has also been discussed.

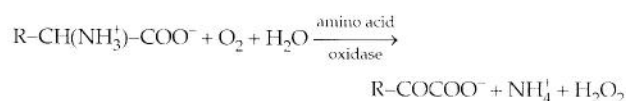
On the other hand a bioelectrode for the quick determination of D(-) and L(+) lactate in foodstuffs is pointed out by Mazzei *et al.* (20). Such a bioelectrode consists of a combination of three different enzymes: a) L(+)-lactate oxidase (LOD); b) D(-)-lactate dehydrogenase (D-LDH); and c) horseradish peroxidase (HPO). These enzymes are immobilized on the tip of an oxygen electrode. Amperometric measurements are possible for the determination of the oxygen concentration according to the following reactions:



The consumption of oxygen, monitored by the O_2 electrode, is therefore proportional to the concentration

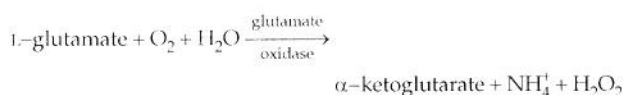
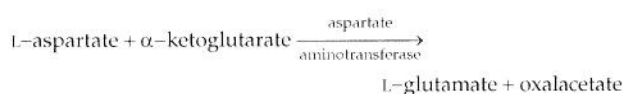
of total lactate in foodstuff samples. As a first practical application, the biosensor has been used for the direct determination of the levels of lactate in tomatoes. Indeed, the value of the total lactate concentration is considered as an indicator and reliable index of the tomato quality, since many fermentation processes lead to the production of D(-) and/or L(+) lactate. This bioelectrode multienzymatic system appears suitable for quick quality control procedures, that can be easily automated and extended also to the on-line control of production processes.

The importance of amino acids as protein constituents in the analysis of foodstuffs is self-evident. There are several reasons why their determination is necessary: for instance, the difficulty often encountered in analysing protein hydrolysates, or the need to determine peptides and free amino acids in different foodstuffs after the latter have been subjected to different technological and industrial treatments and processes, in order to monitor the effects of such procedures on the treated foodstuffs. It is thus logical that a large number of amino acid sensors ought to be developed. One classical amino acid sensor is the one in which the enzyme amino acid oxidase, which catalyses the following reaction:



is coupled with a sensor for H_2O_2 (21). This sensor has an extremely sensitive response to cysteine, but also responds quite well to other amino acids, such as methionine. The enzyme can be entrapped by means of a cellophane membrane on a platinum electrode maintained at a potential of 0.35 V. The observed signal is a combination of the response to the cysteine and H_2O_2 . Alternatively, the same enzyme may be immobilized by means of a cross-linking procedure on an oxygen-electrode (22) consisting of platinum foil polarised at -0.6 V, *vs.* a calomel reference electrode. According to researchers that tested the system, the detection of oxygen is more sensitive than the H_2O_2 based method, since the H_2O_2 formed may react with the ketoacid formed as one of the enzymatic reaction products. In this way, some of the free H_2O_2 released in the enzymatic reaction is consumed in this redox reaction, and therefore is not detected by the H_2O_2 sensor. In this case the system is specific for amino acids, but still comparatively unselective to individual amino acids. One construction model that has been found to give good results in the case of amino acid-sensors, involves the use of an amino acid oxidase enzymatic membrane placed over the head of an NH_3 gaseous diffusion electrode, which operates in an alkaline buffer. For instance, the authors of this paper recently tested the response of biosensors obtained using commercially available ammonia-electrodes, suitably modified by means of amino acid oxidase, immobilized in special cellulose triacetate films and superimposed on the NH_3 potentiometric gaseous diffusion electrode. We have experimentally verified that these sensors display a somewhat variable selectivity *vs.* the various amino acids of the L-series. Another sensor developed by our

group consists of a sensor for aspartate, obtained by coupling a gaseous diffusion electrode for ammonia with an enzymatic membrane based on the aspartase enzyme, immobilized by means of polyazetidine on a dialysis membrane. This biosensor was used to determine aspartic acid in drugs, but also aspartame in sweeteners (23). Fatibello-Filho *et al.* (24) constructed an enzyme electrode for the determination of aspartate, built with the same geometry, but immobilizing the aspartase enzyme by means of the cross-linking method. More recently Villarta *et al.* (25), reported the features of an amperometric enzyme electrode for aspartate analysis, based on the coimmobilization of aspartate aminotransferase and glutamate oxidase on preactivated »immobilon-AV affinity membrane«. The reactions involved are:



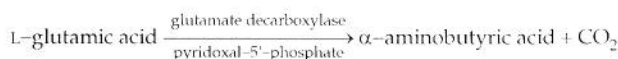
The hydrogen peroxide produced in the second reaction was oxidized at a platinum electrode held at +650 mV (*vs.* Ag/AgCl) and the current change was linearly correlated to the concentration of aspartate present in solution. In order to make a correct estimation of a foodstuff nutritional value, it is very important to know its content in a single amino acid. It can be achieved in an extremely specific manner using an enzymatic sensor obtained employing the cross-linking method to immobilize for instance the enzyme L-lysine decarboxylase on the gas permeable membrane of a CO₂ sensor (26). The carbon dioxide released during the enzymatic reaction is determined using a classical CO₂ electrode. Before being analyzed, the food sample (i.e. grains) is hydrolysed in a hydrochloric acid solution (6 mol/L) at 110 °C, for about twenty hours and then filtered, reduced in volume by evaporation, after which a suitable buffer is added and the solution analyzed.

Even though the available methionine in food is more appropriate in terms of nutritional value, the total methionine value is still widely used in the food industry. The possibility of using the enzyme methionine-lyase for the construction of a methionine sensor has been studied by Fung *et al.* (27).



The biosensor was prepared by depositing a layer of the immobilized enzyme, using cross-linking method, onto an ammonia gas sensing electrode.

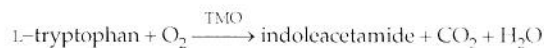
An excellent glutamate sensing electrode was constructed by Kuriyama *et al.* (28), by immobilizing slices of yellow squash tissue on a CO₂ gas sensor. The biocatalytic activity of this electrode arises from the fact that yellow squash tissue contains glutamate decarboxylase, which catalyses the decarboxylation of L-glutamic acid to produce carbon dioxide:



L-tryptophan is one of the essential amino acids needed for human growth. A number of human diseases results from disruption of L-tryptophan metabolism. L-tryptophan also plays a very important role in the metabolism of mammalian cells, microorganisms and plants. Several different sensors have been used in the past for tryptophan determination in food. But these biosensors were found to be non-specific to different amino acids. A short description of a biosensor for specific determination of L-tryptophan has been reported by Vineke *et al.* (29). Tryptophan determination was based on deamination reaction with tryptophanase synthesized by *E. coli*:



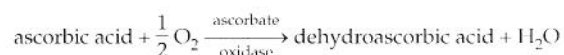
Two types of devices have been described. One of them was an enzyme electrode based on a crude *E. coli* extract containing tryptophanase and the other was a bacterial electrode based on the same *E. coli* cells. The amount of ammonia was measured using an ammonia gas sensing electrode during the deamination reaction. Pyridoxal phosphate as cofactor was necessary for both enzyme and cell based electrodes. A recent research (30) described a new amperometric biosensor for L-tryptophan determination, based on immobilized L-tryptophan-2-monoxygenase (TMO). The last enzyme catalyses the oxygen dependent decarboxylation of tryptophan and therefore, a Clark type oxygen electrode could be used as a transducer for the detection of oxygen consumption.



The stoichiometry between L-tryptophan and oxygen allows the development of an amperometric method for the quantitation of L-tryptophan, based on the measurement of oxygen concentration changes during the decarboxylation reaction. As with other FAD containing oxidoreductases, the tight binding of FAD to TMO provides an analysis procedure which does not require any additional co-factor, in contrast to many other classes of enzymes.

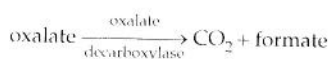
There are also biosensors for peptides or proteins analysis. The most classical type operates in flow conditions. The peptides are first hydrolysed in a small flow cell containing the protease enzyme entrapped on agarose or immobilized covalently, connected in flow conditions through Nylon tubes containing immobilized amino acid oxidase (31) and using an NH₃ gaseous diffusion electrode.

One classical enzymatic sensor for analyzing food substrates is that designed for ascorbic acid. Several different versions have been constructed. One of the more classical ones uses the enzyme ascorbate oxidase, covalently immobilized on a Nylon membrane and a Clark electrode to measure the oxygen (32). The enzymatic reaction, illustrating the functioning of the sensor, is the following:



Ascorbic acid concentration is found to be proportional to the reduction of the partial pressure of the dissolved oxygen, which is determined using the Clark electrode. A similar procedure was implemented also by Matsumoto *et al.* (33) who immobilized the enzyme on a collagen membrane using a classical glutaraldehyde cross-linking procedure. These authors applied the method to ascorbic acid determination in different fruit samples. The results were excellent both in terms of accuracy and agreement with other analytical methods.

Oxalate often has to be determined in extremely complex substrates, foodstuffs or other biological material. For this reason current analytical methods of oxalate assay normally require separation techniques to be used to isolate the analyte prior to determination. Several studies are reported in the literature on the subject of developing classical membrane sensors for oxalate determination, but a number of oxalate biosensors have also been described. In some cases the enzyme immobilized is oxalate decarboxylase, which catalyses the reaction:

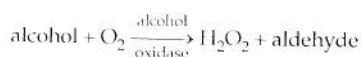


For example, a sensor of this type was obtained by immobilizing the enzyme through cross-linking (34) using glutaraldehyde and bovine serum albumin on a CO₂ electrode. Alternatively, a number of sensors have been made using the enzyme oxalate oxidase (35), which catalyses the reaction:



Vegetable (36) or animal tissue biosensors can be used for oxalate determination. Assolant-Vinet *et al.* (37) developed a reliable enzymatic sensor for oxalate, which they used to make accurate determinations of the analyte in a large number of food substrates. In this case the enzyme oxalate oxidase was immobilized on commercially available preactivated polyamide membranes superimposed on an H₂O₂ amperometric electrode. In this way it was possible to determine the oxalate content of spinach, chocolate, orange juice, etc., with a good degree of accuracy and in excellent agreement with the results of parallel determinations carried out using conventional spectrophotometric methods.

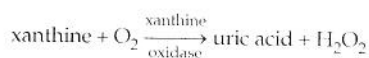
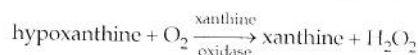
There have also been proposals for different types of biosensor for alcohol determination for several purposes, ranging from bioclinical applications to fermenter monitoring and determinations in food beverages (wine, etc.). In some cases the enzyme alcohol oxidase was immobilized on collagen or Nylon membranes coupled with an oxygen sensor (38).



However, results have not been very promising because this type of sensor has a too short lifetime and above all comparatively poor specificity. Nevertheless, some more highly specific types of biosensor have been proposed. One of these involves the immobilization of cells (of the bacterium *Gluconobacter suboxydans* adsorbed onto a nitrocellulose filter) on an O₂ electrode (39).

Many foods contain lecithin either naturally or added as emulsifying compound. An enzymatic amperometric system for the determination of lecithin as an additive in foods and as a component of commercial drugs was proposed by the authors of this paper (40). The method is based on a detector realized with two enzymes, choline oxidase and phospholipase D and by an oxygen Clark electrode. The former enzyme was immobilized and the latter was free in solution. The experimental conditions were investigated in order to obtain wide applications with different samples. Extraction or dissolution of the samples in ethanol proved satisfactory. Correlation between the proposed enzymatic-amperometric method and an enzymatic-spectrophotometric reference method was good. Lecithin content was determined in several food samples, e.g. flour, chocolate, egg yolk, biscuits, oil and cakes, all of commercial grade.

One recent development in biosensor applications to food control is represented by the possibility of determining, also in continuous process mode, certain analytes that can be used as indicators of the state of preservation of important foodstuffs (*i.e.* biosensors developed for the purpose of the second type of our introducing considerations). For example, the concentrations of trimethylamine, or hypoxanthine, or inosine, have been suggested as indicators of the state of freshness of fish. Watanabe *et al.* (41) proposed for the first time an enzyme sensor specific for hypoxanthine using immobilized xanthine oxidase membrane and an oxygen probe:



and claimed that the enzyme sensor was utilisable for the simple, rapid and cheap determination of hypoxanthine in several fish meats.

The same research group (42) developed a multielectrode enzyme sensor for simultaneous determination of adenosine-5'-phosphate, inosine-5'-phosphate, inosine and hypoxanthine in fish meat. The following development was a hypoxanthine sensor based on an amorphous silicon field effect transistor. Tamiya *et al.* (43), used the pH response of an ISFET with xanthine oxidase immobilized on a thick poly(vinylbutyral) membrane over the gate insulator, to detect the uric acid produced by enzyme-catalysed oxidation of hypoxanthine. Recently Haemmerli *et al.* (44) produced an hypoxanthine biosensor using a polarographic electrode for the determination of both the generated hydrogen peroxide, and the consumed oxygen, in combination with immobilized xanthine oxidase.

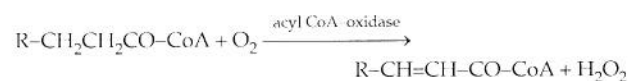
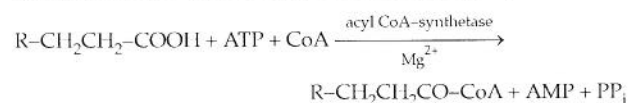
Another approach of the Japanese research group (Watanabe *et al.*) (45) for the detection of fish freshness was a microbial sensor consisting of immobilized living spoilage bacterium (*Alteromonas putrefaciens*) and an oxygen electrode. This system was applied to the continuous determination of fish freshness, like bluefin tuna and yellowtail meats.

On the other hand, the concentration of amines, such as cadaverine, or putrescine, have been proposed as indicators of the state of deterioration of fresh butch-

ered meat. Since several different amines are produced during the decarboxylation of amino acids making up the protein fraction of meat, it is possible to evaluate the degree of freshness of the latter from its amine content. This type of determination is neither fast nor easy and it has been proposed to monitor the freshness of butchered meat by means of routine determination of monoamine content using a special enzymatic sensor obtained by immobilizing monoamine oxidase in collagen and coupling the immobilized enzyme with an oxygen gaseous diffusion amperometric sensor (46).

A method developed in the Cranfield Institute of Technology (47) is based on the observation that the meat glucose is depleted by the microbial surface flora and a gradient of glucose concentration from the surface to the bulk of the meat develops. For lamb inoculated with a controlled bacterial culture, this gradient has been shown to be correlated with the microbial surface counts. It may be possible to develop an instrument for the assessment of the shelf-life of chilled meat joints, based on a measurement of this glucose profile. The sensor produced for this purpose, consists essentially of a piece of carbon foil modified with the enzyme and the mediator, i.e. ferricinium ion, that replaces oxygen as the one electron acceptor in the oxidation of glucose catalysed by glucose oxidase. Based on this direct biosensor principle, a planar glucose sensor array, designed to be mounted in a knife-type instrument, which can be inserted into meat joints, with the help of a cutting edge, has been reported by Kress-Rogers and D' Costa (47).

Biosensors have also been developed to monitor the freshness of food oils. A first approach involved measuring the free fatty acids content in the sample by means of the enzyme acyl coenzyme A (CoA) synthetase, in the presence of ATP, and determining the adenosine-5'-monophosphate (AMP), produced during the enzymatic reaction (48). More recently a simpler enzymatic sensor was built using in series the two enzymes (acyl coenzyme A synthetase and acyl coenzyme A oxidase) (49):



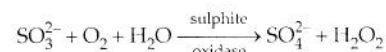
To test the degree of freshness of a milk sample a lactate biosensor was used by Mascini and coworkers (50). This biosensor was obtained by immobilizing lactate oxidase on a Nylon membrane which was superimposed on an oxygen electrode. The aerobic decomposition of the lactose contained in the milk produces lactic acid. Therefore, the ageing of milk simultaneously involves a decrease in pH, a decrease in lactose concentration and an increase in lactate concentration.

In order to monitor the process in which fats turn rancid, research is under way to find a biosensor-detectable »chemical indicator« whose concentration can be correlated with that of one of aldehydes or alkanes, which are produced as the food turns rancid. Their presence is currently detected using costly equipment such as GC-MS.

Also, inorganic species can be determined in food-stuffs using biosensor. For instance, the inhibiting effect of phosphate on acid phosphatase activity, can be used to determine phosphate by a biosensor (51). This is based on the acid phosphatase (AP) contained in thin slices of a *Solanum tuberosum*, in which also a second enzyme, glucose oxidase (GOD), is immobilized by adsorption. The reaction catalyzed by the two enzymes operating in series leads to an oxygen consumption in solution, which is detected by a Clark oxygen electrode. In the presence of phosphate, due to its inhibiting effect on the acid phosphatase enzyme, the O₂ consumption is reduced; from the difference between the oxygen consumptions, in absence and in presence of phosphate, the phosphate concentration can be evaluated. Alternatively, hydrogen peroxide, produced during the second enzyme reaction, can be detected by an amperometric hydrogen peroxide sensor. In this case the phosphate concentration in solution is evaluated from the decreased production of H₂O₂.

In the past years our group developed an inhibition biosensor for phosphate analysis of the type hereby described and applied it to the determination of phosphate contained in fresh and powdered milk, red wine and tomato puree (51).

The biosensors built for the purpose of the third group of our considerations in food analysis, have been recently in the limelight. One typical example is the biosensor for sulphite analysis. The sulphite is used as an antioxidant and antibacterial substance in the food industries. It is consequently to be found in many of the products of these industries, as well as in the effluents from their industrial plants, thus representing a source of environmental pollution. There is therefore a growing interest in finding simple, fast and cheap sulphite assay methods. Recent technological developments have allowed biosensors to be developed also for sulphite assay on the basis of the following reaction:

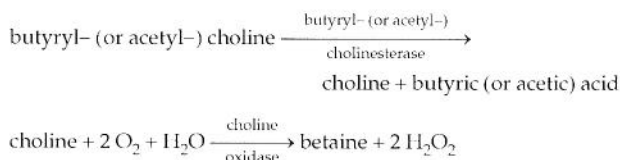


which takes place spontaneously in the presence of atmospheric oxygen and is accelerated by sulphite oxidase. Karube *et. al.* (52,53) developed a first sensor for sulphite determination by coupling subcellular organelles (hepatic microsomes), used as an enzymatic source, to an oxygen electrode. Alternatively, by entrapping the sulphite oxidase in gel and fixing the whole assembly by means of a dialysis membrane to an oxygen electrode it was possible to construct electrodes having a greater selectivity to sulphite, bisulphite and metabisulphite (54). On the other hand, Fonong (55) decided to exploit the relationship between the sulphite consumed and the hydrogen peroxide produced in the enzymatic reaction cited above. In this case the sensor was obtained by coupling a chemically activated collagen membrane (immobilizing the sulphite oxidase) with an H₂O₂ electrode. Also our group recently performed a series of tests on enzymatic sensors for sulphite and applied these sensors to the determination of the sulphite contained in ordinary and sparkling wine with good results (56).

In recent years we have also developed other biosensors of interest in the determination of chemical compounds in foods, like phenols (57), pesticides (58), preservatives, toxics and so on. Work was also performed to develop total toxicity respirometric biosensor (59,60).

The polyphenols content in wine has been determined by us using a new enzyme sensor (61). Two different immobilization methods for the enzyme are proposed and discussed on the basis of the experimental results: several red and white commercial and non-commercial wines were analysed. The first method is based on physical immobilization of the tyrosinase enzyme in kappa-Carrageenan gel while the second method is based on chemical immobilization of the same enzyme in Nylon-functionalised membrane. Results are compared with those obtained by a classical spectrophotometric method and the agreement was satisfactory.

In the field of organophosphorus pesticides analysis, some years ago (1988), our research group proposed and then published, a first operating system (62), based on a choline oxidase sensor, as indicating one, consisting of an oxygen Clark electrode, modified by a cellulose triacetate membrane, containing the immobilized enzyme choline oxidase. This sensor was dipped into a buffer solution, containing dissolved enzyme butyryl- (or acetyl-) cholinesterase. The addition of butyryl- (or acetyl-) choline to this thermostated buffer solution, results in an oxygen consumption, quantitatively due to the two in series enzymatic reactions of hydrolysis of the choline esters and oxidation of produced choline and monitored by Clark electrode. As butyryl- (or acetyl-) cholinesterase activity is inhibited by organophosphorus pesticides, in their presence, less choline is formed and less oxygen consumed. Compounds, such as Malathion or Paraoxon, can be determined, by a calibration procedure and by measuring the decrease of the reaction rate, on adding increasing amounts of pesticides.



A development of the method was carried out by our group (63), some years later (1991), including the assembly of a sensor with the two enzymes butyryl- (or acetyl-) cholinesterase and choline oxidase, both immobilized in a dialysis membrane and superimposed on the oxygen sensor. The new method was characterised by a good handiness, shorter operating times and small amounts of enzyme needed. In this method both enzymes were immobilized in a dialysis membrane, overlapping that one of the Clark electrode. Nevertheless, by the new method, minimum detection limit and linearity range were shifted to higher concentrations (about one decade) in comparison with the ones found in the previous research described above. In addition, the lifetime of the sensor was not satisfactory.

For these reasons we recently worked on the development of a new enzyme sensor (64), using a hydrogen peroxide electrode as an indicating electrode, modified by superimposing to it a Nylon membrane, containing

the two enzymes (choline oxidase and butyrylcholinesterase) properly immobilized. This new sensor shows a longer lifetime and a better response, in comparison with those described above.

In recent years, our research group has developed, also a number of other (enzymatic, vegetable tissue, or inhibition) biosensors and used them for purposes of the first and third group of our introductory considerations. That is to say they were applied to analytical determinations of a bioclinical, pharmaceutical, environmental and of course alimentary interest. Some of the biosensors investigated by the present authors and already used for determination of food matrices, or else having real potential for use in these fields, are briefly summarized in Tables 1 and 2.

The use of enzymatic reactors and enzyme sensors as detectors for flow-injection systems, or for HPLC analysis, is also still increasing (10,68). For instance Ehrendorfer *et al.* (69) proposed an interesting amperometric biosensor coupled with a FIA system for salicylate detection in apple juice, orange juice, dark beer, table wine, etc., while, Jao and Wasa (70) simultaneously determined L(+) and D(-) lactic acid by use of immobilized enzymes in a flow injection system. The same authors pointed out an amperometric detection of acetylcholine and choline, in a liquid chromatographic system with an immobilized enzyme reactor. Other authors stereoselectively determined L-amino acids using column liquid chromatography with an enzymatic solid-phase reactor (71). Shimada *et al.* recently reviewed (72) a good number of enzyme reactors for detection systems in high-performance liquid chromatography and described several applications in this field: analysis of sterols, bile acids, androgens, oestrogens, acetylcholine, choline and other compounds. A number of studies about the application of immobilized enzymes, as post column reactors in the HPLC, was published (73). The proposed method combines the separation efficiency of HPLC, the selectivity of enzyme and the high sensitivity of electrochemical detection. The sample is isolated from a complex matrix like food or body fluids by HPLC with a buffer solution as eluent and on-line converted by the immobilized enzyme. In most cases a simple dilution is sufficient for sample pretreatment. After the enzyme reaction, the product is electrochemically oxidized and the signal is proportional to the analyte concentration. Using this technique the simultaneous separation and determination of several compounds in one run is possible. Determination of different mono- and oligosaccharides, organic acids, amino acids, alcohols and sulphur dioxide has been published (68).

Finally, the on-line coupling of reverse-phase high performance liquid chromatography (HPLC) with bioassays, such as immunoassays, or immunosensors (74), allows a combination of the separation power and automation potential of LC with the high selectivity and sensitivity of biochemical detection principles, but important problems, related to LC and immunoassays, or immunosensors, have to be overcome. For this aim different strategies to implement immunochemical interactions in on-line post-column detection techniques are in course of study.

Table 1. Main biosensors developed by the authors for the analysis of some components in foods and beverages

Enzymes	(Ref.)	Type of biosensor	Substances determined in beverages and foodstuffs
glucose oxidase	(14)	enzymatic-amperometric	glucose in several beverages and foodstuffs: fruit juice, drinks, rhubarb candies
(invertase+ mutarotase + glucose oxidase)	(14)	enzymatic-amperometric	sucrose in: cakes, jams, ketchup, milk tablets
(choline oxidase + phospholipase D)	(40)	enzymatic-amperometric	lecithin and phospholipids containing choline in several foodstuffs: egg, yolk, soya derivatives, cakes, chocolate, biscuits
amino acid oxidase	–	enzymatic-potentiometric	amino acids in proteic hydrolysates (research in progress)
aspartase	(24)	enzymatic-potentiometric	aspartame in sweeteners and aspartic acid in diet products
(acidic phosphatase + glucose oxidase)	(51)	(vegetal tissue + enzymatic) -amperometric-inhibition	phosphate content of milk, powdered milk, tomato puree, red wines
lactate oxidase	(18)	enzymatic-amperometric	lactate in milk (research in progress)
urease	(65)	enzymatic-potentiometric	urea in milk (research in progress)
(cholesterol esterase + cholesterol oxidase)	(66)	enzymatic-amperometric	cholesterol in eggs and other foodstuffs (research in progress)

Table 2. Main biosensors developed by the authors utilised for the analysis of additives, pollutants and preservatives in beverages

Enzymes	(Ref.)	Type of biosensor	Substances determined in beverages
sulphite oxidase	(56)	enzymatic-amperometric	sulphite in wines and sparkling wines
polyphenol oxidase	(61)	enzymatic-amperometric	polyphenols in red and white wines, waters and olive oil
(butyrylcholine esterase + choline oxidase)	(58) (64)	enzymatic-amperometric-inhibition	organophosphorus-pesticides in waters, in agricultural soils aqueous extracts, in fruit washing aqueous solutions (research in progress)
butyrylcholine esterase	(67)	enzyme-FET-inhibition	Paraoxon in waters (application to agricultural-foodstuff matrices, to be performed)

Enzymatic conversion and accordingly biocatalytic sensing have been traditionally performed in aqueous environments. However, relatively recent research, especially performed by Reslow (75), Laane (76), Zacks (77), Dordick (78), Narayan (79) and coworkers, has resulted in the remarkable finding that many enzymes can function in organic solvents (with or without a small amount of added water). As a consequence, the use of enzymatic biocatalysts in non-aqueous solvents represents another possible opportunity for biosensor techniques. The attempts currently being made in this direction are justified by advantages that can be obtained: 1) the possibility that organic phase enzyme electrodes (OPEEs) can be applied to assay new challenging substrates e.g. foodstuffs, or toward additional hydrophobic substrates, 2) the simplification of immobilization technique and 3) the decrease of microbial contamination, that can improve the operational lifetime of biosensor (80).

Some examples of organic phase enzyme sensors (OPEEs) reported in literature are the following: a typical OPEE is the sensor developed by Phillips Petroleum (81), using the alcohol oxidase enzyme and capable of determining alcohol concentrations in organic medium. Miyabayashi *et al.* (82) described a potentiometric OPEE that monitored chymotrypsin-catalysed ester synthesis

in organic media and having a high response in solvents characterised by a lower hydrophobicity, e.g. diisopropylether. Recently Hall and Turner (83) described an amperometric OPEE using cholesterol oxidase enzyme for determining cholesterol concentration in chloroform/hexane mixture. Schubert *et al.* (84) carried out the determination of peroxide levels in organic solvents (chloroform) by coadsorption of potassium hexacyanoferrate(II) and horseradish peroxidase on a carbon electrode, while Mannino *et al.* (85) used a similar biosensor, working in organic phase, for measuring the peroxide value in vegetable oils. The authors of this communication have recently developed an OPEE for hydrogen peroxide determination in several polar (chloroform) and low polar (toluene) organic solvents using immobilized catalase enzyme (86).

An OPEE with good potential applications that has captured the attention of several working groups is the tyrosinase enzyme sensor. Hall *et al.* (87) described an enzymatic-amperometric electrode for determining *p*-cresol in water saturated chloroform. In this sensor, the enzyme catalyses *p*-cresol conversion to 4-methyl-1,2-benzoquinone, which can then be reduced electrochemically using a graphite working electrode. Because of the high resistance to the passage of current through the water

saturated chloroform solution, a supporting electrolyte, tetrabutylammonium-toluene-4-sulphonate (TBATS), is added to the chloroform (88). Wang (89) described several kinds of tyrosinase sensors with the enzyme immobilized by different methods. More recently, Wang *et al.* (90) used a biosensor, obtained by adsorbing an aqueous solution containing tyrosinase onto a suitably polished graphite disk, for determinations in water saturated chloroform medium. The electrochemical basis of these systems is not very different from that one we described above for Turner's biosensor and the supporting electrolyte (TBATS) is the same.

Starting from this knowledge, our group (91) recently made an enzymatic sensor of relatively simple design and able to provide excellent service in *n*-hexane for the analysis of phenol and phenol derivatives. The tyrosinase enzyme is immobilized in this case on the head of an amperometric gas diffusion oxygen sensor by means of a dialysis membrane. Recently, we also measured the reaction rate in different organic solvents and therefore we calculated the immobilized specific activity of the enzyme (92). Results confirmed the low activity of the enzyme in chloroform, while in hexane the activity is very high. In addition, we developed a new method for immobilizing the tyrosinase enzyme in κ -Carrageenan gel, which has been shown to be capable of solving the problem concerning the true lifetime of the enzyme when operating in non-aqueous medium and so we obtained a more efficient biosensor. Using the new biosensor, we performed polyphenols determination in olive oil (93) and other fats, using, as organic solvent, *n*-hexane, rather than chloroform, as recently proposed by other authors, because of the excellent solubility of olive oil in the former, the greater sensitivity displayed by the sensor in *n*-hexane and the very low detection limit in this solvent, but above all the longer working life and reliability of the biosensor when *n*-hexane rather than chloroform is the adopted solvent. Nevertheless, the determination in water saturated chloroform could be interesting when it is required to assay samples with high phenol concentration, without the possibility of prior dilution, in view of the wider linearity displayed by the sensor in chloroform and in water saturated chloroform. For the determination of the polyphenol content in different commercial samples of olive oil, or other edible oils and fats (94), the various oil samples were analysed, using the enzymatic sensor dipped in *n*-hexane. In practice, each sample was added directly to the non-aqueous solvent in which the biosensor was immersed, either as such, or after proper dilution with the same solvent. The obtained results show a good correlation with the values found using traditional methods able to detect the polyphenols content in different olive oils and in several edible oils and fats. We were also able to confirm, that the products denoted as »olive oil«, or »olive oil residues«, are found to contain less polyphenols than the samples denoted as »extra-virgin« and »virgin« respectively. This is indicative of the corrective treatments to which the first type of commercial samples are subjected. As an application of the biosensor is the simple way of determining the polyphenol content of the oils, the use of the biosensor is to check rapidly the authenticity of an olive oil.

Another well known characteristic of the »virgin« olive oil is the high degree of stability to oxidative processes. A good correlation has been found between polyphenols content and the product's stability to oxidation. This supports the important antioxidant role played by polyphenols in »virgin« olive oils and of course the great interest in the development of suitable and inexpensive methods for their analysis, like the one based on a biosensor able to operate directly dipped in organic solvents.

In conclusion, the rapid development of biosensors, particularly those using the electrochemical transduction, is one of the most significant lines of modern biotechnologies. The reason for their rapid spread is to be sought in the potential that biosensors offer for determining even species of considerable molecular complexity and for extending the already large area of chemical compounds that can be determined by classical electrochemical sensors (95), which essentially comprises cationic or anionic electrolytes, both inorganic and organic. Furthermore, determinations are fast and cheap, and can be carried out using simple portable equipment so that testing is not limited to standard laboratories, but can also be performed in »the field« and *in situ*.

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Biosenzori za kontrolu kakvoće namirnica u vodenim i nevodenim medijima

Sažetak

Zbog svoje jednostavnosti, malih troškova, mogućnosti automatizacije te stalnog motrenja i nadzora, biosenzori kao analitički uređaji pobuđuju sve veću pozornost. Uobičajena je njihova uporaba za analizu uzoraka hrane. U radu je dan kratak opis najčešćih biosenzora koji se koriste u kontroli kakvoće namirnica pri utvrđivanju svježine, udjela konzervansa ili pesticida.

Također su ukratko prikazani biosenzori koji mogu djelovati u nevodenim otopinama, a imaju stvarnu ili moguću vrijednost pri analizi namirnica. Konačno, opisana su vlastita novija istraživanja autora na tom području.