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Biosensors Based on Enzyme Inhibition

Fabiana Arduini and Aziz Amine

Abstract The present chapter describes the use of biosensors based on enzyme inhibition as analytical tools. The parameters that affect biosensor sensitivity, such as the amount of immobilized enzyme, incubation time, and immobilization type, were critically evaluated, highlighting how the knowledge of enzymatic kinetics can help researchers optimize the biosensor in an easy and fast manner. The applications of these biosensors demonstrating their wide application have been reported. The objective of this survey is to give a critical description of biosensors based on enzyme inhibition, of their assembly, and their application in the environmental, food, and pharmaceutical fields.

Keywords Biosensor · Enzyme · Inhibition

Contents

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1 Introduction

According to the IUPAC (International Union of Pure and Applied Chemistry) definition, a biosensor may be defined as a device incorporating a biocomponent (antibody, enzyme, microorganism, tissue, nucleic acid) in intimate contact with a suitable physicochemical transducer. The transducer converts the biochemical signal into a quantifiable electronic signal, which is proportional to the concentration of a specific analyte or group of analytes. As reported in a technical report in the *Biosensor and Bioelectronics Journal*,

> Biosensors may be classified according to the biological specificity-conferring mechanism or, alternatively, to the mode of physico-chemical signal transduction. The biological recognition element may be based on a chemical reaction catalyzed by, or on an equilibrium reaction with macromolecules that have been isolated, engineered or present in their original biological environment. In the latter cases, equilibrium is generally reached and there is no further, if any, net consumption of analyte(s) by the immobilized biocomplexing agent incorporated into the sensor. Biosensors may be further classified according to the analytes or reactions that they monitor: direct monitoring of analyte concentration or of reactions producing or consuming such analytes; alternatively, an indirect monitoring of inhibitor or activator of the biological recognition element (biochemical receptor) may be achieved [1].

In the case of an enzymatic biosensor, in which the enzyme is the biocomponent, we can summarize that the measurement of analytes can be performed by means of two different approaches: if the enzyme metabolizes the analyte, the analyte can be determined measuring the enzymatic product, or if the analyte inhibits the enzyme, the decrease of the enzymatic product formation can be measured and correlated to the analyte concentration. In the latter case, this type of biosensor is called a "biosensor based on enzyme inhibition." Nowadays, several reviews of biosensors based on enzyme inhibition have been published. Taking into consideration the papers published from 2000, there are just three reviews in the literature. The first one in chronological order was reported in the *Biosensor and Bioelectronics* Journal by Luque de Castro and Herrera, in which an overview of biosensors and biosensing systems was given. In this case the authors highlighted that the inhibition effect is not selective, and for this reason the title is "Enzyme Inhibition-Based Biosensors and Biosensing Systems: Questionable Analytical Devices" [2]. Another review was reported by our research group, also published in the *Biosensors and Bioelectronics* Journal with the title, "Enzyme

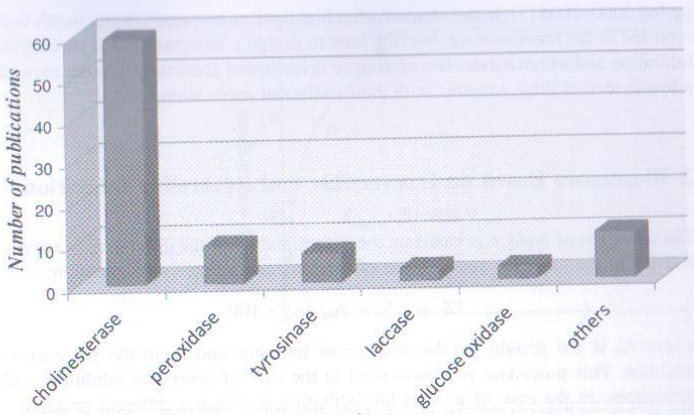

Fig. 1 Distribution of enzymes used to construct biosensors based on enzyme inhibition (period 2006–2012)

Inhibition-Based Biosensors for Food Safety and Environmental Monitoring" [3]. The review highlighted research carried out during 2000–2005 on biosensors that are based on enzyme inhibition for determination of pollutants and toxic compounds in a wide range of samples. Here the different enzymes implicated in the inhibition, different transducers forming the sensing devices, and the different contaminants analyzed were considered. The last one was reported in the *Analytical Letters* Journal also by our research group with the title of, "Reversible Enzyme Inhibition Based Biosensors: Applications and Analytical Improvement Through Diagnostic Inhibition," in which research carried out from 2000 up to 2009 on biosensors based on *reversible* enzyme inhibition for determination of drugs, pollutants, and toxic compounds was reported [4].

We highlight that, to our knowledge, these reviews are the only ones totally dedicated to biosensors based on enzyme inhibition; in fact, in other reviews such as, "Determination of Pesticides Using Electrochemical Enzymatic Biosensors" [5] or "Design and Development of Biosensors for the Detection of Heavy Metal Toxicity" [6], only a section dedicated to the biosensor based on enzyme inhibition is present. However, taking into consideration the three reviews mentioned above, it seems that the world of biosensors based on enzyme inhibition is confined to a restricted field of applications in analytical chemistry. However, this is not true if we consider that the cholinesterase biosensor is a biosensor based on cholinesterase inhibition; in fact, including the period between 2000 and 2012, there are eight reviews concerning the cholinesterase biosensor [7–14]. In Fig. 1 we show the distribution of enzymes used for the design of biosensors in the period 2006–2012 demonstrating that the cholinesterase biosensor is the most used enzyme for developing enzyme-based biosensors. We have found the same trend also in the

period 2000–2005 [3]. In this chapter, after highlighting research activity in this field reported in the literature, we describe how to design a biosensor based on enzyme inhibition and which parameters need to be investigated accurately in order to reach a low detection limit, together with their analytical application.

2 Biosensors Based on Irreversible and Reversible Inhibition

The detection of analyte is based on the determination of the difference in enzyme activity in the presence and absence of inhibitor according to the equation:

$$I\% = (A_0 - A_i/A_0) \times 100$$

where A_0 is the activity in the absence of inhibitor and A_i in the presence of inhibitor. This procedure is always used in the case of reversible inhibition and, sometimes, in the case of irreversible inhibition in which a different protocol is usually adopted in order to obtain an increase in sensitivity, as described in detail in the successive protocol of the measurement section. The different protocol is due to a different kind of inhibition: reversible inhibition is characterized by noncovalent interaction between inhibitor and enzyme, with the consequent restoration of the initial activity after the inhibition measurement. On the contrary, in the case of irreversible inhibition characterized by covalent bonding between the enzyme and the inhibitor, the restoration of the initial activity requires a reactivation of the enzyme using specific compounds. Thus, in order to choose the best measurement protocol, it is important to know the type of inhibition. The research study should start with the calculation of the Michaelis–Menten constant (K_{Mapp}) using the Michaelis–Menten equation

$$V = \frac{V_{max}[S]}{K_M + [S]}$$

where V is the reaction rate, V_{max} is the maximum reaction rate, $[S]$ is the substrate concentration and K_{Mapp} is the Michaelis–Menten apparent constant, apparent because the enzyme is immobilized. The enzymatic activity is measured as a function of the substrate concentration and a typical response is reported in Fig. 2. The substrate concentration that produces exactly half of the maximum reaction rate (Fig. 2) is numerically equal to the K_{Mapp} and gives information about the affinity of the enzyme towards the substrate: a low value of K_{Mapp} indicates a high affinity for the substrate. A good biosensor should have the K_{Mapp} value near the K_M value of the enzyme free in solution.

To understand if the enzyme is inhibited by the analyte in an irreversible or reversible way, the procedure should be the one described in Fig. 3; if the enzyme restores the initial enzymatic activity after the measurement, then the inhibition is of reversible type (Fig. 3a); if the enzyme does not restore its initial enzymatic activity instead, the inhibition is of irreversible type (Fig. 3b).

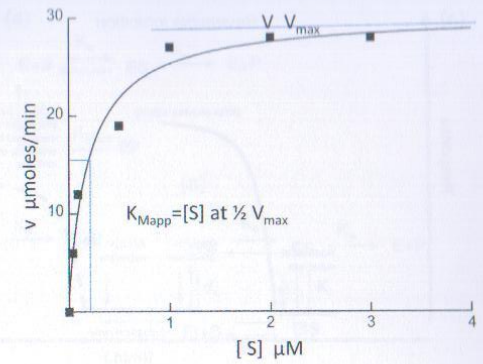

Fig. 2 Typical Michaelis–Menten plot

In the case of irreversible inhibition, the reaction scheme is reported in Fig. 4a. In the case of reversible inhibition, there are different mechanisms involved in interactions between enzyme and inhibitor. Most of the developed biosensors are based either on a *competitive* or *noncompetitive* mechanism. In the first case (Fig. 4b), the inhibitor may bound to the active site center and compete with the substrate for the active site. This equilibrium is regulated by the inhibition constant that describes the affinity of the inhibitor for the enzyme. In competitive inhibition, a high concentration of substrate competes with the inhibitor and prevents the detection of a low concentration of inhibitor. For this reason, in order to reach a low detection limit, the substrate concentration should be chosen as a compromise between a good analytical signal and an inhibition effect still detectable for the needed level. In the case of noncompetitive inhibition (Fig. 4c), the inhibitor binds to both the enzyme and the enzyme–substrate complex, most likely at a site other than the active site, such that the inhibitor does not compete directly with the substrate. In this case, the degree of inhibition is not dependent on the substrate concentration, and this format permits reaching a low detection limit of the analyte/inhibitor more easily, because there is no limitation on using an amount of substrate giving the optimal analytical signal.

The other two mechanisms of inhibition are *uncompetitive inhibition* and *mixed inhibition*. For the case of uncompetitive inhibition (Fig. 4d), the inhibitor binds only the enzyme–substrate complex, so that the degree of inhibition is independent of the substrate when the substrate concentration is higher than the Michaelis–Menten constant value. For the latter case of a mixed inhibition (Fig. 4e), the inhibitor binds the enzyme and enzyme–substrate complex with different affinities as regulated by K_i in the case of the inhibitor–enzyme complex, and αK_i for the inhibitor enzyme–substrate complex. The inhibition is designated *mixed, predominantly competitive* when $\alpha > 1$, or *mixed predominantly uncompetitive* when $\alpha < 1$, and *noncompetitive* when $\alpha = 1$. Thus, noncompetitive inhibition is one of the cases of mixed inhibition. For the evaluation of the inhibition type and the determination of K_i, an exact

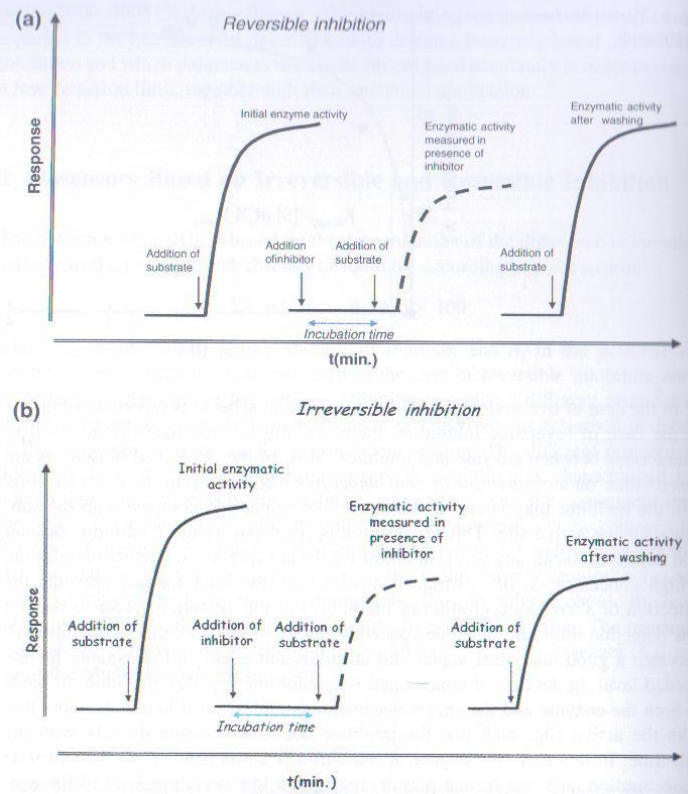

Fig. 3 **a** A typical response before and after inhibition in the case of a biosensor based on reversible inhibition, or **b** irreversible inhibition

evaluation of the mechanism [15–17] can be made by use of the Lineawever–Burk plot, Dixon plot, and Cornish–Bodwen plot. Often in pharmacological and toxicological fields, the I_{50} is commonly reported; it designates the inhibitor concentration required for 50 % of inhibition. I_{50} is often regarded as simply equal to K_i, albeit this is only true for purely noncompetitive inhibition.

Table 1 shows the relations between I_{50} and K_i and calculates the values of I_{50}/K_i ratios when [S] increases from 0.1 to 10 km. Under competitive inhibition, the ratio I_{50}/K_i increases by increasing [*S*], particularly when $[S] > K_m$. In the opposite case, under uncompetitive inhibition, the ratio I_{50}/K_i decreases by increasing [*S*],

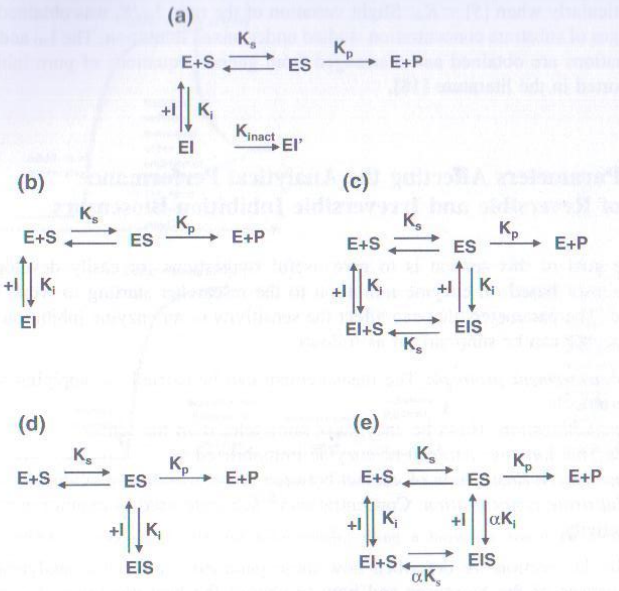

Fig. 4 **a** Scheme of enzyme inhibition in the case of irreversible inhibition, and **b** reversible inhibition competitive, **c** noncompetitive, **d** uncompetitive, and **e** mixed type inhibition

Table 1 Relations between I_{50} and K_i for pure inhibition and for different ratios of $[S]/K_m$

	Competitive inhibition	Noncompetitive inhibition	Uncompetitive inhibition	Mixed inhibition
I_{50}	$(K_m + [S])$ K_i/K_m	K_i	$(K_m + [S])$ $K_i/[S]$	$(K_m + [S])\alpha K_i/$ $(\alpha Km + [S])$
I_{50}/K_i	$1 + ([S]/K_m)$	1	$1 + (K_m/[S])$	$\alpha\ (1 + ([S]/K_m))/$ $(\alpha + ([S]/K_m))$
I_{50}/K_i when $[S]/K_m = 0.1$ and $\alpha = 2$	1.1	1	11	1.05
I_{50}/K_i when $[S]/K_m = 1$ and $\alpha = 2$	2	1	2	1.33
I_{50}/K_i when $[S]/K_m = 10$ and $\alpha = 2$	11	1	1.1	1.83

particularly when $[S] < K_m$. Slight variation of the ratio I_{50}/K_i was obtained in all ranges of substrate concentration studied under mixed inhibition. The I_{50} and I_{50}/K_i equations are obtained and rearranged from general equations of pure inhibition reported in the literature [18].

3 Parameters Affecting the Analytical Performance of Reversible and Irreversible Inhibition Biosensors

The goal of this section is to give useful suggestions for easily developing a biosensor based on enzyme inhibition to the researcher starting to work in this field. The parameters that can affect the sensitivity of an enzyme inhibition-based biosensor can be summarized as follows.

- *Measurement protocol:* The measurement can be carried out applying several protocols.
- *Immobilization:* How the enzyme is immobilized on the sensor.
- *Enzyme loading:* Amount of enzyme immobilized.
- *Incubation time:* Time of contact between enzyme and only inhibitor.
- *Substrate concentration*: Concentration of substrate used to monitor enzymatic activity.

In this section we described how these parameters affect the analytical performances of the biosensor and how to choose the best conditions in order to obtain a sensitive measurement.

3.1 Measurement Protocol

In the case of *reversible* inhibition, the most used approach is based on the measurement of initial enzymatic activity by adding the substrate to the working solution in which the biosensor is immersed (first protocol). Subsequently, the biosensor reaction reaches the steady state and the initial enzyme activity is evaluated (A_0). Next, the inhibitor is added to the same working solution. The addition of inhibitor leads to a decrease of enzyme activity, and, in fact, the signal immediately decreases; after a certain time, the steady state is again reached and corresponds to the enzyme residual activity (A_i; Fig. 5a). Knowing the initial activity (A_0) and residual activity (A_i), it is then possible to calculate the degree of inhibition correlated to inhibitor concentration.

The second protocol is characterized by two different measurements (Fig. 3a). The first one is carried out in the absence of inhibitor (A_0), after which the working solution is replaced with a new working solution to which the inhibitor is then added. Subsequently, the substrate is added and the steady-state signal is recorded

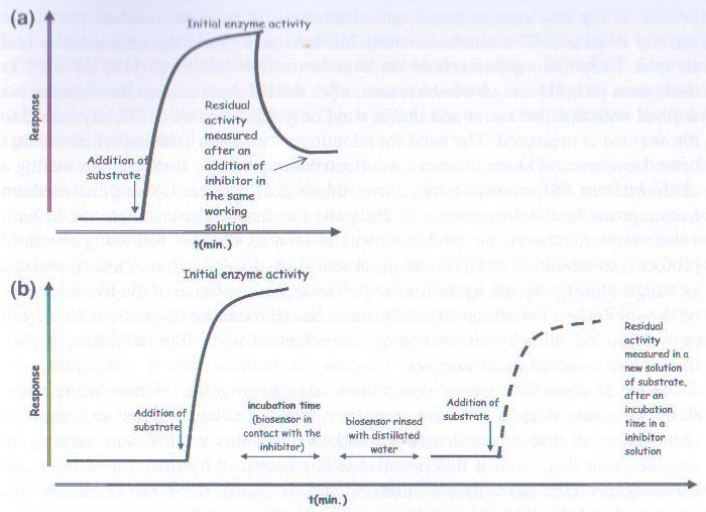

Fig. 5 Different protocols of inhibitor measurement using a biosensor based on enzyme inhibition

and corresponds to the residual activity (A_i). In this case the incubation step is absent. In the case of irreversible inhibitor, the first and second protocols are usually not recommended because they are characterized by a high detection limit. The protocols usually used for the detection of an irreversible inhibitor are the third and fourth protocols. In the third protocol (Fig. 3a), the biosensor is immersed in a buffer solution; the substrate is then added and the signal registered. The biosensor is then immersed in the inhibitor solution for a certain period (incubation time) and afterward, in the same inhibitor solution, the substrate is added and the residual activity measured, registering the signal. The fourth one (also called the "medium exchange method;" Fig. 5b) is performed as follows: the biosensor is immersed in a buffer solution, the substrate is then added, and the signal registered. Afterwards, the biosensor is immersed in the inhibitor solution for a certain period (incubation time). Next, the biosensor is rinsed several times with distillate water. The biosensor is then immersed in a new buffer solution and the substrate added, and the residual activity measured.

In the case of reversible inhibition the first protocol is usually suggested because it is faster and can be carried out in the same step. In the case of irreversible inhibition the fourth protocol is suggested because it allows reaching lower detection limits than the ones obtained using the first and second protocols, and, with respect to the third protocol, it allows avoiding (1) electrochemical and (2) enzymatic interferences. The electrochemical interferences, which can be

present in the real sample tested, are eliminated because the residual enzymatic activity is measured in a new substrate buffered solution in the absence of a real sample. Enzymatic interferences such as reversible inhibitors [19] as well as detergents [20, 21] are avoided because, after the incubation step, the biosensor is washed with distilled water and in this way, only the inhibitor covalently linked to the enzyme is measured. The need for adopting a "medium exchange method" has been demonstrated in the literature as effective for pesticide measurements using a cholinesterase biosensor enzyme, for example [22]. In detail, using the medium exchange method in the presence of 200 ppb of sodium dodecyl sulfate (SDS, limit value for wastewater), no inhibition was observed, whereas following the third protocol, an inhibition of 88 % was observed. With this procedure, the enzyme acts as a high-affinity capture agent for the pesticide, and, because of the irreversibility of the inhibition, the successive enzymatic reaction can be carried out in a fresh buffer solution, thereby circumventing the effect of reversible inhibitors of cholinesterase present in real samples.

However, there is an exception: in the case of irreversible inhibitor nerve agent detection using a cholinesterase biosensor, rapid measurement is an important issue, thus the first protocol can be used to reach this requirement taking into consideration that, even if this protocol is characterized by lower sensitivity, the nerve agents are very strong inhibitors [23]. Recently the same approach was reported by Alonso et al. in whose paper the slope of the biosensor response measurement in the presence of inhibitor is reported, coupled with an artificial neural network and arrays of biosensors with acetylcholinesterase from *Drosophila melanogaster* wildtype and genetically for a rapid determination of a pesticide mixture [24].

3.2 Immobilization

Immobilization is a key step to obtaining a robust and sensitive biosensor. There are several methods for immobilizing the enzyme, such as physical adsorption, cross-linking method, and self-assembled monolayer (SAM) formation. Physical immobilization such as adsorption is one of the simplest procedures to immobilize the biocomponent onto the transducer [25]. Acetylcholinesterase (AChE) was immobilized by adsorption on screen-printed electrodes modified with multiwall carbon nanotubes (MWCNTs): some μL of AChE solution were dropped on the MWCNT modified electrode surface and allowed to dry at room temperature under a current of air. The electrode was then rinsed twice with buffer to remove the loosely adsorbed enzyme molecules [26]. In order to avoid enzyme leakage during the measurement, a Nafion membrane can also be added [27]. An interesting approach was also the one based on the layer-by-layer electrostatic self-assembly of AChE on MWCNTs modified glassy carbon electrodes [28]. The CNTs were initially NaOH treated in order to assume a negative charge and then dipped into a solution of cationic poly(diallyldimethylammonium chloride) (PDDA), which

leads to the adsorption of a positively charged polycation layer (CNT–PDDA). Afterward, the negatively charged AChE was adsorbed on CNT–PDDA to obtain CNT–PDDA–AChE. Finally, in order to avoid the leakage of AChE from the electrode surface, another PDDA layer was absorbed, resulting in a sandwich structure of PDDA/AChE/PDDA. This system allows a low detection limit for paraoxon (4×10^{-13} M).

One of the most common methods of enzyme immobilization is chemical immobilization by means of cross-linking with glutaraldeyde. This method confers high working stability to the biosensor even if it usually causes a decrease of the enzymatic affinity towards its substrate. This behavior is due to the distortion of the enzyme structure with a consequent $K_{M\text{app}}$ higher than K_M obtained for ChE in solution [22, 29]. A valuable approach to immobilize the enzyme is the use of layered double hydroxides, a layered structure built on a stacking of positive layers ($[M^{II}_{1-x}\ M^{III}_{x}(OH)^2]^{x+}$). Their intercalation properties were used for an easy and biocompatible immobilization of enzyme molecules; however, in order to strengthen the resulting inorganic biocoating, glutaraldehyde can be added inducing a partial covalent binding between adjacent enzyme molecules. This approach was used, for example, to assemble a xanthine oxidase biosensor and to investigate the inhibitory effect by allopurinol [30].

The SAM can be an alternative method to immobilize the enzyme close to the electrode surface with a high degree of control over the molecular architecture of the recognition interface. An example can be the biosensor based on AChE enzyme immobilization via glutaraldehyde on a preformed cysteamine (SAM) on gold-screen printed electrodes (Au–SPEs). This method allows the detection of 2 ppb of paraoxon pesticide [31]. Another approach can be step-by-step self-assembly, such as in the case of horseradish peroxidase incorporated into a laponite/chitosan modified glassy carbon electrode for sulfite detection [32]. The enzyme can also be immobilized by means of a polymerization reaction such as the case of peroxidase immobilized on a screen-printed electrode in which acrylamide was used as monomer and the polymerization reaction was initiated by adding $K_2S_2O_8$ [33].

Recently an alternative way to realize a biosensor is enzyme immobilization on magnetic beads. The measurement was performed by the retention of the enzyme-functionalized magnetic beads onto a magnetized electrode. An example reported in the literature is the case of tyrosinase immobilized onto glutaraldehyde-activated streptavidine magnetic particles with a carbon paste electrode for the evaluation of the inhibitory potency of the most frequently used active substances in a cosmetic product [34].

How does the immobilization type affect the kinetic inhibition? In the case of irreversible inhibition, the different types of immobilization used can change the $K_{M\text{app}}$ or the sensitivity towards the inhibitor. In the case of a reversible inhibitor, the immobilization can change the sensitivity such as in the case of an irreversible inhibitor; moreover, the immobilization can also change the type of inhibition as in the case of the inhibition of the polyphenol oxidase [35] immobilized in conducting polymer matrices. The immobilized enzyme modifies the inhibition type

when compared with the free enzyme in the case of cinnamic and sorbic acid, and also changes the inhibition constant (affinity of the inhibitor for the enzyme) as a function of the characteristics of the polymeric films. A clear example about the sensitivity change in the enzyme immobilization function was reported in the research work carried out by Vidal et al. [36], in which the different analytical performances using different immobilization types are reported in Table 1 of the paper for dichlorvos detection using tyrosinase as the enzyme. This means that although the type of inhibition in solution is well known, it is better to restudy the type of inhibition when using a biosensor, in order to know the type of inhibition exactly and easily optimize the parameters for obtaining a sensitive biosensor.

3.3 Effect of Incubation Time

The *incubation time* is the reaction time between the enzyme and the inhibitor. For irreversible inhibition, it is possible to achieve lower detection limits using longer incubation times; in fact, the degree of enzyme inhibition increases with the incubation time [37] until reaching a plateau [38]. The incubation time is usually chosen as a compromise between a sensitive measurement and a measurement carried out in a reasonable time. In the case of reversible inhibition, the degree of inhibition is theoretically independent of the incubation time, which means that the analysis time can be very short because no incubation time is required. The term "theoretically" is required because in the case of an enzyme free in solution, this behavior is always confirmed, whereas in the case of an enzyme immobilized on a sensor, such behavior is not always observed. An example could be the dependence on incubation time reported by Cosnier et al. [39] in the case of As(V) detection through the reversible inhibition of acid phosphatase and polyphenol oxidase entrapped in anionic clays. In this case the use of incubation time was useful to increase the sensitivity only due to specific electrostatic interactions between the positively charged layers of the clay and the As(V) anion that allows a preconcentration fo As(V) in the clay.

3.4 Effect of Enzyme Loading

Enzyme loading is another critical factor to be evaluated. In the case of reversible inhibition using enzyme free in solution, the enzyme concentration does not affect the degree of inhibition if the enzyme concentration is lower than the concentration of the inhibitor. Instead, for irreversible inhibition, the degree of inhibition depends on the enzyme concentration. Specifically, the enzyme concentration should be chosen taking into consideration that (1) the amount of enzyme immobilized should give a measurable signal and (2) the lowest amount of enzyme is necessary to achieve the lowest detection limit. In this view, it is very useful to have a highly sensitive enzymatic product detection and an enzyme immobilization that does not decrease enzymatic activity. In this approach, the use of nanomaterial can be an added value. In fact, using sensors modified with nanomaterials such as carbon nanotubes, carbon black, and gold nanoparticles [40–43] it is possible to reach a lower detection limit for the enzymatic product, because of the promoted electron transfer reaction catalyzed by the nanomaterials. The high sensitivity for the enzymatic product allows the use of a lower amount of enzyme and consequently in the case of irreversible inhibition, better sensitivity of the biosensor. In the case of reversible inhibition, the amount of enzyme should not affect the degree of inhibition; however, for an immobilized enzyme, this behavior is not so strictly respected, such as in the case of a tyrosinase biosensor reported by Shan et al. [44]. In this case the authors studied a set of five membranes with different amounts of enzyme loading, observing an increase of sensitivity up to 30 μg of tyrosinase loading, and then a gradual decrease due to the film thickness effect [44].

3.5 Effect of Substrate Concentration

In the case of irreversible inhibition, a high substrate concentration can be selected in order to obtain a higher output signal; usually the minimum substrate concentration to have the maximum reaction rate is chosen [22]. In the case of reversible inhibition, the choice of substrate amount is not so predictable. For reversible competitive inhibition, the best analytical performance can be obtained using (1) substrate at a concentration lower than that of inhibitor or (2) using a substrate with low affinity for the enzyme. In the first case, as demonstrated by Benilova et al., the degree of inhibition decreases at a high concentration of substrate for butyrylcholinesterase inhibited by α-chaconine [45], whereas in the second case, analytical performance can be improved by choosing the best substrate as demonstrated by Shan et al. [44]. In their study, the inhibition of tyrosinase by benzoic acid was investigated as a function of five different phenolic compounds as substrate (catechol, p-cresol, m-cresol, phenol, and p-chlorophenol). It was found that the highest degree of inhibition was obtained using catechol as the substrate, which showed the highest value of K_M among the five phenolic compounds tested, corresponding to a lower affinity of the enzyme for this substrate.

3.6 Biosensor Stability

As reported by Gibson [46], "One of the main problems of many biosensors is their intrinsic stability.... However, the majority of enzymes used in biosensors are labile and require stabilization to produce viable devices." This means that in order to develop a successful biosensor, it is important to reach low detection

limits but it is also true that the biosensor should be characterized by a satisfactory working and shelf stability where (1) the working stability may be defined as the retention of activity of an enzyme when the biosensor is in use and (2) shelf stability is defined as the enhancement or improvement of activity retention of an enzyme when stored under specified conditions after manufacture [46]. The problem of low shelf stability at room temperature in dry conditions is probably the reason for the gap between academic research and the biosensor presence in the market. This is a relevant problem; in fact, the reduction of the gap between the real market and on-going research is one of the policy priorities of the Horizon 2020 strategy. In this context, for example, we have performed a study to develop a biosensor for organophosphate detection characterized by high working and storage stability [47]. In this study we have investigated several immobilization types also with stabilizers using both AChE and BChE. Briefly, for the construction of AChE or BChE biosensors we have used three approaches: in the first approach, the enzyme was adsorbed onto the working electrode surface, storage at 4 °C overnight, and after that some μL of several stabilizers were added to the working electrode surface. The biosensors were tested when the working electrodes of biosensors were completely dry.

An another approach a similar protocol to the aforementioned was adopted but followed by a final step of Nafion® coverage by simple addition of some μL of Nafion on the working electrode surface and waiting to be dry. The last approach was made using the cross-linking method by means of glutaraldehyde, Nafion, and bovine serum albumin (BSA). In the case of AChE the best result in terms of shelf stability was obtained in the case of AChE immobilized by gelatin, retaining its activity up to 5 months when maintained at RT in dry conditions; however, we observed the possibility of using this biosensor no more than five times because after that, there is a probable solubilization of the membrane. In the case of BChE, the best shelf life stability was obtained using the biosensor constructed immobilizing the BChE by means of glutaraldeyde, Nafion, and BSA. This type of BChE biosensor demonstrated a shelf stability of at least 6 months and a working stability of 10 h, confirming that this biosensor is characterized by high working and storage stability; for this reason this biosensor was chosen to be assembled to a prototype for nerve agent detection for future commercialization [48]. However, in the case of inhibitor measurement the working stability can also be affected by the change of pH, temperature, and matrix composition [49]. In the case of a decrease of enzymatic activity due to the change of these parameters, the degree of inhibition is overestimated, and thus is very important during enzyme inhibition to evaluate the matrix effect in order to avoid false-positive results. In addition, in the case of irreversible inhibition, the enzyme can't recover its original activity and thus only few experiments are carried out with the enzyme. The addition of activator can allow 60–90 % of the initial activity [50–52], but after a few generations the enzyme is totally inhibited. That is why it is recommended to adopt a procedure for the single use of an enzymatic biosensor using, for example, a disposable biosensor [22, 29, 31].

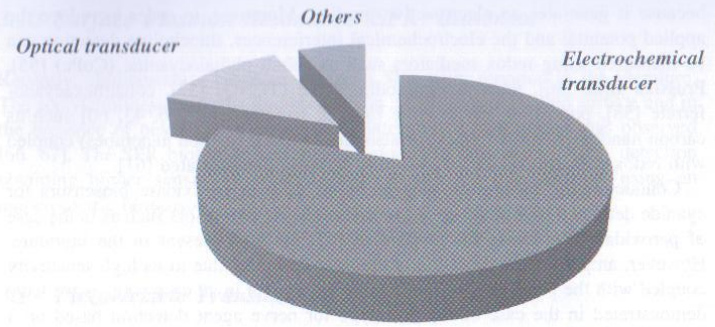

Fig. 6 Distribution of transducers used to construct biosensors based on enzyme inhibition (period 2006–2012)

3.7 Transducer

The choice of transducer is also important in order to have a sensitive, robust, and cost-effective system. As reported in Fig. 6, the most used type of transducer is the electrochemical one (81 % of the biosensors based on enzyme inhibition reported in the literature are electrochemical biosensors) for several reasons, because it is robust, cost-effective, fast, miniaturizable, and used also in the case of colored solutions. In addition, the possibility of using screen-printed electrodes renders this kind of sensor suitable for an easy, fast, and cost-effective measurement for each type of inhibitor both reversible and irreversible, avoiding reactivation in the latter case.

3.8 Electrochemical Transduction

In our first review we discussed the transducer for a cholinesterase biosensor and we divided the electrochemical biosensors into bienzymatic and monoenzymatic ones; on the contrary, in the 2006–2012 period we found only two systems based on the bienzymatic approach, confirming that the monoenzymatic system is the preferable one [53, 54]. As reported in the literature [13], it is possible to obtain a monoenzymatic biosensor employing both a potentiometric or conductimetric transducer, using the natural substrate acetylcholine with acetylcholinesterase. However, in the case of a cholinesterase amperometric monoenzymatic biosensor, the nonnatural acetylcholinesterase substrate acetylthiocholine should be used,

because it generates an electroactive product. Moreover, in order to reduce the applied potential and the electrochemical interferences, thiocholine detection can be measured using redox mediators such as cobalt phthalocyanine (CoPc) [55], Prussian Blue [56], tetracyanoquinodimethane (TCNQ) [57], cobalthexacyanoferrate [58], potassium ferricyanide [59], or nanomaterial [26, 43, 60] such as carbon nanotubes, carbon black, or also nanomaterial (carbon nanotubes) coupled with redox mediator (CoPc), as we have recently demonstrated [61].

Conductimetric biosensors such as in the case of peroxidase biosensors for cyanide detection [62] or square wave voltammetric biosensors such as in the case of peroxidase biosensors for glyphosate [63] are also present in the literature. However, amperometric detection is the most common, due to its high sensitivity coupled with the possibility to miniaturize the system in an easy way, as we have demonstrated in the case of the prototype for nerve agent detection based on a butyrylcholinesterase amperometric biosensor [48].

3.9 Optical Transduction

Optical transducers were also utilized in the case of a biosensor based on enzyme inhibition. A colorimetric method was developed by Pohanka et al. for nerve agent and organophosphate pesticide detection. The principle of the assay is based on the enzymatic hydrolysis of acetylcholine into acetic acid and choline by acetylcholinesterase. Acidification of the reaction medium due to accumulation of acetic acid was visible using a pH indicator strip. The achieved limit of detection was 5×10^{-8} M for paraoxon-ethyl and 5×10^{-9} M for sarin and VX [64].

A biosensor composed of nanostructured multilayers of the enzyme AChE and photoluminescent CdTe QDs was reported in the literature for organophosphate detection [65]. The decrease of enzymatic activity in the presence of organophosphate leads to the decrease of thiocholine production and then the photoluminescent quenching rate of QDs. By measuring the quenching rate before and after an incubation step with the pesticide, one can calculate the concentration of organophosphate with a very low detection limit, 2.89 ppt.

An interesting approach was reported by Li et al. although the format is a bioassay and not a biosensor because the enzyme is used in solution. The system is based on acetylcholinesterase inhibition by an organophosphate compound, and citrate-coated Au nanoparticles were used with a colorimetric probe. The catalytic hydrolysis of acetylthiocholine into thiocholine by acetylcholinesterase induces the aggregation of Au nanoparticles and the color change from claret-red to purple or even grey; in the presence of pesticides the aggregation and the relative color change is absent, whereas in the absence of pesticides there is an AuNP aggregation. The linear range value found was from 0.02 to 1.42 ppm [65].

3.10 Surface Plasmon Resonance (SPR) Biosensor

Recently, cholinesterase biosensors using a SPR were reported in the literature. The acetylcholinesterase was immobilized on a SPR biosensor chip surface and in the presence of pesticides, a changing of intensity of SPR angles was observed [66, 67]. The SPR biosensor was also developed in the case of AFB_1 detection obtaining higher sensitivity when compared with the one obtained using an amperometric biosensor [68].

3.11 Piezoelectric Transduction

The insecticide inhibitors can also be measured by means of piezoelectric biosensors [69, 70]. The detection of organophosphate and carbamate was carried out measuring the precipitation of an enzymatic reaction product over a quartz crystal microbalance (QCM) [71].

3.11.1 Analytes

Pesticides

The detection of pesticide residues in food, water, and soil is one of the major issues of analytical chemistry. Pesticides are, in fact, among the most important environmental pollutants because of their increasing use in agriculture. Among the several pesticides used, organophosphorus and carbammic insecticides are often used due to their high toxicity coupled with low persistency in the environment. This type of insecticide has the power to inhibit the cholinesterase enzyme in an irreversible way, thus they can be detected by means of a cholinesterase biosensor. The amperometric monoenzymatic biosensor based on acetyl or butyrilcholinesterase was principally applied in water samples (drinking water, river water, and wastewater) with good recovery values [22, 31, 61]. An adopted procedure and the typical response for pesticide (paraoxon) detection using a butyrilcholinesterase biosensor based on screen-printed electrode modified with Prussian Blue are shown in Fig. 7a, b, respectively, using the portable instrumentation shown in Fig. 7c. The electrochemical bienzymatic AChE–ChOx was used to detect coumaphos in honey samples [52].

The cholinesterase biosensor was also applied to detect organophosphorus and carbammic insecticides in fruit, vegetables, and dairy products as reported in the literature before 2006 [72]. An interesting application published in 2008 reported the detection of insectides in tomato without any previous manipulation of the sample; in fact, the amperometric biosensor was immersed directly in the tomato pulp during the incubation time, obtaining a recovery of 83.4 % for 50 μM

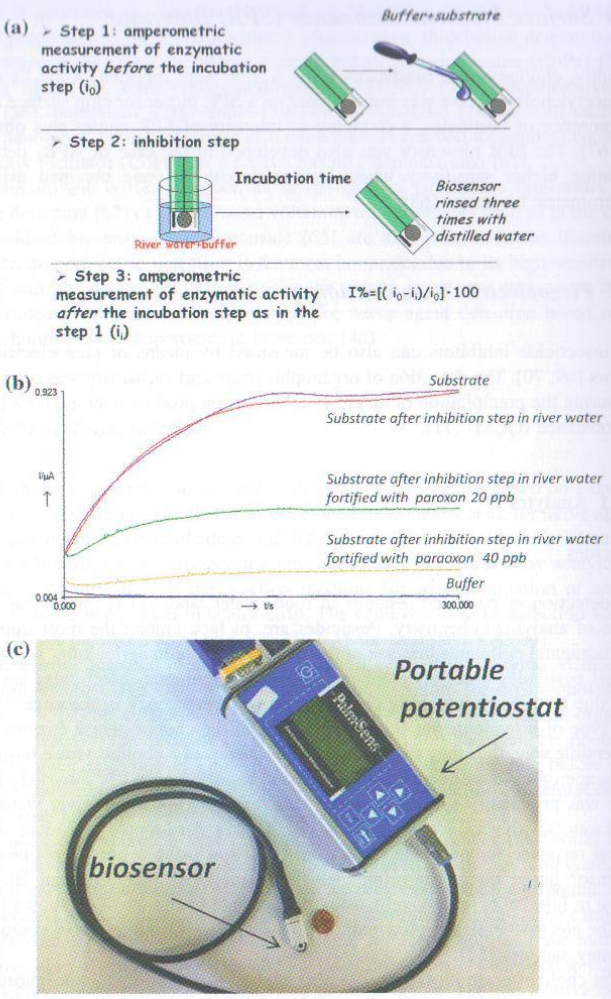

Fig. 7 **a** Procedure used to detect pesticides using the "medium exchange method;" **b** amperograms obtained using the following conditions: applied potential + 0.2 V versus Ag/AgCl, phosphate buffer 0.05 M + KCl 0.1 M, pH 7.4 using BChE biosensor based on Prussian Blue modified screen-printed electrode; and **c** the portable instrumentation used (unpublished data)

carbaryl and showing very low interference from the matrix components [73]. The results reported showed the real possibility to detect organophosphorous and carbamic insecticides using an AChE biosensor in real samples. In the case of organothiophosphate, it is important to stress that these insecticides usually were electrochemically oxidized before the analysis because the oxidized form is able to inhibit the enzyme more strongly [74]. This means that organothiophosphate insecticides themselves are weak inhibitors of ChE: in fact, only their oxo-form is highly toxic. However, in the literature sometimes low detection limits using organothiophosphate are shown, and this could be ascribed to (1) a possible mistake using parathion spontaneously oxidated to paraoxon, (2) the use of organothiophosphate coupled with chemical or electrochemical oxidation in order to have organophosphate–oxo, or (3) the use of a very sensitive biosensor.

Another approach to detect pesticides is the use of tyrosinase, laccase, or peroxidase enzyme. The herbicide glyphosate [N-(phosphonometyl)glycine] was detected using a biosensor with atemoya peroxidase [63]. Carbamate methomyl was measured using a biosensor based on laccase obtained from a genetically modified fungus (*Aspergillus oryzae*) immobilized in a new supported ionic liquid phase formed by platinum nanoparticles/1-butyl-3-methylimidazolium tetrafluoroborate ionic liquid/montmorillonite [75]. A tyrosinase biosensor was used to detect the herbicides atrazine and diuron with a conductimetric biosensor [76]; the insecticide dichlorvos was detected by means of a chronocoulometric biosensor [77]. In the case of tyrosinase, the inhibition is of reversible type with the advantage of using the same biosensor for several measurements, but without the possibility of (1) the use of the medium exchange method in order to avoid electrochemical interferences, and (2) to increase the sensitivity increasing the incubation time.

Heavy Metals

For heavy metal detection, several enzymes can be used such as alkaline phosphatase, glucose oxidase, and acetylcholinesterase. However, in the period 2006–2012, an interesting system for Hg^{2+} was presented by Cosnier et al. [39] using an amperometric bienzymatic system based on the competitive activities of glucose oxidase and laccase [78]. The As^{3+} was detected using acetylcholinesterase immobilized on screen-printed electrodes. As reported by the authors, the As^{3+} is a powerful inhibitor, more so than other metal ions such as mercury, nickel, and copper. The biosensor was challenged in spiked tapwater samples with good recovery values [79]. The same authors instead used acid phosphatase immobilized on screen-printed electrodes for As^{5+} detection at the μM level [80]. The amperometric biosensor based on glucose oxidase for the detection of metallic cations, cadmium, copper, lead, and zinc was developed by Ghica and Brett [81] or coupled with a flow-injection system by Guascito et al. for heavy metal ion (Hg^{2+}, Ag^{+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}) measurements [82].

Toxins

Okadaic acid is a toxin that accumulates in bivalves; this toxin can be detected by means of a biosensor based on protein phosphatase-2A (PP2A) due to the ability of okadaic acid to inhibit this enzyme [83]. The same enzyme was also used to realize a biosensor for microcystin detection, applied to detect this cyanobacterial toxin with a 50 % inhibition coefficient (IC50) of 83 ppb and a limit of detection (LOD; 35 % inhibition) of 37 ppb. Real samples of cyanobacterial blooms from the Tarn River (France) have been analyzed using the developed amperometric biosensor and the toxin contents have been compared to those obtained by a conventional colorimetric protein phosphatase inhibition assay and high-performance liquid chromatography (HPLC). The authors found higher values of R.S.D. (35 %) in the case of the biosensor due to the less steep slope of the curve (which induces a higher uncertainty on the concentration values) coupled with reproducibility problems associated with the electrode construction and with fouling phenomena [84].

Recently we have demonstrated the possibility of detecting the toxin AFB_1 by means of acetylcholinesterase [85]. Prior to developing a biosensor, a bioassay using a choline oxidase biosensor was applied to detect AFB_1 in olive oil obtaining recovery values higher than 75 % [86]. In the case of a biosensor in which the enzyme was immobilized, a higher detection limit was found. For example, Hansmann et al. developed an AChE biosensor depositing 3 μL of a 1:1 mixture of polyvinylalcohol and enzyme on cobalt–phthalocyanine-modified screen-printed electrode and polymerized the mixture under neon light at 4 °C for 3 h. This sensor allows the detection of a minimum concentration of 3 μM of AFB_1 corresponding to 1 ppm [87]. We have developed an amperometric biosensor for detecting AFB_1 at ppb levels using AChE immobilized on Prussian Blue-modified screen-printed electrodes by means of physical immobilization. The AChE immobilized in a gelatin layer allows obtaining a LOD of 100 ppb. Pohanka et al. have developed a biosensor immobilizing the enzyme with a gelatin layer, obtaining IC_{50} = 100 ppb [88]. Recently Puiu et al. have immobilized AChE via its primary amine groups (lysine residues) through 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) hydrochloride and N-hydroxysuccinimide on the (SAM) functionalized surface of the surface plasmon resonance chip. The LOD was 0.94 ppb for AFB_1 which is lower than the ones previously reported [68].

Drugs and Clinical Application

In the case of drugs, the application of a biosensor is rather limited to academic research because, at this time, for quality measurement the US Food and Drug Administration requires highly selective methods. However, this does not mean that in the future the US Food and Drug Administration could not also accept the biosensor as a screening method in the pharmaceutical industry.

In order to detect amaryl and acorbose, which are therapeutic drugs that prevent high blood glucose levels, α-glucosidase enzyme was immobilized on bismuth-

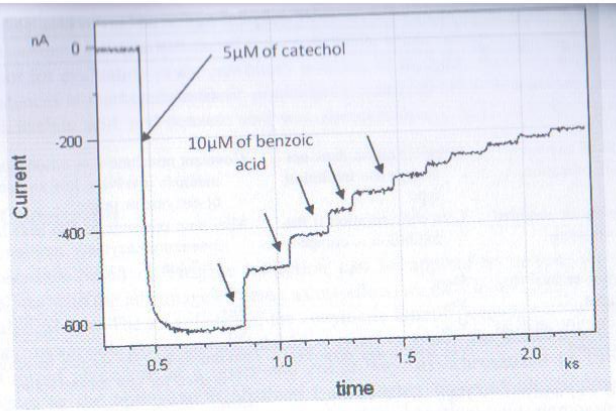

Fig. 8 A typical amperogram in the case of benzoic acid measurement using a tyrosinase biosensor. Enzyme tyrosinase cross-linked with glutaraldehyde and deposited on a carbon paste electrode. Applied potential is −0.15 V versus Ag/AgCl, phosphate buffer pH 7.0. After stabilization of the catechol signal, successive additions of benzoic acid were added (unpublished data)

modified glassy carbon electrodes, obtaining a linear range of 0.002–0.24 mg/mL and 0.0008–0.0066 mg/mL for amaryl and acorbose, respectively [89]. Allopurinol is used to treat hyperuricemia and it was measured by means of an amperometric xanthine oxidase biosensor [30]. Glutathione is detected by means of an amperometric biosensor based on pyranose oxidase inhibition [90]. Several thiols such as glutathione, N-acetylcysteine, and cysteine were detected using amperometric biosensors based on peroxidase inhibition [91, 92].

Others

The chemical warfare nerve agent can be detected by means of a cholinesterase biosensor. Our group developed a butyrylcholinesterase biosensor immobilizing the enzyme on screen-printed electrodes modified with Prussian Blue. The system was challenged towards two different concentrations of Sarin gas (0.1 mg/m^3 and 0.5 mg/m^3) at different incubation times (from 30 s up to 10 min) demonstrating that it is possible to detect the Sarin at a concentration of 0.1 mg/m^3 with only 30 s of incubation time [29]. A butyrylcholinesterase biosensor was also integrated into a miniaturized prototype composed of the cell in which the biosensor is inserted, a little fan for sampling the air, and an electronic circuit. The circuit was able to

Table 2 Practical considerations of biosensors based on reversible and irreversible inhibition

	Reversible inhibition	Irreversible inhibition
Enzyme loading	Medium	Very low
Time of incubation of enzyme with inhibitor	No incubation is required	10–60 min
Method of enzyme immobilization	Immobilization does not change the inhibition type	Covalent attachment or adsorption methods involving low amount of enzyme/gr or/cm^2
Concentration required of substrate	Low concentration if the inhibition is competitive	Minimum concentration to have the maximum enzymatic reaction rate ($\approx$2 km)
Total time of analysis	Short	Long
Batch analysis	Yes	Yes
Flow injection analysis	Yes	No
Regeneration	Yes with water or buffer	No unless specific reagent (reactivator) is used for few regenerations
Frequency of analysis per hour	High	Low
Limit of detection	Very low if K_i is very low	Very low
Matrix effect	Exposed to interfering species of the matrix	Less sensitive to matrix effect using the "medium exchange method" (see Fig. 5b)

apply the potential, to register the current, to turn the fan on and off, and eventually give an alarm [47].The acetylcholinesterase electrochemical biosensor was also developed to measure Tabun, Sarin, Soman, Cyclosarin, and VX in solution [93].

Cyanide is a substance commonly used worldwide in industrial applications often resulting in contamination of groundwater. Biosensors prepared through immobilization of bovine liver catalase in a photoreticulated poly(vinyl alcohol) membrane on the surface of a conductimetric transducer was reported in the literature. The biosensor allows cyanide detection with LOD of 6 μM [94].

Fluoride is a reversible inhibitor of tyrosinase, thus it can be detected by means of an amperometric tyrosinase biosensor as reported by Asav et al. reaching a linear range from 1.0 to 20 μM [95].

Sulfide is able to inhibit peroxidase and thus, a biosensor using horseradish peroxidase has been developed characterized by a detection limit of 5 μM [32], whereas using a biosensor based on *Coprinus cinereus* peroxidase, a detection limit of 0.3 μM was found [96].

Benzoic acid is extensively used as a preservative in food, beverages, mouthwashes, cosmetics, and pharmaceuticals. Amperometric tyrosinase-based biosensors for benzoic acid determination with the use of a flow-batch monosegmented sequential injection system were reported in the literature with the detection limit of 0.03 μM [97]. Highly sensitive biosensors based on the immobilization of tyrosinase by calcium carbonate nanomaterials were applied from the determination of benzoic acid in real beverage samples [46]. A typical amperogram of benzoic acid measurement using a tyrosinase biosensor is shown in Fig. 8. The tyrosinase biosensor for evaluation of the inhibitory potency of the most frequently used active substances in marketed cosmetic products against hyperpigmentation such as kojic acid, azelaic acid, and benzoic acid was also reported in the literature [34].

4 Conclusion

Biosensors based on enzyme inhibition can be applied to monitoring several analytes with the advantage of being a cost-effective, easy to use, and miniaturized analytical tool. The knowledge of the enzymatic kinetic (knowing if the inhibition is an irreversible or reversible inhibition type) can help the researcher reach the optimized procedure in a faster and easier way, as schematized in Table 2 in respect to the optimization obtained by changing one variable at time. Wide application in the environmental, food, and pharmaceutical fields demonstrates the suitability of this tool also with real samples.

We also highlight that in the case of organophosphate detection, low detection limits using organothiophosphate could be ascribed to a possible mistake using parathion spontaneously oxidated to paraoxon, thus the presence of the oxo-form in the standard solution used should be checked.

In this overall scenario, it is important to highlight the low selectivity to the biosensor based on enzyme inhibition. This characteristic can be an advantage as in the case of the cholinesterase inhibition that can be considered a "family doctor," whereas in the presence of inhibition, a specialist doctor such as an HPLC instrument can be used with a decrease in terms of time analysis and cost. However, sometimes the selectivity of the biosensor can be improved by optimizing the procedure knowing the enzymatic kinetic [13] or also optimizing the sample treatment; thus it can be very useful during biosensor-based inhibition development to evaluate interferences or demonstrate selectivity using a sample fortified with several analytes, because very often in the papers based on enzyme inhibition reported in the literature the authors focused their attention on a single analyte detection instead of which the biosensor is able to detect a class of compounds instead of a single compound. In this direction, we propose using the biosensor based on enzyme inhibition as a first alarm system in order to give, even if not specific, a fast and cost-effective response.

Development of an array of enzyme electrodes for multianalyte detection and the use of the chemiometric method for interpretation of experimental data in the analysis of these mixtures of inhibitors may allow in the near future the conversion of biosensing systems to marketable devices suitable for large-scale applications.

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