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Chapter 29

Rapid detection of organophosphates, Ochratoxin A, and Fusarium sp. in durum wheat via screen printed based electrochemical sensors

D. Compagnone, K. van Velzen, M. Del Carlo, Marcello Mascini and A. Visconti

29.1 INTRODUCTION

29.1.1 Biotic and abiotic contaminants in durum wheat

Durum wheat safety is affected by different threats comprising both abiotic and biotic agents. In the first class, pesticides are widely represented including neurotoxic molecules such as organophosphates and carbamates. Organophosphorus (OP) compounds are substances widely used in agricultural practices as pesticides having low environmental persistence and high efficacy. These compounds act by inhibition of acetylcholinesterase (AChE) activity as neurotoxic agents via an excessive stimulation of the cholinergic receptors in both insects and mammals, including humans [1]. This can lead to various clinical implications and high acute toxicity [2]. Among organophosphates dichlorvos (2,2-dichlorovinyl dimethyl phosphate) and pirimiphos methyl (*O*-[2-(diethylamino)-6-methyl-4-pyrimidinyl]*O,O*-dimethylphosphorothioate) are important contaminants for the durum wheat industry. Dichlorvos is one of the most widely used pesticides worldwide in the storage of many products, such as corn, rice, and durum wheat, finding widespread use in most European countries [3]. European Union regulation foresees a maximum residue limit (MRL) for dichlorvos in durum wheat at $2.0 \, \mu g/g$ [4]. Dichlorvos is also classified as a probable human carcinogen on the basis of the effects observed in mice and rats. Therefore, the U.S. Environmental Protection Agency (EPA) proposed cancellation of most uses of dichlorvos and proposed

restrictions on retained uses. In particular, because of dietary cancer risk, the EPA proposed cancelling uses of dichlorvos in processed agricultural commodities that are stored in bulk, packages, or bags including durum wheat and its derivates such as flour and pasta [5]. Pirimiphos methyl is an organothiophosphate pesticide that is active by contact, ingestion, and vapor action, and causes inhibition of AChE of tissues, determining accumulation of acetylcholine at cholinergic neuro-effector junctions (muscarinic effects), and at skeletal muscle myoneural junctions and autonomic ganglia [6]. As well as other thiophosphate molecules, pirimiphos methyl is active in its oxon product that appears to be the active metabolic intermediary. It is mainly used for storage of grain products such as corn, rice, durum wheat, and sorghum where it shows an excellent persistence as reported in the IPCS-INTOX databank of the World Health Organization (WHO) [7].

Among biotic agents fungi that produce toxins are an important and widespread threat that can contaminate cereals.

Fusarium species, including *Fusarium culmorum*, are universal fungal contaminants of maize, wheat, barley, and other small cereals. *F. culmorum* is a pathogen causing "foot rot" and "head blight" diseases and can produce mycotoxins such as zearalenone, deoxynivalenol, and other trichothecenes that can enter the food chain [8–10]. The early identification of this fungal pathogen is therefore crucial in order to avoid crop losses and protect consumer health [11–13]. Other fungi belonging to *Aspergillus* and *Penicillium* species can contaminate cereals including durum wheat resulting in a possible contamination with ochratoxin A (OTA), (7-(L-β-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocumarin). OTA is a mycotoxin produced by several *Aspergillus* and *Penicillium* species growing in different agricultural commodities in the field or during storage [14,15]. OTA has been shown to be nephrotoxic, teratogenic, carcinogenic, and immunotoxic to several animal species. The International Agency for Research on Cancer (IARC) has classified OTA as possibly carcinogenic in humans (Group 2B) [16]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), after evaluation of OTA nephrotoxicity, proposed for this mycotoxin a provisional tolerable weekly intake (PTWI) of 0.1 µg/kg body mass (equivalent to 14 ng/kg body mass/day) [17].

Recently, the European Commission fixed the maximum levels for OTA at 5 µg/kg in cereals, derivative products, and roasted coffee, 3 µg/kg in all cereal products intended for direct human consumption, 10 µg/kg in dried vine fruits and soluble coffee, and 2 µg/kg in wine, grape juice,

and must [18]. Lower levels (0.5 µg/kg) have been established in foods for infants and young children [19].

29.1.2 Screen-printed electrochemical sensors for the detection of acetylcholinesterase inhibitors

OP compounds and carbamate are widely used as insecticides, pesticides, and warfare agents [20,21]. Detection of pesticides is usually carried out by multiresidue methods (MRMs) of analysis, which are able to detect simultaneously more than one residue and have been developed mainly based on chromatographic techniques. Two groups of MRMs are used: (i) multiclass MRMs that involve coverage of residues of various classes of pesticides, and (ii) selective MRMs, which concern multiple residues of chemically related pesticides (e.g., *N*-methyl carbamate pesticides (NMCs), carboxylic acids, phenols, etc.). As foods are usually complex matrices all of the pre-analytical steps (matrix modification, extraction, and clean-up) are often necessary.

The anticholinesterase activity of organophosphate and carbamate has been used as the basis to build up a number of detection schemes for these classes of compounds. A large number of applications in pure standard solution or in environmental samples have been published [22] but only few applications on real food samples have been reported. In spite of the significant demand for sensing pesticides in food samples, it seems that the use of biosensors penetrate very slowly in the food industry. Since the inhibition of AChE by OP is irreversible and because of the matrix fouling of the sensing devices, a cheap, disposable electrode is foreseen as a simplification in the analytical procedure. Single-use biosensors, based on thick- or thin-film technology, are of high interest for real sample analysis due to their low cost, which make them disposable, and their compatibility with portable instrumentation for in-loco measurements.

Most of the inhibition bioassays or biosensors for organophosphate and carbamate pesticides are based on the amperometric detection of the enzymatic product of the reaction.

Some applications of amperometric biosensing strategies for pesticide detection in real or spiked food samples have been recently reported. Most of the applications have been developed for vegetable matrices. Different formats of biosensors have been used: disposable screen-printed choline oxidase biosensors [23] using AChE in solution were utilized to detect pesticides in real samples of fruit and vegetables.

Potato, carrot, and sweet pepper samples spiked with aldicarb, propoxur, carbaryl, carbofuran, and methomyl tested with this AChE biosensor, exhibited acceptable recoveries (79–96%) [24].

An interesting application on spiked (aldicarb, carbaryl, carbofuran, methomyl, and propoxur) fruit and vegetable samples was based on screen-printed electrodes (SPEs) chemically modified with a carbon paste mix of cobalt(II)phtalocyanine and acetylcellulose [25]. In this paper, an appealing solvent-free extraction procedure of the spiked samples is reported. This was performed by mixing the fruits and passing the resulting juice through a sieve. No effect of the matrices pH on the biosensor performance is reported. Screen-printed sensor developed using photolithographic conducting copper track, graphite–epoxy composite, and either AChE or butirrylcholinesterase was also used in the analysis of spiked (paraoxon and carbofuran) samples of tap water and fruit juices at sub-nanomolar concentration [26].

A recent application of a tetracianoquinodimethane (TCNQ)-modified SPE for the development of a biosensing device for chlorpyrifos methyl was also reported. This method was demonstrated to detect the active molecule both in standard solution and in commercial products (Reldan®22) with comparable sensitivity. The analytical protocol was then applied to grapes and vine leaf samples in order to improve safety in wine-making process [27].

Other food samples that have been recently investigated with electrochemical biosensor based on AChE inhibition are infant foods. The European Union has set a very low limit (10 µg/kg) for pesticides in infant food [28]. An amperometric biosensor that met these limits both for infant food and orange juice has been reported. The method included an oxidation step of phosphorothionates pastiches to produce the oxygenated derivative, which represent the active pesticide molecule. Moreover, the biosensors could be regenerated via the recovery of AChE activity through a chemical activation with pyridine-2-aldoxime methochloride (PAM). The biosensors performed well in solvent extract containing water, though they exhibited a reduced recovery in food with a lower water content [29].

Following work by the same group addressed some of the major problems arising when electrochemical biosensors are in contact with food matrices: pH effect and particle effect. Both problems were solved treating the biosensor surface with a Tween20®/phosphate buffer solution (pH 7.5) after the incubation with pesticide. The treatment was successful in removing the particulate, the correct pH for enzyme activity measurement was attained and the pesticide enzyme inhibition

retained. A large number of samples were analyzed and the results were in agreement with reference standard methods [30].

To our knowledge, there are only few reports on application of screen-printed cholinesterase-based biosensors to food samples. Many other amperometric detection schemes and chemistries have been recently investigated that could enhance the overall analytical performance of screen-printed devices. Nevertheless, the possibility to successfully apply these approaches to real food samples is strictly linked to their ability to surmount the matrix effect that leads either to complicated multi-step sample preparation or to poor recovery.

Carbon nanotubes (CNTs) are an emerging class of material due to their exceptional structural and electronic characteristics [31]. The possibility of the promotion of electron transfer at a lower overpotential and their high surface area provide the basis for improving biosensing systems [32]. They catalyze the electrochemical reaction of important analytes involved in biosensor development such as NADH [33], thiols [34], and hydrogen peroxide [35]. Moreover, CNTs appear to be an interesting material for the immobilization of biological molecules [36–38] in biosensor applications [39–41]. The fabrication and evaluation of CNT-derived screen-printed disposable electrochemical sensors based on a CNT ink has been reported [42]. CNTs have recently been used in the development of a disposable biosensor based on thick film-strips for the sensitive detection of OP pesticides. In this work, the dual role of CNT, electrocatalytic activity toward thiocholine and immobilization matrix for the enzyme, is demonstrated leading to the development of a redox mediator-free, simple, and robust single-enzyme biosensor able to detect 0.5 nM of paraoxon in solution [43].

An interesting and emerging technology that has led to astonishing results in OP determination is the use of sonochemical fabrication of SPE to obtain micro-electrode array. This technology result in the ablation of non-conductive polymer films that coat, and so insulate, underlying conductive surfaces. Sensors were fabricated via the electro-polymerization of an insulating film at planar electrode surfaces and then ablated with ultra-sound to expose microdiameter scale areas of underlying conductor. A second polymer of polyaniline with conducting properties, carrying co-entrapped AChE, may then be electrodeposited *in situ* at the micro-electrode cavities forming an enzyme micro-electrode array. With this device the authors obtained a stir-independent response, as expected for micro-electrodes able to detect as low as 10^{-20} M of dichlorvos. The authors claim that this astonishing result is due to the combined effect of enzyme inhibition amplification because of the

biosensor geometry and the use of recombinant AChE selective for the particular pesticide used [44].

To complete this overview on the strategies that are pursued to enhance the sensitivity and stability of screen-printed biosensors for the detection of pesticides new immobilization approaches have to be mentioned. SPE modified with concanavalin A have been used to bind, via a high affinity interaction, AChE. The obtained biosensors, optimized for manufacturing conditions, were able to detect 10^{-8} M of clorpyrifos [45].

Vakurov *et al.* [46] evaluated a strategy to improve the covalent binding of AChE to screen-printed carbon electrodes modified with polyamines. To improve the extent of dialdehyde modification, electrodes were aminated. Initially, this was performed by electrochemical reduction of 4-nitrobenzenediazonium to a nitroaryl radical permitting attachment to the carbon surface; subsequent reduction of the 4-nitrobenzene yielded a 4-aminobenzene-modified carbon surface. The obtained biosensors resulted in very sensitive devices measuring as low as 10^{-10} M of OPs.

The application of such interesting technologies to real food samples has to overcome the difficulties of extraction strategies and the problems arising from matrix components that can affect the biosensor performance. In the recent years, our group have been involved in the realization of biosensing strategies based on SPEs for the detection of dichlorvos and pirimiphos methyl in durum wheat. The proposed methods were all based on a choline oxidase biosensor obtained by modification of carbon SPEs able to determine AChE activity in solution. In order to avoid cumbersome regeneration steps, we decide to use free cholinesterases rather than immobilized on the choline oxidase biosensor surface.

We successfully applied an AChE inhibition assay to the detection of dichlorvos in durum wheat samples using a simplified extraction procedure. The aqueous extraction solvent (phosphate buffer) was used as the assay buffer, thus simplifying the overall procedure and limiting the negative effects of organic solvents on enzyme activity. The total assay time, including the extraction step, was 30 min. The choline oxidase biosensor exhibited an excellent stability: after 20 days from preparation, the blank measurement lost only 10% of the signal intensity. The calculated limits of detection (LODs) in buffer solution were 10 and 0.05 ng/mL, respectively, using either electric eel AChE (eeAChE) or a recombinant AChE specifically engineered to be inhibited by dichlorvos (rAChE) [47].

The developed assay was also applied to milled samples. An optimized extraction protocol using exane as extraction solvent exhibited

recoveries of dichlorvos at 0.25–1.50 µg/g from 96.5% to 100.9%, with a LOD of 0.02 µg/g. An aliquot of the filtered hexane extract was partitioned with phosphate buffer solution, and the organic layer was evaporated prior to electrochemical analysis. An LOD of 0.05 µg/g of dichlorvos was obtained with mean recoveries of 97–103% at spiking levels of 0.25–1.50 µg/g. A good correlation (0.9919) was found between the results obtained with the electrochemical and those obtained with the gas chromatographic methods. The electrochemical method was peer-validated in two laboratories that analyzed 10 blind samples (five duplicates), including a blank and four spiked samples with dichlorvos at levels of 0.25, 0.60, 1.00, and 1.50 µg/g. Within-laboratory repeatability (RSD_r) and between-laboratory reproducibility (RSD_R) ranged from 5.5% to 7.8% and from 9.9% to 17.6%, respectively [48].

The possibility of detecting a phosphotionate pesticide in durum wheat has been also investigated. The determination was accomplished via chemical oxidation of the phosphothionate molecule both in buffer and in matrix extract solution optimizing the experimental parameters (reagents concentration and reaction time). The procedure was then applied to standardize the pirimiphos methyl detection obtaining the calibration curves under different conditions. The LOD with matrix extract was 50 or 100 ng/mL, depending on the extract % addition, which allowed the detection of samples contaminated at the MRL = 5 mg/kg. The samples mean recovery was 70.3% and no false positive samples were detected [49].

29.1.3 Screen-printed electrochemical DNA sensors for identification of microorganisms

In the last 20 years, there has been a continuous increase in the use of nucleic acid combined with electrochemical transducers to produce a new kind of affinity biosensor. Among the different kind of electrochemical sensor formats available, SPE based on thick and thin film technology have played an important role. This is surely due to their recognized advantages in terms of cost that allow their disposable use.

As well as other molecular-based biosensors DNA biosensors rely on highly specific recognition events to detect their target analytes. The role of the transducer is to provide a suitable platform that facilitates formation of the probe-target complex in such a way that the binding event triggers a usable signal for electronic readout. According to IUPAC definition, a biosensor includes a molecular recognition layer and a signal transducer that can be coupled to an appropriate readout

device. DNA is especially well suited for biosensing applications, because the base-pairing interactions between complementary sequences are both specific and robust.

Different strategies of electrochemical sensing have been used for DNA electrochemical detection: (1) direct DNA electrochemistry, (2) indirect DNA electrochemistry, (3) DNA-specific redox indicator detection, (4) DNA-mediated charge transport, and (5) nanoparticle-based electrochemistry amplification.

Direct DNA electrochemistry allows highly sensitive detection of DNA, down to femtomoles of target sequence without a labelling step, which simplifies the overall detection protocol, the main limitation of this approach being the high background signals that often limits a simple readout. This strategy has been applied to different kinds of electrodes in terms of geometry and material ranging from the hanging mercury drop electrode [50], carbon paste electrode [51], to screen-printed carbon transducers [52]. Methods to oxidize target DNA indirectly through the use of electrochemical mediators have also been explored. An especially attractive approach uses polypyridyl complexes of Ru(II) and Os(II) to mediate the electrochemical oxidation of guanine [53]. This detection approach provide high sensitivity without complex instrumentation through redox-mediated indirect DNA oxidation, the main limitation consisting in the electrode preparation that can be difficult to handle. Several applications have been described in which target DNA sequences are labelled with redox-active reporter molecules. Appearance of the characteristic electrochemical response of the redox reporter signals the occurrence of the hybridization event. Using physical separation methods to isolate the labelled sequences, LODs of the order of $\sim 10^{-10}$ molecules have been reported [54,55]. As an alternative to chemical labelling schemes, DNA-mediated charge transport electrochemistry has been exploited as detection scheme using redox-active molecules that noncovalently associate with the double helix. In these analyzes, rather than serving as a reactant, the DNA is the mediator. These assays can provide high sensitivity and simplicity. Nanoparticle-based electrochemistry amplification is another detection scheme that has been exploited. In this case, intercalative probe molecules are used taking advantage of the electronic structure of double-helical DNA, using intercalated redox probe molecules to report the perturbations occurring in base stacking [56]. In this format the intercalator is not used to report the amount of DNA or whether it is double stranded versus single stranded; here the DNA base pair stack mediates charge transport to the intercalator bound to the DNA. The method relies on the switch of inherent characteristic of

duplex DNA and its ability to mediate charge transport is used for the detection. Assays of DNA-mediated electrochemistry, using both DNA-mediated charge transport, and nanoparticle-based electrochemistry amplification, are therefore uniquely suited to sense changes in DNA: damage, mistakes, mismatches, and even protein binding [57,58].

DNA biosensors, based on the summarized schemes, have been proposed in different area of analysis: environmental, clinical, food control, biotechnology, and pharmaceutical.

In this report, we review some recent applications of electrochemical DNA screen printed based biosensor for the detection of microorganisms including, viruses, bacteria, and fungi. An electrochemical hybridization biosensor based on the intrinsic oxidation signals of nucleic acids (guanine oxidation) and proteins (tyrosine and tryptophan) has been designed, that makes use of the unique binding event between *Escherichia coli* single-strand binding (SSB) protein and single-stranded DNA (ssDNA). The voltammetric signal from guanine oxidation significantly decreased upon binding of SSB to single-stranded oligonucleotides (probe), anchored on a single-walled CNT-modified screen-printed carbon electrode. Simultaneously, oxidation of the tyrosine and tryptophan residues of the SSB protein increased upon binding of the SSB protein to ssDNA and ss-oligonucleotides. The LOD of 0.15 µg/mL of target DNA can be applied to genetic assays [59]. Some work devoted to food safety resulted in the realization of an electrochemical biochip able to specifically detect *Bacillus cereus*. *B. cereus* constitutes a significant cause of acute food poisoning in humans. The DNA biosensor was specifically designed using a capture probe for the toxin-encoding genes. A bead-based sandwich hybridization system was obtained in conjugation with electric chips for detection of both vegetative cells and spores of *Bacillus* strains. The system consisted of a silicon chip-based potentiometric cell, and utilizes paramagnetic beads as solid carriers of the DNA probes. The specific signals from 20 amol of bacterial cell or spore DNA were achieved in less than 4 h. The method could be also applied directly to unpurified spore and cell extract samples. The developed method can offer a contribution in the rapid identification of *Bacillus* strains without preceding nucleic acid amplification [60]. A recent advancement in DNA biosensor consisted in the realization of an electrochemical microfluidic biosensor with an integrated minipotentiostat for the quantification of RNA based on nucleic acid hybridization and liposome signal amplification for dengue virus detection. The detection scheme was ensured by short DNA probes that hybridize with the target RNA or DNA sequence. A reporter probe, coupled to liposomes entrapping the electrochemically active redox couple

potassium ferri/ferrohexacyanide, was used to amplify the recognition reaction. The capture probes were bound to superparamagnetic beads that were isolated on a magnet in the biosensor. Upon hybridization reaction, the liposomes were lysed to release the electrochemical markers. The detection occurred at interdigitated ultramicroelectrode array. The system was completed by a miniaturized instrumentation (miniEC). The functionality of the miniEC was successfully demonstrated with the detection of dengue virus RNA [61]. A biosensor for virus detection, particularly for SARS (severe acute respiratory syndrome) virus, has been described by Abad-Valle *et al.* [62]. The functioning scheme was a typical hybridization biosensor constructed on a gold thin-film electrode. The selected target, a 30-mer sequence, was chosen in a lysine-rich region, unique to SARS virus and the complementary strand, the probe, was immobilized to the gold surface. Hybridization reaction with the biotin-conjugated SARS strand (at the 3′-end) was obtained and monitored using alkaline phosphatase (AP)-labelled streptavidin that permitted amplified indirect electrochemical detection. The analytical signal is constituted by an electrochemical process of indigo carmine, the soluble product of the enzymatic hydrolysis of 3-indoxyl phosphate. The use of a sensitive electrochemical technique such as square wave voltammetry allowed an LOD of 6 pM to be obtained for this DNA sequence [62]. A multiple pathogen detection system based on hybridization coupled with bioelectronic detection was described by Vernon *et al.* [63]. The method was developed using papillomaviruses as a model system. In this case, two chips were spotted with capture probes consisting of DNA oligonucleotide sequences specific for HPV types and two electrically conductive signal probes were synthesized to be complementary to a distinct region of the amplified HPV target DNA. The measuring system was able to successfully detect 86% of the HPV types contained in clinical samples demonstrating the feasibility of integrated detection of multiple pathogens.

Recently, our group has devoted some research activity to the realization of DNA screen-printed biosensors for the detection of fungi [64]. To our knowledge there is no evidence in the literature of analysis of fungi via electrochemical detection of DNA or RNA.

In our research, we proposed a post amplification analytical method to detect *F. culmorum*, a pathogen causing "foot rot" and "head blight" diseases in cereals, and can produce mycotoxins such as zearalenone, deoxynivalenol, and other trichothecenes that can enter the food chain. The early identification of this fungal pathogen is therefore recommended in order to avoid crop losses and protect consumer health [65].

The sensing principle of this work is based on the hybridization reaction between a probe and the complementary target sequence. The hybridization is followed by the electrochemical detection using a square wave voltammetry (SWV). The direct label-free detection was accomplished by monitoring the guanine oxidation peak of the target sequence relying on the use of inosine-modified (guanine-free) probes [66–68]. The specificity and selectivity of the methods aroused from the selection of four different probes immobilized on sensor surface, complementary to different regions of the amplified sequence. A sequence was identified that is able to grant selectivity and specificity to the sensing system.

29.1.4 Screen-printed electrochemical immunosensors for the detection of toxins

Immunosensors are analytical devices, which selectively detect analytes and provide a concentration-dependent signal [69]. Electrochemical immunosensors employ either antibodies or their complementary binding partners (antigens) as biorecognition elements in combination with electrochemical transducers [70]. Among electrochemical transducers, SPE appears as the most useful due to their characteristics [71]. SPEs are mass produced and therefore the single device has a low cost as compared to conventional electrodes, hence they can be used in a disposable manner, avoiding cumbersome regeneration steps following the sample measurement. They have a small size thus reducing the volume of sample that can be analyzed (20–100 μL).

Some work has been carried out from Prof. Palleschi's group in Rome leading to the development of disposable immunosensor for the determination of domoic acid (DA) in shellfish [72]. DA is neurotoxic amino acid responsible for the human syndrome known as "amnesic shellfish poisoning" (ASP). The method involves the use of disposable SPEs for the immunosensor development based on a "competitive indirect test". DA conjugated to bovine serum albumin (BSA–DA) was coated onto the working electrode of the SPE, followed by incubation with sample (or standard toxin) and anti-DA antibody. An anti-goat IgG–alkaline phosphatase (AP) conjugate was used for signal generation via the use of an electrochemical substrate, naphtyl phosphate. The enzyme product, naphtol was detected by differential pulse voltammetry (DPV). The immunosensors allowed the detection of 20 μg/g of DA in mussel tissue. Moving toward toxins produced by fungi, the same group has devised an electrochemical immunosensor for aflatoxin M1. The analyzed matrix was milk [73]. The immunosensors were fabricated by

immobilizing AFM1 antibodies directly on the surface of SPEs, and allowing the competition to occur between free AFM1 and that conjugated with peroxidase (HRP) enzyme. The electrochemical technique used was the chronoamperometry, performed at -100 mV. Results have shown that using SPEs aflatoxin M1 can be measured with an LOD of 25 ppt and with a working range between 30 and 160 ppt. Moreover, the authors claim that interference problems have been addressed for the direct analysis of aflatoxin M1 in milk.

Another micotoxin that is widely present in food commodities is OTA, 7-(L-b-phenylalanylcarbonyl)-carboxyl-5-chloro-8-hydroxy-3,4-di-hydro-3R-methylisocumarin. It is produced by several *Aspergillus* and *Penicillium* species. It is considered as secondary toxic metabolite with nephrotoxic, teratogenic, carcinogenic, and immunotoxic activity in several animal species. Immunosensing strategies have been proposed to be able to detect OTA in different matrix, such as wine [74] and durum wheat [75]. In the former matrix, the assay was carried out on carbon-based SPEs. The immunosensors were developed using polyclonal antibodies. The assay gave an LOD of 180 pg/mL and sensitivity of 6.1 ± 0.1 ng/mL. The immunosensors were challenged with wine to assess a matrix effect. Recoveries obtained were in the 70–118% range.

A more challenging matrix was faced in the latter research [75] carried out in collaborations with authors' groups. In this case, the immunosensors were realized using monoclonal antibodies, and two formats of immunosensors compared. The immunosensor in the direct format was then used for the determination of OTA in wheat. Samples were extracted with aqueous acetonitrile and the extract analyzed directly by the assay without clean up. The I_{50} in real samples was 0.2 μg/L corresponding to 1.6 μg/kg in the wheat sample with a LOD of 0.4 μg/kg (calculated as blank signal—3σ). Within- and between-assay variability were less than 5% and 10%, respectively. Results obtained on naturally contaminated wheat samples were compared with a reference method with a good correlation ($r = 0.9992$).

29.2 APPLICATION

In this report, we describe the application of SPEs to the determination of different contaminants in durum wheat samples. All the reported applications have been developed using PalmSens hand-held potentiostat equipped with PalmSens PC software for the elaboration of current data (PalmSens, Amsterdam, The Netherlands) (Fig. 29.1).

Fig. 29.1. Hand-held potentiostat by Palmsens used in all the optimization protocols described in the text.
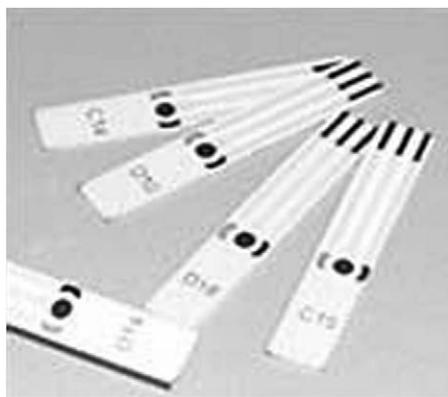

Fig. 29.2. Screen-printed electrodes used in all the proposed applications.

The different biosensors were obtained using thick-film SPEs produced by Biosensor Laboratory, University of Florence and commercialized by PalmSens. The electrochemical cell, consisting of a graphite working electrode and silver counter and pseudo-reference electrodes, was printed on a planar polyester substrate (Fig. 29.2).

The applications (Screen-printed electrochemical sensors for the detection of AChE inhibitor, Screen-printed electrochemical DNA sensors for identification of microorganisms, and Screen-printed electrochemical immunosensors for the detection of toxins) have been finalized to

Fig. 29.3. The Palmsens hand-held potentiostat equipped with the dedicated multiplexer for eight-channel measurements.

the assembly of a dedicated instrumentation and software able to handle delocalized analysis of OPs, *Fusarium* sp. DNA, and OTA.

The instrumentation consisted of the hand-held potentiostat interfaced with a CH8 multiplexer (PalmSens) (Fig. 29.3) that allow different sensor configuration:

a. Sensorarrays with eight working and eight combined reference/counter electrodes
b. Sensorarrays with eight working electrodes sharing a reference and a counter electrode
c. Sensorarrays with eight working electrodes sharing a combined reference/counter electrode

The software front page reported in Fig. 29.4 enables the choice of the analytical application among pesticides, *Fusarium* sp., and ochratoxin.

Each application allows the use of eight independent channels that depending on the procedure can be used to perform individual or duplicate analysis. For instance, in the OTA application the default setting of the software permit to dedicate two channels for the blank measurement and two channels for each of the three calibrators or samples whereas in the OPs protocol the user can utilize single channel or multiple channels. In each application, a check biosensor option is available to test the correct functioning and positioning of the sensors.

In OTA and OPs protocols calibration and determination windows are present. The user can either create his/her own three-point experimental calibration, which can be saved for further use, or load external data to obtain the calibration curve. The concentration levels of the standard solutions are then used by the software to classify the sample result according to a "traffic light scheme" (red light for sample exceeding the legal limit, yellow for samples that need analytical confirmation, and green light for negative samples).

The *Fusarium* sp. DNA application consists of a check biosensor tool and a PCR sample window. The check biosensor enables the control of

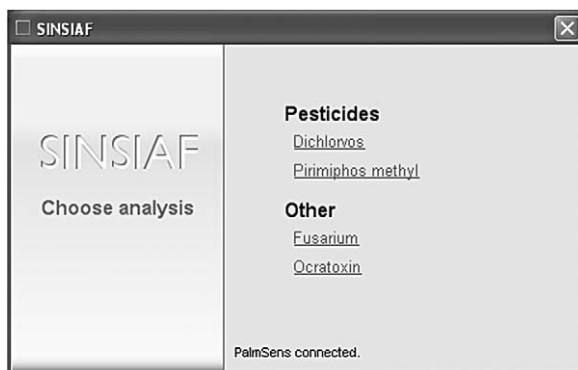Rapid detection of organophosphates, Ochratoxin A, and Fusarium sp.

Fig. 29.4. Dedicated software for durum wheat safety control, the front page enables the choice of the experimental procedure to be carried out.
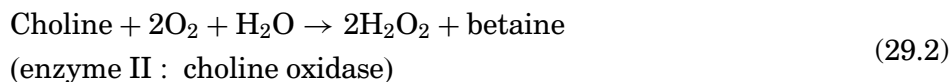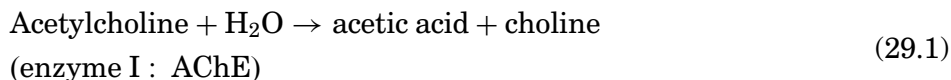
the probe immobilization procedure, then in the PCR sample window the amplified samples are measured. Four out of the eight channels are used as "negative" control and four for sample measurement. The software, using an internal algorithm, allows the discrimination between positive and negative samples.
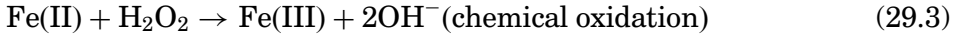
29.2.1 Screen-printed electrochemical sensors for the detection of dichlorvos and pirimiphos-methyl

Experimental details are reported in Procedure 42 (CD accompanying this book).

Both the determinations reported here rely on the inhibition activity of OPs pesticides toward AChE combined with the detection of the AChE enzyme product choline at the surface of a mediator-modified screen-printed choline oxidase electrochemical biosensor.

The biochemical–electrochemical pathway used to determine the inhibition consisted of two enzymatic reactions (Eqs. (29.1) and (29.2)) generating a chemical oxidation (Eq. (29.3)) that was determined by cathodic chronoamperometry (Eq. (29.4)). The used iron containing electrochemical mediator was the widely used Prussian blue.

$$\text{Acetylcholine} + \text{H}_2\text{O} \rightarrow \text{acetic acid} + \text{choline}$$
$$\text{(enzyme I : AChE)}$$
(29.1)

$$\text{Choline} + 2\text{O}_2 + \text{H}_2\text{O} \rightarrow 2\text{H}_2\text{O}_2 + \text{betaine}$$
$$\text{(enzyme II : choline oxidase)}$$
(29.2)

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + 2\text{OH}^- \text{(chemical oxidation)} \tag{29.3}$$

$$\text{Fe(III)} + e^- \rightarrow \text{Fe(II)}\text{(electrochemical reduction)} \tag{29.4}$$

Standard and sample extract solutions were analyzed according to the following experimental scheme: first, the current intensity of a blank sample extract was measured, and then the current intensity of either the standard or the sample extract was measured. The current intensity of the blank sample ($I0$) and of the contaminated sample or standard solution (II) was used to calculate the percent inhibition according to the following formula:

$$I(\%) = 100\frac{(I0 - II)}{I0} \tag{29.5}$$

Electrochemical experiments were carried out using a hand-held potentiostat equipped with dedicated software for the elaboration of current data (Fig. 29.5). The current was sampled 2 min after the reaction started. Figure 29.5 shows the chronoamperogram where current versus time is plotted. The current shape was reproducible from time to time and hence the precision assured sampling the current, 2 min after the start of the measurement. After 2 min the current was continuously increasing due to the residual activity of AChE in solution. The biosensor
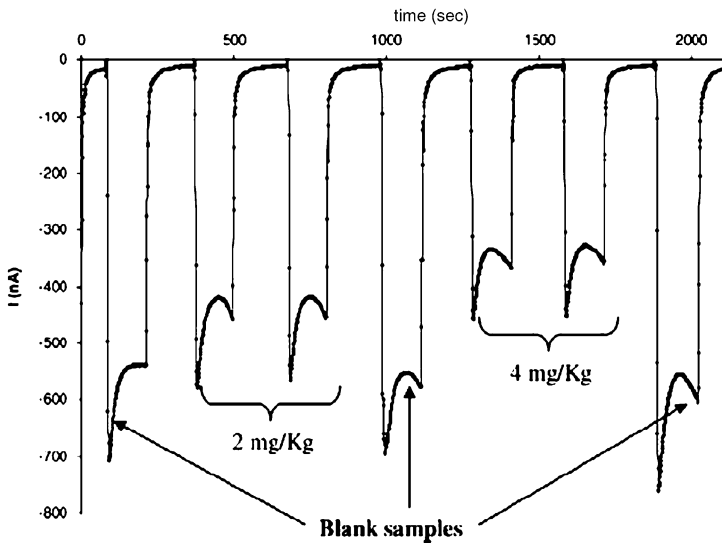

Fig. 29.5. Typical amperogram recorded for durum wheat extract at 2 and 4 mg/kg using the hand-held potentiostat. Reprinted with permission from Ref. [47].

used to detect the extent of the AChE inhibition was a choline oxidase biosensor assembled on thick-film electrode.

29.2.1.1 Dichlorvos determination in durum wheat samples

Here we report experimental data obtained using the proposed electrochemical assay with screen-printed choline oxidase biosensor for the detection of dichlorvos in durum wheat samples. As described in P1E9, two different extraction approaches have been optimized: the former using whole wheat kernels and aqueous extraction and the latter using grounded samples and hexane extraction. Both studies were carried out using free cholinesterases because the aim of the protocol was to devise a rapid procedure that did not include cumbersome regeneration steps, which were unavoidable with immobilized AChE.

The former approach led to a simplified extraction protocol where the extraction solvent was then used as assay buffer.

In this application, the use of wild-type electric eel AChE and a recombinant AChE, specifically selected as very sensitive to dichlorvos, was compared. The effect of the matrix extract was determined by using various sample: solvent ratios, 1:2.5, 1:5, 1:10, and 1:20. The optimal extraction ratio, considering the electrochemical interferences and the effect on enzyme activity and bioavailability of the pesticide, was 1:10.

The method was calibrated both in buffer and durum wheat extract. The LODs in durum wheat samples were 0.45 mg/kg for the wild-type AChE and 0.07 mg/kg for rAChE. These characteristics allowed the detection of contaminated samples at the legal MRL, which is 2 mg/kg [4]. Moreover, fortified samples of durum wheat were obtained with both dichlorvos and the commercial product Didivane, which contains dichlorvos as active molecule. At all the tested levels, the occurrence of contaminant was detected with an average recovery of 75%. The total assay time, including the extraction step, was 30 min. Because several extractions as well as most of the assay steps can be run simultaneously, the throughput for one operator is 12 determinations per hour. In Table 29.1, we summarize the results obtained for the fortified samples.

Samples ($n = 55$) fortified at different levels (4, 2, 1, and 0.5 mg/kg) were extracted and analyzed with the proposed electrochemical assay using both eeAChE and rAChE. For eeAChE, the recovery for dichlorvos-spiked samples ranged between 74 and 78%. The incomplete recovery may be due to concurrent causes, such as the use of whole kernels as sample, water-based extraction solvent, and adsorption

TABLE 29.1

Recovery study on wheat samples fortified at various levels with dichlorvos and Didivane using both eeAChE and rAChE

Fortification level (mg/kg)	eeAChE			rAChE		
	Inhibition (%)[c]	Calc. (DDPV) (mg/kg)	Recovery (%)	Inhibition (%)[c]	Calc. (DDPV) (mg/kg)	Recovery (%)
4.0 ($n = 15$)[a]	42 ± 3	3.1 ± 0.1	78 ± 3	NA[d]	NA	NA
2.0 ($n = 15$)[a]	27 ± 1	1.5 ± 0.1	77 ± 5	70 ± 1	1.5 ± 0.2	74 ± 2
2.0 ($n = 15$)[b]	26 ± 1	1.5 ± 0.1	75 ± 5	68 ± 1	1.4 ± 0.1	69 ± 2
1.0 ($n = 5$)[a]	16 ± 1	0.8 ± 0.1	77 ± 10	46 ± 2	0.7 ± 0.1	74 ± 4
0.5 ($n = 5$)[a]	8 ± 2	0.4 ± 0.1	74 ± 20	26 ± 1	0.4 ± 0.1	72 ± 3

Reprinted with permission from Ref. [47].
[a]Fortified with dichlorvos.
[b]Fortified with Didivane.
[c]Inhibition (*I*%) is the analytical signal.
[d]NA, not available.

exerted by the matrix. Although the advantage of using the extraction solvent as measuring buffer has an important influence on the speed of the assay, in order to detect dichlorvos at 0.5 mg/kg with better accuracy, it was necessary to use the rAChE; in that case, an $I\%$ of 26.3% was obtained, which is well above the minimum detectable inhibition of the method (8%). Moreover, the recovery of the samples spiked with Didivane was comparable to that obtained with the pure molecule, dichlorvos. The recovery data suggest that the buffer used in the extraction protocol allowed a repeatable recovery at an applicable contamination level of both dichlorvos in pure form and dichlorvos in commercial formulation (Didivane), thus avoiding the use of a polar organic solvent such as methanol or acetonitrile, which may influence enzyme activity and sensor stability.

Despite aqueous-based extraction using whole wheat kernels has been demonstrated as effective to detect dichlorvos, the procedure might lead to inadequate homogeneity of testing samples. Moreover, aqueous solvents cannot be used for extraction of ground wheat samples due to the formation of a slurry, preventing filtration of adequate amounts of extract. To apply the electrochemical method to ground wheat samples, as generally required in food analysis, a non-aqueous extraction solvent was required.

We have hence tested a number of extraction solvents that could be used with the grounded samples for dichorvos extraction and then easily coupled, via liquid–liquid partitioning with the assay procedure.

To perform electrochemical analysis with the choline oxidase biosensor, dichlorvos needed to be transferred to PBS solution, thus avoiding any electrochemical interference by organic solvents.

Therefore, the filtered hexane extract was submitted to liquid–liquid partitioning with PBS and the upper organic layer was removed by evaporation. Finally, the buffer solution containing dichlorvos was analyzed by the biosensor. Dichlorvos was easily measured in ground wheat by electrochemical bioassay at levels as low as 0.05 µg/g. The method was peer-validated by two laboratories, and the results of the validation test are reported in Table 29.2.

As shown in Table 29.2, mean recoveries of dichlorvos ranged from 97% to 108%, RSD_r values ranged from 5.5% to 7.8%, and RSD_R values ranged from 9.9% to 17.6%. The dichlorvos mean recovery was calculated for each spiking level as the mean of four measurements, $n = 4$ (Table 29.2). No false negative or false positive results were obtained by the electrochemical assay. A good correlation between dichlorvos concentrations obtained by electrochemical biosensor and GC analysis was

TABLE 29.2

Results of the validation test performed by two laboratories for determination of dichlorvos in ground durum wheat by electrochemical bioassay

	Spiked level				
	Blank	0.25 µg/g	0.60 µg/g	1.00 µg/g	1.50 µg/g
Lab 1, rep 1	Nd[a]	0.27	0.54	1.03[b]	1.71[b]
Lab 1, rep 2	Nd	0.24	0.48	1.18[b]	1.64[b]
Lab 2, rep 1	Nd	0.29	0.67	0.99[b]	1.38[b]
Lab 2, rep 2	Nd	0.29	0.63	0.93[b]	1.48[b]
Mean recovery (%)	–[c]	108	97	103	103
S_r	–	0.015	0.036	0.081	0.061
RSD_r (%)	–	5.5	6.2	7.8	3.9
S_R	–	0.027	0.102	0.117	0.179
RSD_R (%)	–	9.9	17.6	11.4	11.5

Reprinted with permission from Ref. [48].
[a]Not detected, LOD = 0.05 µg/g.
[b]Data calculated with calibration curve obtained in diluted (1:3) blank extract.
[c]Not applicable.

also found as shown in Fig. 29.6. The correlation coefficient (r) was 0.9919.

29.2.1.2 Pirimiphos-methyl determination in durum wheat samples
The electrochemical assay for the detection of AChE inhibitors outlined above has been optimized for the detection of pirimiphos methyl-pirimiphos-methyl in durum wheat. Pirimiphos methyl is a phosphotionate insecticide and therefore it requires to be transformed in the corresponding oxo-form to act as an effective AChE inhibitor, in fact it did not show inhibition of AChE in a concentration range 50–5000 ng/mL (data not shown).

The procedure for the oxidation of pirymiphos methyl via N-bromosuccinimide (NBS) and AChE inhibition was optimized in buffer solution for reagents, concentration, and inhibition time (see Procedure 43 in CD accompanying this book for more details).

As a compromise, between analytical performance and overall assay length, a 10-min incubation of NBS and ascorbic acid (AA) and 30-min AChE incubation was selected. A statistical analysis, using stepwise general least squares analysis (SGLSA), of the data demonstrated that only AChE incubation time had a significant and positive influence on the inhibition of AChE.
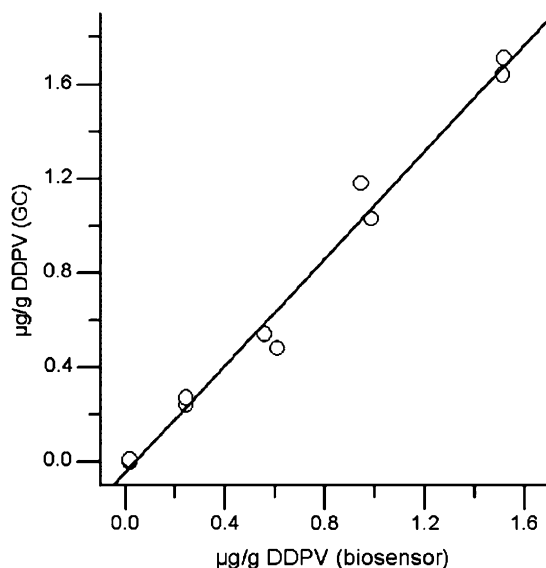
Fig. 29.6. Comparison of dichlorvos (DDPV) contents in spiked durum wheat samples analyzed by GC and electrochemical biosensor. Reprinted with permission from Ref. [48].

The method was calibrated both in buffer solution and in durum wheat extract. The intra-electrode RSD (%) ranged between 1.6 and 15.0, whereas the inter-electrode RSD (%) was comprised between 4.6 and 15. The LOD was 38 ng/mL, and the $I50\%$ was 360 ng/mL. The assay conditions were then re-optimized to work with durum wheat extracts and calibrations were obtained under different experimental conditions such as sample pretreatment (milled or whole grains) and extract concentration (2% or 4%). The calibrations were slightly affected by the sample matrix resulting in an increased LOD (65–133 ng/mL) and $I50\%$ (640–1650 ng/mL). The LOD referred to the sample, determined using the best operational condition, was 3 mg/kg.

Spiked samples were prepared at the EU regulated level (5 mg/kg) and analyzed with the optimized protocol resulting in an average recovery of 70.3%.

Acetone and methanol were tested for the extraction and the effect on the assay compared (Fig. 29.7).

Acetone appeared to strongly affect the enzyme activity and was therefore discarded, whereas methanol did not strongly influence the enzyme and hence was used for sample extraction.
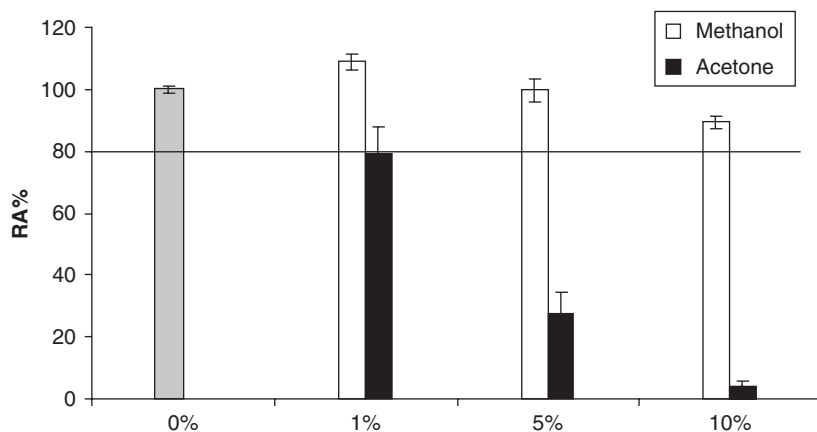
707

Fig. 29.7. Effect on the residual enzyme activity of methanol and acetone at different concentration (0–10%) in phosphate buffer. Redrawn with permission from Ref. [49].

TABLE 29.3

Recovery data of spiked samples ($n = 9$)

	I%	Recovery (%)
S1	15.6	80.0
S2	12.7	69.0
S3	14.3	75.0
S4	12.7	69.5
S5	13.5	72.5
S6	11.3	63.5
S7	14.8	76.5
S8	12.7	69.0
S9	9.9	58.5
Mean	13.1 ± 1.7	70.3 ± 7.0

I% values are the mean of three measurements for each sample, $8 < CV < 15$. The recovery has been calculated with respect to the spiked level (5 mg/kg). Reprinted with permission from Ref. [49].

Once the oxidation of the phosphothionate pesticide was optimized, the assay was applied to nine spiked samples resulting in acceptable recovery (Table 29.3). The spiking concentration was 5 mg/kg for all the samples, which correspond to the legal limit settled by the European Union.

In this application, the samples were extracted using pure methanol, which was then directly diluted in the assay buffer.

The assay scheme used to detect AChE inhibitors is not selective, as a great number of molecules inhibit AChE.

The methods here proposed are "target oriented"; that is the sought for analyte is known to the analyst. This occurs in the situations where the grower must use a certain production protocol, including pesticide treatments, as stated by contract. In the case where the analyte is not known, the assay can be used as a toxicity test able to detect the presence of a total anticholinesterase activity (TAA).

29.2.2 Screen-printed electrochemical sensors for the detection of ochratoxin in durum wheat

Competitive electrochemical enzyme-linked immunosorbent assays based on disposable SPEs have been developed for quantitative determination of OTA.

Indirect and direct formats of immunosensors-based assay were developed. A total of 6 µL of OTA–BSA in buffer (indirect format) and 6 µL of goat IgG (anti-mouse IgG) (direct format) in buffer were dispensed on the graphite-based screen-printed working electrodes and kept overnight at 4°C. Another 6 µL of 1% PVA solution was used to block the surface for 30 min at room temperature. Also, 6 µL of OTA monoclonal antibody were added to the electrode surface for 30 min at room temperature. Binding or competition was run with 6 µL of OTA–AP conjugate or conjugate+standard for 30 min at room temperature. Washing was then carried out. The activity of the label enzyme was measured electrochemically by the addition of 100 µL of substrate solution (5 mg/mL 1-naphthyl phosphate in DEA buffer; prepared daily), for 2 min at room temperature. The enzymatic product, 1-naphthol, was detected by differential pulse voltammetry (DPV) using the following conditions: potential range 0–600 mV, pulse width 60 ms, pulse amplitude 50 mV, and scan speed 50 mV/s (Fig. 29.8).

The assays were carried out using monoclonal antibodies in the direct and indirect formats. OTA working range, I_{50} and LODs were 0.05–2.5 µg/L and 0.1–7.5 µg/L, 0.35 (± 0.04) µg/L and 0.93 (± 0.10) µg/L, 60 µg/L and 120 µg/L in the direct and indirect assay formats, respectively. The immunosensor in the direct format was selected for the determination of OTA in wheat. Samples were extracted with aqueous acetonitrile and the extract analyzed directly by the assay without clean-up. The I_{50} in real samples was 0.2 µg/L corresponding to 1.6 µg/kg in the wheat sample with an LOD of 0.4 µg/kg (calculated as blank signal—3σ). Within- and between-assay variability were less than 5%
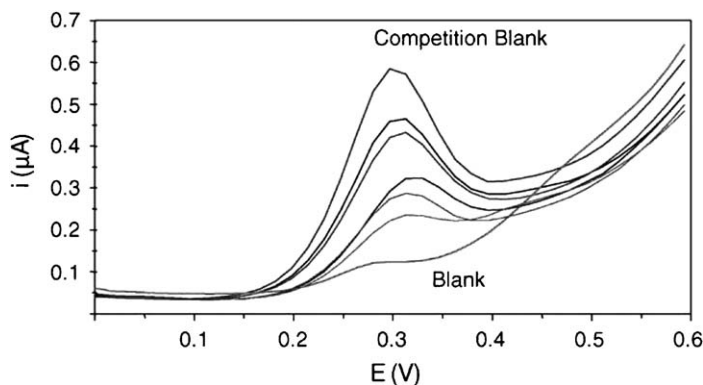
Fig. 29.8. Differential pulse voltammograms obtained for the samples assayed (one representative voltammogram for each of the spiked levels, from 0.5 to 15 µg/kg). Competition blank signal is relative to OTA level = 0, blank signal is OTA-free wheat extract with no OTA–AP conjugate used in the assay. Reprinted with permission from Ref. [75].

TABLE 29.4

Comparison of wheat samples contaminated with OTA determined by electrochemical immunosensor and HPLC

Theoretical OTA level (µg/kg)	HPLC		Immunosensor
	OTA±SD[a] (µg/kg)	RSD[b] (%)	OTA±SD (µg/kg)
0.5	0.44±0.08	18.2	0.6±0.1
1	0.9±0.1	11.1	1.0±0.1
4	3.8±0.4	10.5	3.1±0.4
5	4.5±0.5	11.1	4.2±0.6
15	13.1±0.9	6.9	12±1

Reprinted with permission from Ref. [75].
[a]Mean value±SD ($n = 3$, SD = standard deviation).
[b]RSD = relative standard deviation.

and 10%, respectively. A good correlation ($r = 0.9992$) was found by comparative analysis of naturally contaminated wheat samples using this assay and an HPLC/immunoaffinity clean-up method based on the AOAC Official Method 2000.03 for the determination of OTA in barley as reported in Table 29.4.

29.2.3 Screen-printed electrochemical sensors for the detection of *Fusarium* sp. DNA

DNA electrochemical sensors were developed for the detection of specific sequences of *F. culmorum*. The sensing principle of the proposed application was based on the affinity interaction between complementary strand of nucleic acids: the probe was immobilized on the sensor surface and the target analyte was free in solution. The hybridization is followed using an SWV. The direct label-free detection was accomplished by monitoring the guanine oxidation peak of the target sequence. This approach rely on the use of inosine-modified (guanine-free) probes. In fact, the non-electroactive base inosine still forms a specific base pair with the cytosine residue. This results in a flat baseline (in the potential region $+0.9$–$1\,$V versus pseudo Ag/AgCl reference electrode) for the probe-modified electrode. The duplex formation was thus detected through the appearance of the guanine oxidation peak of the target sequence, following hybridization. The use of such a label-free biosensor for the detection of post PCR samples can improve the safety, since the indicators are usually toxic or carcinogenic compounds, and save time. The sensors were first applied to synthetic complementary oligonucleotides, and then to PCR-amplified samples of *F. culmorum*. The specificity of the genosensors was tested in the presence of different amounts of non-complementary PCR sample. The crucial part of this work was to understand how to increase the occurrence of the hybridization reaction between the probe and the denatured single strand of PCR product. In this application, the probe selection was carried out using a computational approach. Computational tools can greatly improve the rational design of the ligands by exploiting different knowledge about the structure and the chemical properties of the target molecule. The computational run was performed by fixing the folding temperature at $37°$C with a concentration of sodium of $330\,$mM to fit the experimental conditions. The other parameters were left as default. An important purpose of this work was to evaluate the relation between experimental and computational data. Therefore the probe selection was focused on the study of the steric hindrance of the simulated secondary structure by emphasizing as primary parameter the intra-strand hydrogen bonds within the PCR single strand. For that reason, we selected the probes complementary to the PCR single-strand regions with different intra-strand hydrogen bonds as reported below (Fig. 29.9):

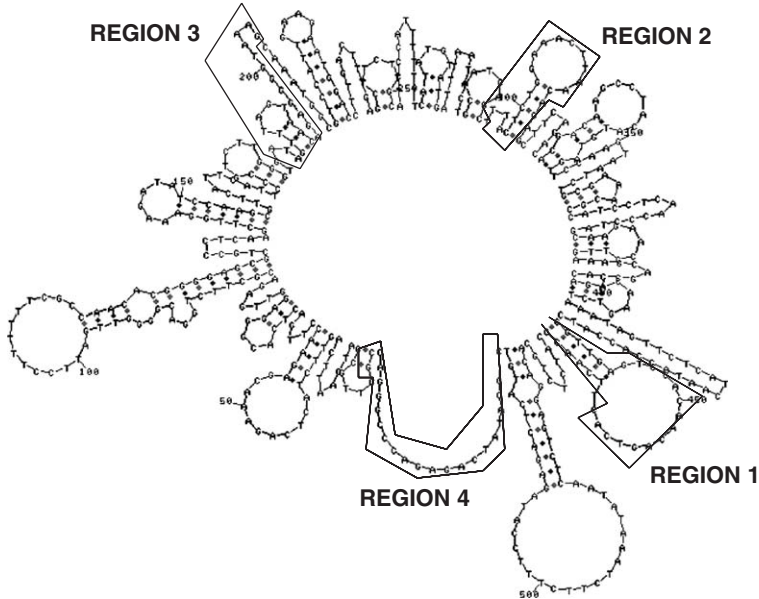– probe 1 complementary to a region with six intra-strand hydrogen bonds (3 G-C; 3 A-T)

Fig. 29.9. Dots indicate intra-strand hydrogen bonds. Folding conditions: $T = 37°C$, $[Na^+] = 330\,mM$.

– probe 2 complementary to a region with five intra-strand hydrogen bonds (3 G-C; 2 A-T)
– probe 3 complementary to a region with three intra-strand hydrogen bonds (1 G-C; 2 A-T)
– probe 4 complementary to a region with two intra-strand hydrogen bonds (2 G-C; 0 A-T)

The different probes were used to obtain four independent sensors that were then tested for their selectivity and specificity toward synthetic complementary sequences and PCR samples. In Fig. 29.10 we report the response curve obtained for probe 3 DNA sensor versus different concentration of the complementary strand and versus three non-complementary sequences.

Thereafter, the different DNA biosensors were used to test PCR samples obtained from *F. culmorum*. The same DNA biosensor was also challenged with completely non-complementary DNA (Table 29.5). The results exhibited a dose-dependent response up to 7 µg/mL; for higher concentrations a hook effect was observed.
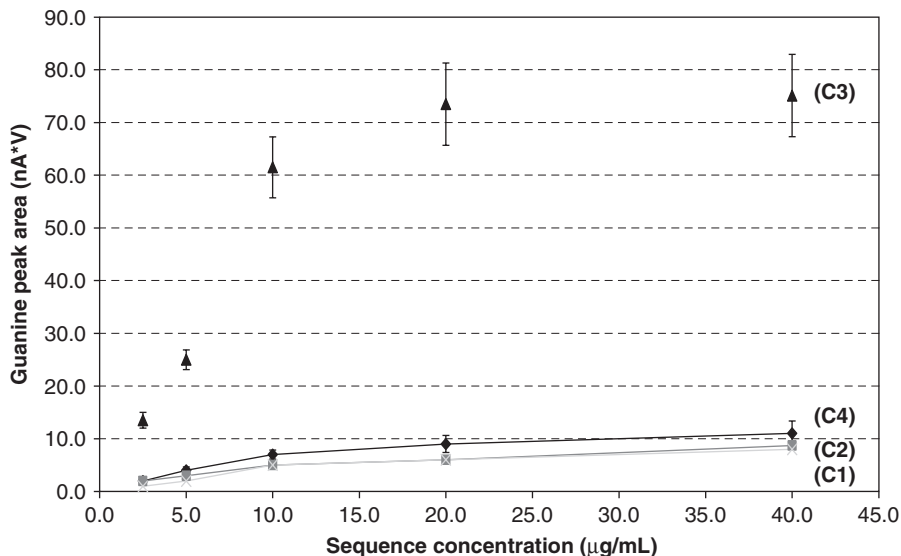
Fig. 29.10. Correlation between analytical signals observed with probe 3 genosensor and concentrations of the oligonucleotide complementary to the probe 3 (C3) and of the oligonucleotides complementary to the probes 1, 2, and 4 (C1, C2, and C4, respectively).

TABLE 29.5

Analytical signals observed with different concentrations of *F. culmorum* PCR product and of a non-complementary DNA fragment

PCR concentration (µg/mL)	*F. culmorum* PCR product ($\pm$SD) (nA V)	Non-complementary PCR product ($\pm$SD) (nA V)
30	14.03 ± 1.96	0.32 ± 0.04
15	24.16 ± 3.14	0.05 ± 0.02
7	36.40 ± 6.55	0.03 ± 0.01
3	10.10 ± 2.12	0.10 ± 0.02

PCR products were thermally denatured prior to analysis.

In order to avoid false negatives, a control procedure was introduced. The PCR samples were split into two aliquots, one of which was thermally denatured, for the analysis.

It was experimentally evaluated that if the denatured/non-denatured signal was higher than 3, the sample could be considered as positive, while for lower ratio the sample has to be classified as

negative. Particularly, interesting results were obtained using the probes complementary to the regions 3 and 4. The SPE modified with the probe complementary to region 3 exhibited acceptable repeatability (CV within 20% on three consecutive measurements) with no measurable analytical signal in the presence of different amounts of non-complementary PCR sample.

29.3   CONCLUSIONS

The applications described above, coupled with the realization of a dedicated portable instrumentation and software, represent a user-friendly analytical tool dedicated to durum wheat safety. Moreover, all the applications are based on the use of one single type of thick-film SPE facilitating the overall procedure for the final user that has to store and handle one single type of transducer. The developed device, which consists of the hand-held potentiostat, the multiplexer for eight-channel control and a dedicated software, can be used to detect OPs pesticides, such as dichlorvos and pirimiphos methyl at contamination level below the MRL settled by the European Union, OTA, and also amplified DNA of *F. culmorum*.

The software has a user-friendly interface that informs the user on the procedural steps that must be performed (e.g., ''add 20 μL of Vial A to Vial B, mix and transfer on the sensor surface'') allowing the realization of the measurement even for non-trained personnel. All the internal controls result in a ''pass or fail'' message as well as all the incubation times are controlled by the software. At the end of every protocol the user receives a screen message that contains a self-explanatory result via a color code according to a ''traffic light scheme''.

For dichlorvos detection, the major achievements were the development of two alternative extraction methods using alternatively the assay buffer or organic solvent as extraction mean. The former procedure allowed a simplified protocol, using whole kernels, that could readily be used in field to measure as low as 0.45 mg/kg using wild-type eeAChE and 0.07 mg/kg using a specifically designed engineered rAChE. The latter procedure performed using solvent extraction allowed a complete recovery of the pesticide from grounded kernels. The procedure that can be used in a laboratory environment as screening method allowed the detection at one order of magnitude lower (0.05 mg/kg) than in the former protocol using wild-type eeAChE. The method has been peer-validated in an inter-laboratory experiments. A second challenging pesticide was

investigated for method development resulting in a procedure, finally transferred on the developed software, for the analysis of pirimiphos methyl in durum wheat. The phosphotionate pesticide was oxidized and transformed in the active oxo form using a dedicated optimized procedure able to function in durum wheat extract. An extraction protocol carried out in methanol was used as this solvent could then be used in the electrochemical assay protocol. The LOD referred to the sample, determined using the best operational condition, was 3 mg/kg. Spiked samples were prepared at the EU-regulated level (5 mg/kg) and analyzed with the optimized protocol resulting in an average recovery of 70.3%.

For OTA detection, the optimized immunosensors and the protocol that was implemented on the electrochemical device allowed the detection of 0.4 µg/kg, with within- and between-assay variability less than 5% and 10%, respectively. The method was evaluated with respect to a reference instrumental method (HPLC/immunoaffinity clean-up method based on the AOAC Official Method 2000.03) obtaining good agreement ($r = 0.9992$).

Finally, an electrochemical DNA biosensor able to discriminate between positive and negative PCR-amplified samples of *F. culmorum* was shown.

In conclusion, the overall device enables a thorough control on durum wheat samples in a user-friendly manner, at detection levels that allow an improvement in the control protocols that have to be carried in field by non-specialized personnel.

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