Review

A review on novel developments and applications of immunosensors in food analysis

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ABSTRACT

The present review deals with novel developments in immunosensors destined for final application in food analysis. In this perspective particular emphasis will be given to the most important approaches which recently have been used for immunosensor construction and assembling. For this reason, electrochemical, surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) techniques will be explored in detail and recent and practical examples on food matrices will be reviewed. Objective of this survey is to give a general overview of the possible application of immunosensors to the food analysis field.

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1. Introduction

The analysis of foods to assess the presence of both biological (pathogenic bacteria) and chemical contaminants is a practice of crucial importance for ensuring food safety and quality. Conventional bacteriological testing methods rely on specific microbiological media to isolate and enumerate viable bacterial cells in food. These traditional methods are very sensitive and inexpensive, but require several days to generate results because they rely on the ability of microorganisms to multiply and to produce visible colonies. For example, the current official method for enumerating Salmonella bacteria in food (ISO/DIS 6579, 2001) involves a series of time-consuming sequential cultural steps, taking more than 4–5 days for the detection. In general these steps include: (a) pre-enrichment (b) selective enrichment (c) isolation and (d) biochemical and serological confirmation if presumptive positive Salmonella colonies develop.

The majority of chemical contaminants are commonly analysed using separative techniques coupled to various detectors such as GC-FID, GC-ECD, GC–MS, HPLC-UV, HPLC-FL, HPLC-MS.

Over the past decade, the control of food safety has been mainly carried out through product testing rather than process control. The main problem with end-product testing is the high number of samples to be examined before one can decide on the safety of the product batch, especially when contaminants are expected to be heterogeneously distributed in the batch. Moreover, end-product testing detects only failures and does not identify causes. The HACCP (Hazard Analysis Critical Control Point) system, which identifies specific hazards and measures for their control, is now generally accepted as the most effective system to ensure food safety [1]. HACCP can be applied throughout the food chain from primary production to final consumption rather than relying mainly on end-product testing. As well as enhancing food safety, the application of HACCP can provide another benefit: to aid inspection by regulatory authorities.

With the implementation of the HACCP system for control of the process line at CCPs (Critical Control Points) the demand for rapid, sensitive and accurate methods to detect biological and chemical contaminants has increased. In particular, tests that can be completed within minutes or hours would enable processors to take quick corrective actions when contaminants are detected [2]. Hence, the development of portable, rapid and sensitive biosensor technology is crucial for this purpose [3–5].

The importance of biosensors relies on their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex samples with minimum sample pre-treatment [6–9]. Biosensors generally involve biological recognition components such as enzymes, nucleic acids, antibodies, animal or vegetable tissues in intimate contact with an appropriate transducer [10–13].

When antibodies or antibody fragments are used as molecular recognition element for specific analytes (antigens) to form a stable complex, the device is called immunosensor. Depending on the method of signal transduction, immunosensors may be divided into four basic groups: electrochemical, optical, piezoelectric and thermometric [14]. The transducers chosen are directly related to the labelling, enzymatic or not, performed on the antigen or on the antibody. For each particular detection type, a specific labelling is usually performed, even though some labels can be used with different detection methods (i.e. horseradish peroxidase can be employed for an electrochemical immunosensor and for fluorescent and chemiluminescent detection using a fibre optic sensor).

Most of the developed immunosensors are based either on a competitive or sandwich assay, when applied to the detection of low (i.e. herbicides, toxins) and high (proteins, cells) molecular weight molecules, respectively [15]. Two approaches can be followed for the development of competitive immunosensors: a first one in which immobilised antibodies (Ab) react with the free antigens (Ag) in competition with labelled antigens (Ag*) (Fig. 1A) or a second one in which immobilised antigens compete with free antigens for labelled free antibodies (Ab*) (Fig. 1D). In order to favour the immobilization and the interaction with the antibody, the antigens, especially those with a small molecular weight, are usually conjugated with a protein (i.e. Ag–BSA, Ag–KLH, Ag–OVA). Both these approaches are defined as direct competitive immunosassay [16].

The second format is generally preferred and circumvents all the problems related to antibody immobilisation (loss of affinity and correct orientation of the antibody) and is also used when enzyme conjugated primary antibodies are not available for the selected analyte. In this case an anti-species IgG–enzyme (commonly named secondary antibody) is used as label after binding with the Fc region of the primary antibody and the format is denoted as indirect competitive immunosassay (Fig. 1E) [16].

In a sandwich assay, after interaction between immobilised antibodies (Ab) and free antigens, labelled antibodies (Ab*, directed toward a second binding site of the antigen) are added; at this point, Ag is “sandwiched” between two antibodies (Ab and Ab*) (Fig. 1B).

Transducers, such as piezoelectric sensors (quartz crystal microbalance, QCM) and modern optical sensors based on
Novel development and applications of immunosensors in food analysis

Fig. 1 – Immunoassay formats. (Top) Antibody ( gif] immobilised on the support: (A) direct competitive assay using antigen labelled with enzyme; (B) sandwich assay using detecting antibody labelled with enzyme; (C) direct assay (for SPR and QCM). (Bottom) Antigen ( gif] immobilised on the support: (D) direct competitive assay using primary antibody labelled with enzyme; (E) indirect competitive assay using a secondary antibody labelled with enzyme. (F) Direct assay (for SPR and QCM). In the case of electrochemical immunosensors the support is a working electrode while for SPR and QCM the support is a chip or a quartz crystal, respectively. For competitive and sandwich assays (A, B, D), label is not used for SPR and QCM measurements.

surface plasma resonance (SPR), allow the label-free detection with a direct quantification of the immunocomplex (Ab–Ag). For SPR and QCM, only a few examples are based on the use of a direct assay (not to be confused with the direct competitive assay) where the simple binding between antigen and an antibody is detected (Fig. 1C and F). In fact, for low-molecular weight analytes such as antibiotics, hormones and veterinary drug residues the sensitivity of SPR is usually not sufficient when a direct antibody/antigen interaction is adopted. For this reason competitive and sandwich formats (without any labelled molecules) are highly preferred [17]. Since in SPR there is no need for a label, the indirect competitive format is never performed with this technique.

In any case, the general strategy for immunosensor construction is to place the biological material in close contact with the transducer in order to obtain high sensitivity and to minimise the time of measurement. Several immunosensors for the detection of biological and chemical contaminants are reported in the literature, but only a few have actually been applied to food analysis. The main applications are based on the use of electrochemical, optical (especially SPR) and piezoelectric immunosensors. In the present review we will describe the principles and food analysis applications of those immunosensors which have been employed over the past 5 years with real food samples in order to give a valuable tool for those researchers interested in this kind of application.
2. Electrochemical immunosensors

Several electrochemical techniques (potentiometric, amperometric, and conductimetric) can be applied for analytical purposes; however, amperometric detection systems have been demonstrated to be the most suitable means for immunosensor construction due to their high sensitivity, low cost and the possibility of instrument miniaturisation. Amperometric detection is based on the measurement of a current at a fixed (potentiostatic technique) or variable (voltage-sweep technique) potential and classically involves a three-electrode system, although this is often reduced in practice to two electrodes in many devices. By applying a certain potential between the working and the reference electrode, the species of interest is either oxidised or reduced at the working electrode causing a transfer of electrons which ultimately results in a measurable current that is directly proportional to the concentration of the electroactive species at the electrode surface over a wide dynamic range.

Electrodes are commonly made of inert metals such as platinum, gold, or carbon, either in the form of graphite, glassy carbon or pyrolytic graphite, as a solid or as a paste. These electrodes are commonly used to detect chemical compounds produced or consumed by binding or catalytic reactions. Therefore, for the construction of amperometric immunosensors, electrochemically active labels (directly [18,19] or as product of an enzymatic reaction) are needed.

Since enzyme labels provide great signal amplification in the assay and also a large number of antibody–enzyme or antigen–enzyme conjugates are commercially available, the majority of amperometric immunoassays is based on the use of specific enzyme/substrate couples. Table 1 summarises the label enzyme/substrate couples typically used to detect the formation of the antigen–antibody complex with electrochemical measurements [20–27].

The main disadvantage of the immunosensors based on the use of conventional electrodes is the regeneration (between each standard solution or sample to analyse) of the binding sites of the antibodies bound to the immunosensor surface, given that the Ag–Ab interaction is reversible. This regeneration using acidic or alkaline solutions, guanidinium chloride, or ionic strength shock is time-consuming and potentially harmful to the binding capacity, and it may lead to a diminished lifetime of the immobilised antibodies and potentially very useful device that offers the unique possibility of combining the high sensitivity of electrochemical SPE-based immunosensors with the favourable characteristics of high throughput ELISA procedures. These advantages consist in rapidity of analysis and the possibility to carry out the calibration and the analysis of several unknown samples (in replicate) at the same time. In this way, it becomes possible to overcome the most significant drawback of electrochemical disposable immunosensors, due to the fact that they have to be handled singly, thus increasing the overall time of analysis. Moreover, the MEI system allows the cre-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Potential (working electrode)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>P-Aminophenyl phosphate (PAPP)</td>
<td>+150 mV (carbon)</td>
<td>Trau et al. [20]</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>1-Naphthyl phosphate</td>
<td>+550 mV (glassy carbon)</td>
<td>Cardosi et al. [21]</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Phenyl phosphate</td>
<td>+800 mV (platinum)</td>
<td>Brooks et al. [22]</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Glucose/O₂</td>
<td>+650 mV (platinum)</td>
<td>Carter et al. [23]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Potassium iodide/hydrogen peroxide</td>
<td>+127 mV (carbon)</td>
<td>Krishnan et al. [24]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Hydroquinone/hydrogen peroxide</td>
<td>−300 mV (platinum, gold, graphite)</td>
<td>Kalab et al. [25]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Ferrocenesic acid/hydrogen peroxide</td>
<td>+300 mV (glassy carbon)</td>
<td>Del Carlo et al. [26]</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Tetramethylbenzidine/hydrogen peroxide</td>
<td>+100 mV (glassy carbon)</td>
<td>Volpe et al. [27]</td>
</tr>
</tbody>
</table>
Fig. 2 – (a) Schematic view (left) and picture (right) of a common screen-printed electrode. (b) Multichannel electrochemical immunoassays (MEI) (left) with a close-up of a single well and instrumentation reader (right).

ation of a multianalyte array, by immobilising in different rows or columns the various antibodies for different analytes.

Recently, electrochemical sensors have been successfully coupled with immunomagnetic beads (IMBs) in a device called a magneto-electrochemical immunosensor [36–38]. Magnetic beads are particles constituted from a dispersion of magnetic material (Fe₂O₃ and Fe₃O₄) and then covered with a thin shell of polymer which contains the magnetic material and also serves to define a surface area for the absorption or coupling of a large variety of molecules. Antibodies or antigens are immobilised at the surface of magnetic beads and all immunological steps are performed in micro-tubes using a rotation sample mixer. After each incubation or washing step, the IMBs are concentrated on the side wall of the micro-tubes by placing the tubes in a specially designed magnetic particle separator allowing the supernatant to be discarded. Finally, the IMBs are concentrated onto the magnetised working electrode surface and the electrochemical measurements are carried out. Using this approach, which combines the selectivity of the antibodies with the sensitivity of the electrochemical detection and the possibility of concentrating magnetic particles on the electrode surface, it is possible to achieve remarkable enhancement in the performance of classical immunoassays [38].

The cited advantages of amperometric detection, coupled with the advances in sensor technology and the feasibility of “in situ” application with friendly user technology make this technique one of the most used detection systems for electrochemical immunosensors (Table 2). Hence, this review’s section will be devoted to the description of the most recent developments in this type of immunosensors.

2.1. Applications in food analysis

2.1.1. Veterinary drugs

In modern livestock production, veterinary drugs and anabolic steroids (endogenous or synthetic hormones) are being used on a large scale. Incorrect or unauthorised use of these compounds may leave residues in edible tissues, thus causing toxic effects for consumers. In order to protect public health, maximum residue limits (MRLs) of veterinary medical products in foodstuffs of animal origin (liver, milk, egg, kidney, muscle, fat, etc.) have been established according to European Union regulation (2377/90/CEE). For natural steroid hormones (estradiol, progesterone and testosterone) action limits (to discriminate between the endogenous level and illegal animal administration) have been set for biological fluids such as blood, urine and serum [39].

2.1.1.1. Anabolic steroids. Concerns over the use of steroid hormones as growth promoters, have prompted the EU to prohibit their use in food producing animals. Subsequently, rigorous screening procedures have been implemented in all member states to detect the illegal administration of such compounds. Indirect competitive electrochemical immunoassays (Fig. 1E) for the detection of testosterone,
19-nortestosterone and methyltestosterone in bovine urine were developed by Conneely et al. [40,41] using conjugated testosterone-BSA immobilised onto disposable screen-printed electrodes. Undiluted blank bovine urine was tested and a strong matrix effect was observed; for this reason a dilution step (1:20 with buffer) was used to minimise this effect. The authors claim a satisfactory precision (repeatability and reproducibility), accuracy and stability. Only the immunosensor for the detection of testosterone was employed for real sample analysis on urine samples from heifers that have been treated at various time points with testosterone. The results obtained, even if in good agreement with a typical metabolic profile of testosterone in bovine urine, were not confirmed with a reference method.

The same group has developed similar competitive indirect immunoassays (Fig. 1E) for the screening of boldenone and methylboldenone in bovine urine [42]. Fortified urine samples at different levels of boldenone and methylboldenone were prepared and analysed showing good accuracy and precision. Finally, incurred urine samples collected from heifers treated with boldenone and methylboldenone were analysed using the developed immunosensors and in this case the results from the immunoassays were compared with those obtained in another laboratory using a well-characterised and validated method (GC–MS). Some variation between the two different methods was observed and explained by the authors with the consideration that urine samples were determined directly after a single step dilution by immunosensors, while they were first subjected to a hydrolysis step for GC–MS. Moreover, antibodies can recognise individual or several endogenous steroid isomers. Although the authors conclude that the immunosensors developed can provide semi-quantitative information and would be beneficial as screening method to find and select suspicious samples for further analysis, it is our opinion that experiments of cross-reactivity (towards other endogenous steroid isomers) and analysis of incurred urine samples (with and without a hydrolysis step) should be carried out. This could provide a better evaluation of the effective potentiality of the system to avoid false compliant results and to limit the number of false non-compliant results.

A disposable immunosensor for detection of 17β-estradiol (17β-E2) in non-extracted bovine serum has been reported by Volpe et al. [43]. The direct competitive assay (Fig. 1A) is based on the use of screen-printed electrodes and a PampsS portable electrochemical detector. To develop a prototype for field measurements, the entire competitive protocol has been optimised directly in a blank of non-extracted bovine serum having no detectable amount of 17β-E2 by LC–MS/MS.

According to the new EU criteria established by the Commission Decision 2002/657/EC for qualitative and quantitative screening methods, the detection capability (CC3) was determined by analysing 20 blank samples fortified at the action limit (40 pg mL\(^{-1}\)) obtaining a value below 40 pg mL\(^{-1}\) with no false compliant results. Results obtained on real samples were confirmed by LC–MS/MS, demonstrating that the disposable immunosensor was suitable as a screening tool for field analysis of bovine serum estradiol. A disadvantage of this system is the use of a blank bovine serum, not easy available, for the construction of the calibration curve.

2.1.1.2. Antibiotic. A disposable electrochemical indirect competitive assay (Fig. 1E) for the detection of two macrolides (erythromycin and tylosin) in bovine muscle was recently developed using an SPE [44]. The antibiotics were extracted from 2 g of homogenised tissue using 10 mL of 20% (v/v) methanol in PBS by stirring for 15 min. After centrifugation and filtration 1 mL of the filtrate was used for the detection of tylosin, while another aliquot was diluted in PBS (1/9: v/v) for the detection of erythromycin.

To evaluate the matrix effect, calibration curves, obtained using blank meat samples spiked, after extraction, with known amounts of macrolides, were generated. Finally, blank samples spiked with mixtures of the macrolide antibiotics (at concentrations of 0.5 × MRL, MRL, 2 × MRL) and real samples were analysed. The immunosensor system showed good precision and accuracy and the results obtained on real samples were confirmed by micro-LC–MS/MS showing promising features of the developed sensors.

An interesting electrochemical magneto-immunosensor for the detection of sulfonamide antibiotics in milk has been presented by Zacco et al. [45]. Class-specific anti-sulfonamide antibodies were covalently bound on tosyl-activated magnetic beads. The immunological reaction for the detection of sulfonamide antibiotics performed on the magnetic beads (in Eppendorf tubes) is based on a direct competitive assay (Fig. 1A) using a tracer with HRP as the enzyme label. After the immunochimical reactions, the modified magnetic beads can be easily captured by a magneto sensor made of graphite-epoxy composite (m-GEC), which is also used as transducer.

In a first instance the matrix effect was evaluated in raw full cream and UHT milk. In the case of raw and UHT full cream milk, it was necessary a dilution step (1:4) while for UHT skimmed and semi-skimmed milk (with minor fat content) it was possible to avoid the sample dilution step. Five spiked samples prepared with the raw full cream milk (diluted 1:4) were then analysed by the electrochemical magneto-immunosensor using the standard curve as reference. Although spiked values are slightly overestimated, because the competitive curve in buffer is taken as a standard curve, results obtained are very close to the expected values. Because of the simplicity of the immunochimical procedure, this strategy can be suitable for fast semiquantitative and quantitative analysis of sulfa drug residues in milk.

2.1.2. Pathogenic bacteria

Although in these last 5 years several electrochemical immunosensors for the detection of pathogenic bacteria have been developed, their application to food analysis is very poor. Even when the experimentation in food is carried out it does not always demonstrate in a satisfactory way the effective applicability of this kind of immunosensors in food analysis.

Chemburu et al. [46] have developed a flow through amperometric immunoassay system utilising highly-dispersed carbon particles as immobilisation support of antigen specific unlabeled antibodies. A sandwich immunoassay (Fig. 1B) was employed with all steps performed at a fixed flow rate. Detection of E. coli, L. monocytogenes and C. jejuni was demonstrated with detection limits of 50, 10, and 50 CFU mL\(^{-1}\), respectively, and an overall assay time of 30 min. This system was
then applied to milk and liquid surrounding chicken (chicken extracts) samples (diluted 10 times) spiked with known amount of the target bacteria and calibration curves were generated and compared with a standard curve (prepared in buffer). A matrix effect was observed in both diluted media (especially in milk) in terms of working range, but the authors claim that no change in the detection limits was observed for L. monocytogenes in chicken extract (where LOD = 30 CFU mL\(^{-1}\)).

Due to the fact that a variable matrix effect was observed (between milk and surrounding chicken) in our opinion the developed system cannot be applied in cases where it is necessary to quantify the bacterial concentration because it would be necessary to construct one calibration curve for each type of matrix. Moreover, although this system is capable of rapidly detecting bacteria at very low concentrations, taking in account that the samples must be diluted 1:10 before the analysis and that low mandatory limits have been established by the European Community [47] (e.g. L. monocytogenes must be absent in 25 g or at maximum 100 CFU g\(^{-1}\), depending on the kind of the product), a short pre-enrichment phase seems to be necessary prior to the analysis.

More recently Delibato et al. [34] developed a simple and rapid multichannel electrochemical immunosensor (MEI) for the detection of Salmonella enterica, based on the use of a 96-well plate and a “sandwich” format (Fig. 1B). The limit of detection was calculated to be 2 \(\times\) 10\(^6\) CFU mL\(^{-1}\) with a total analysis time of about 3 h. Because the European legislation established that Salmonella must be absent in a defined amount of food products (1 g, 10 g or 25 g, depending on the kind of the product), the immunochemical system was initially applied to meet samples (without endogenous Salmonella) experimentally contaminated with S. Enteritidis (1–10 CFU/25 g) after a 24 h of pre-enrichment. Results obtained by MEI were confirmed by classic cultural method and compared to a specific PCR real-time SYBR-green. The MEI system is economical, rapid (in terms of immunoassay time) and easy to use; however, before a real application of this method for food analysis, it is necessary to extend its application to meet samples experimentally contaminated (with 1–10 CFU of other S. enterica serotypes in 25 g) and not experimentally contaminated. Moreover, also in this case, the pre-enrichment phase represents a serious drawback in terms of overall analysis time.

2.1.3. Toxins

2.1.3.1. Domoic acid. Domoic acid (DA), a neuroexcitatory toxin from marine diatoms, which is found in sea products (especially in filter-feeding molluscs such as clams, oysters and mussels), is the causative agent for amnesic shellfish poisoning (ASP). The presence of DA-producing algal blooms throughout the world has raised concerns with fishery managers and thus detection of DA in shellfish has become essential.

An electrochemical indirect competitive immunoassay (Fig. 1E) for a rapid screening of DA was recently proposed [48] based on the use of a SPE. After the characterisation of the proposed system with standard solutions in buffer, the suitability of the assay for DA quantification in mussels was evaluated. Samples were spiked with DA before and after sample treatment to study the extraction efficiency and the matrix effect, respectively. After treatment of “blank” mussels, the extract was fortified with a known amount of DA and analysed. Using a 1:250 (v/v) dilution in PBS-M (phosphate saline buffer pH 7.4 + CH\(_3\)OH 10%) to minimise the matrix effect the authors claim a detection limit of 20 \(\mu\)g g\(^{-1}\) of DA in mussel tissue which represents the maximum acceptable limit defined by the Food and Drug Administration [49].

2.1.3.2. Mycotoxins. Mycotoxins are defined as “fungal metabolites which, when ingested, inhaled or adsorbed through the skin cause lowered performance, sickness or death in man or animals, including birds”. The most important mycotoxins are the aflatoxins (AFs) and ochratoxin A (OTA) that are produced as secondary metabolites by the fungi Aspergillus and Penicillium and are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. When aflatoxin B1 (AFB1) is ingested by cows, it is transformed into its hydroxylated product, aflatoxin M1 (AFM1), which is then secreted in the milk. Unfortunately, AFM1 is relatively stable during milk pasteurisation and storage as well as during the preparation of various dairy products [50,51].

Analytical methodology must allow the determination of aflatoxins at least below the specific regulatory levels. In fact, the European Committee Regulations (ECR) has established the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits for direct human consumption: 4 ng g\(^{-1}\) for total aflatoxins (AFB1, AFG1, AFB2, AFG2) and 2 ng g\(^{-1}\) for AFB1 alone. The current maximum level for AFM1 in milk is 0.05 ng mL\(^{-1}\), while for OTA is 3 \(\mu\)g kg\(^{-1}\) in all cereal products intended for direct human consumption [52].

2.1.3.3. Aflatoxins. Responding to the need to achieve high sensitivity and move to the use of disposable probes, several electrochemical immunosensors have recently been reported in literature for the detection of AFB1 in corn and barley [35,53,54] and AFM1 in milk [55]. In particular, for AFB1 determination, an indirect competitive electrochemical immunoassay (Fig. 1E) has been developed using disposable screen-printed carbon electrodes. The specificity of the assay was assessed by studying the cross-reactivity of the MAb towards other aflatoxins. The results indicated that the MAb could readily distinguish AFB1 from other toxins, with the exception of AFG1. The proposed system showed a low matrix effect for barley and good recovery when analysing spiked samples that were treated with an easy procedure: extraction of the analyte with 85% methanol:15% PBS, then centrifugation and dilution 1:1 (v/v) with phosphate buffer. The results obtained were confirmed by HPLC coupled with fluorescence detection. The stability of the modified sensor, up to the blocking step, was also evaluated so as to have a strip ready to use directly in the competition step [53].

Recently Fierramini et al. [35] realised an improvement over the previously described immunoprobes for AFB1 determination in corn using an electrochemical immunoplate with multichannel read-out (Fig. 2B). A negligible matrix effect and good recoveries were obtained for spiked corn samples, demonstrating the suitability of the proposed assay for accurate determination of AFB1 in corn samples; however, considering the sample treatment, the method exhibited a detection limit too close to the law limit of AFB1 in corn (2 ng g\(^{-1}\)).
Another disposable electrochemical immunosensors has been proposed by Micheli et al. for the detection of AFM1 in milk [55]. Amperometric immunosensor was based on the use of screen-printed electrodes and performed in a direct competitive format (Fig. 1A). Studies of interference and matrix effects have been performed to evaluate the suitability of the developed immunosensor for AFM1 analysis directly in centrifuged milk without the need of pre-treatment or extraction steps. The proposed system was compared with a conventional method (spectrophotometric ELISA) obtaining similar results but with the advantages of a shorter analysis time and the suitability for “in situ” monitoring.

A biosensing device (a system in which the biological material is immobilised on a solid phase, but not in intimate contact with the transducer) was realised by Badea et al. [56] for AFM1 detection in raw milk. This automated low-cost instrumentation combines the rapidity and reproducibility of the flow-injection technique with the high selectivity and sensitivity characteristics of immunochemical reactions. The proposed method is an adaptation of a generic flow-injection immunoassay (FI-IA) system based on amperometric detection of horseradish peroxidase (HRP), which served as the enzymatic label for tracer analyte in the eluate. The generic scheme of this system (Fig. 3) was based on an off-line incubation of the antigen (Ag), the tracer (Ag*, enzyme-labelled antigen) and the antibody (Ab) until equilibrium was reached. Then, this mixture was introduced into a flow-injection system where the antigen–antibody complex (Ab–Ag and Ab–Ag*) is trapped on the Protein G column, while the free (i.e. unbound) tracer was eluted and detected by amperometric measurement of the enzymatic activity. Different milk samples were analysed and the results were in good agreement with those obtained by HPLC. Both the electrochemical methods (immunosensor and FI-IA) present as disadvantages the fact that fresh untreated milk sample is required for the analysis. Any storage condition of milk (frozen or cooled for more than three days) leads in fact to the binding of the toxin with milk proteins making it not detectable with these proposed methods.

2.1.3.4. Ochratoxin A. A direct competitive immunoassay (Fig. 1A) based on single disposable screen-printed electrodes has been developed for quantitative determination of ochratoxin A (OTA) in wheat [57]. A one-step procedure of extraction of OTA from wheat was used with aqueous acetonitrile (ACN:H2O, 6:4 (v/v)) solution and after filtration the extract was used directly for the analysis. A good correlation was found by comparative analysis of naturally contaminated wheat samples using the proposed immunosensor and the HPLC/immunoaffinity clean up method [58] for determination of OTA in cereals.

2.1.4. GMO

Progress in genetic engineering technology has enabled the introduction and expression of novel genes in crop plants in order to produce agronomically useful traits such as insect and disease resistance. In the contest of this development, three transgenic Lepidoptera-resistant maize lines (Bt-11, MON-810, Bt-176), commonly referred to as Bt-maize, express the genes for the Bacillus thurigiensis toxic proteins Cry 1Ab (Bt-11, MON-810) and Cry 1Ac (Bt-176). In the EU, foods containing ingredients with a content of GMOs >0.9% (for each ingredient) must be labelled. To enforce these regulations, reliable and fast methods for the detection and quantification of GMOs present in food products are needed.

An immunomagnetic electrochemical sensor (IMES) for detection of Bt-Cry 1Ab/Cry1Ac proteins in genetically modified corn samples has been recently developed [59]. The IMES is based on the use of magnetic beads as support for the immunological chain following a sandwich format (Fig. 1B). At the end of all the immunological reactions, the immunobeads were localised onto the surface of a magnetised screen-printed electrode and differential pulse voltammetry (DPV) was used for measurement. The current response was found to be directly proportional to the concentration of GMOs >0.9% (for each ingredient) must be labelled. To enforce these regulations, reliable and fast methods for the detection and quantification of GMOs present in food products are needed.

An immunomagnetic electrochemical sensor (IMES) for detection of Bt-Cry 1Ab/Cry1Ac proteins in genetically modified corn samples has been recently developed [59]. The IMES is based on the use of magnetic beads as support for the immunological chain following a sandwich format (Fig. 1B). At the end of all the immunological reactions, the immunobeads were localised onto the surface of a magnetised screen-printed electrode and differential pulse voltammetry (DPV) was used for measurement. The current response was found to be directly proportional to the concentration of Cry1Ab and Cry1Ac proteins. The scheme of this system is reported in Fig. 4. This method was applied to corn samples treated with an appropriate extraction buffer. After centrifugation the supernatant was used for the IMES test. Certified reference materials (CRMs), having different mass fractions (0.5, 1, 2, 5%) in the dried powder prepared from genetically modified
(MON 810) maize, were treated and analysed multiple times using Cry 1Ab protein as calibrator in order to assess, under these experimental conditions, the correspondence between the % of genetically modified material and the concentration of Cry1Ab protein (ng g$^{-1}$). Once obtained, this linear correlation curve was used for all the successive GMO measurements.

The performances of the immunomagnetic electrochemical sensor, in terms of detection limit and total analysis time, are comparable to those of commercially available spectrophotometric kits and thus the proposed method represents a new approach for GMO analysis.

2.1.5. Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are ubiquitous pollutants widely diffused in the environment. Their residues have been found in air, water, milk, human and animal adipose tissue. A simple protocol for analysing PCBs in real samples [60] has been developed using SPEs and a direct competition assay. With this method, araclor mixture (1242 and 1248) was determined in different matrices including sheep’s milk, bovine adipose tissue, and bovine muscle. Results obtained were compared with the results accredited from ISO 17025 methods for PCB detection (high-resolution gas chromatography and a low-resolution mass spectrometer (HRGC–LRMS) as confirmatory analysis. The extraction procedure used was in accordance with the criteria proposed by the European Directive [61] and was based on extraction with petroleum ether, evaporation under nitrogen stream and purification using alumina glass column. The minimum matrix effect observed indicates that the response of the immunosensor was only weakly influenced by the type of the sample. For this reason, normalisation of the data was possible obtaining a wide applicability of the immunosensor to different typologies of food samples. This broad applicability, coupled with the high sensitivity, may lead to the use of this system as a preliminary indicator before using the HRGC/LRMS analysis in the food laboratory.

2.1.6. Pesticides  

2.1.6.1. 2,4-Dichlorophenoxyacetic acid. 2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most used herbicides in agriculture, and the highest permissible concentration of this herbicide in drinking water was set by the European Union 0.1 ng mL$^{-1}$. Deng et al. [33] proposed an array immunosensor (MED) for simultaneous analysis of several water samples in order to monitor for 2,4-D contamination. A direct competitive immunoassay (Fig. 1A) was carried out in an ELISA microplate, while the enzymatic product was transferred onto another plate and determined amperometrically using pre-equilibrated eight-channel electrodes. The repeatability of individual electrodes was satisfactory, and homogeneity of MED was greatly improved by tuning the electrodes with their corresponding calibration factors.

2.1.6.2. Atrazine. Atrazine has been identified as one of the herbicides that is persistent in groundwater and surface waters because of its polarity. Food and environmental regulatory agencies have established a control program in order to prevent this pollutant from entering the food supply. The European Community has thus established a maximum residue limit (MRL) of 0.1 $\mu$g mL$^{-1}$ for atrazine in potable water and fruit juice. A novel electrochemical immunosensor for the detection of atrazine residues in liquid samples (such as orange juice) has been proposed and realised by Zacco et al. [62]. A graphite–epoxy composite transducer was bulk modified with universal affinity biomolecules, such as avidin (Av–GEB) and Protein A (ProtA–GEB). Using ProtA–GEB in a direct competitive immunological electrochemical assay (Fig. 1A) an excellent linear range, a low detection limit and good sensitivity were achieved with orange juice samples using atrazine–HRP tracer as enzymatic label. Because of
the simplicity of the immunochemical procedure presented, this strategy seems to be well-suited for fast semiquantitative and quantitative on-site analysis of the presence of atrazine (or atrazine immunoreactive herbicide) in real samples.

3. Optical immunosensors

Although electrochemical transduction methods are widely used, optical-based biosensor systems have proved to be the most widely reported for immunosensor applications. Optical biosensors can employ a number of techniques to detect the presence of a target analyte and are based on well-founded methods including chemiluminescence, light absorbance, fluorescence, phosphorescence, light polarization and rotation. Among optical methods, surface plasmon resonance is currently the most used technique [63–71].

3.1. SPR immunosensor

The primary attraction of SPR-based immunosensors is their capacity for highly specific detection of small molecules with low detection limits being realised for a wide variety of analytes in complex matrices. These characteristics make them suitable for application in biomedical, food and environmental fields [72–77]. The main applications of SPR systems are for the determination of both the affinity and kinetics of interactions between two or more biomolecules [65–78], receptor–ligand interactions [79] and detection of nucleic acid hybridisation [80]. However, until now the main analytical application of such systems is based on the use of antibodies as specific reagents [81] and only a small fraction of the possible applications is related to food analysis.

The basic principle and practical operation of SPR have been described in several review articles [14, 68–71, 82]. For the sake of clarity, here we will present a simplified explanation of the SPR approach. A SPR immunosensor is comprised of the following components: a light source, a prism, a transduction surface (usually gold-film), a biomolecule (antibody or antigen), a flow system, and a detector. Plane polarised light is directed through the glass prism to the gold/solution dielectric interface over a wide range of incident angles; the intensity of the resulting reflected light is measured against the incident angle with a detector. Plane polarised light travels, in particular conditions, through an all-fiber optic assembly. In the most common format a particular property of the wave guide makes possible to follow the production of the antibody–antigen complex. In these systems antibodies are usually immobilized on the surface of tethered fiber-optic probes [15, 84, 85]. If an electromagnetic wave travels, in particular conditions, through an all-fiber system to the fiber tip it penetrates as an evanescent wave into the area outside the tip. This wave is then able to excite fluorescently labelled antigens which have reacted with the antibodies present in the tip resulting in a high fluorescent signal related to the concentration of the analyte.

Although the use of fluorescence detection allows a good sensitivity, these optical-based immunosensors still suffer of many drawback mainly related to non-selective adsorptions and interferences due to background fluorescence. This has limited the applicability of these systems in real life and few are the examples appeared in literature regarding their practical application in food analysis [85–90]. Other optical-based immunosensors utilize the chemiluminescent detection and involve the use of label enzymes such as HRP and alkaline phosphatase in conjunction with chemiluminescent substrates or products.

3.3. Applications in food analysis

Both conventional laboratory-based and miniaturised transportable SPR immunosensor systems are being developed for the detection of various analytes of importance in the food-related field. For food borne pathogens and their toxins, a number of assays based on SPR technique and other optical methods have been developed over the last decade. However, many of the reports regarding the optimization of an analytical system for food analysis do not address sample application and method validation. The final demonstration of the real applicability of these systems in food analysis is then usually missing. In fact, although there are many examples of SPR immunosensors developed for several pathogens and toxins, few are the papers dealing with practical applications using food samples (Table 3). Only these examples will be reviewed in the following paragraphs.

3.3.1. Veterinary drugs

3.3.1.1. Chloramphenicol. Chloramphenicol is a broad-spectrum antibiotic with excellent antibacterial and pharmacokinetin properties. However, its use is limited because it is often associated with serious side effects. SPR
Table 2 - Summary of electrochemical immunosensors with food applications

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Assay format</th>
<th>Working range</th>
<th>LOD</th>
<th>Sample</th>
<th>Sample treatment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>17ß-Estradiol</td>
<td>Direct competitive</td>
<td>0.003–40 ng mL⁻¹</td>
<td>0.015 ng mL⁻¹ᵃ</td>
<td>Bovine serum</td>
<td>Whole serum.</td>
<td>Volpe et al. [43]</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>Indirect competitive</td>
<td>0.01–330 ng mL⁻¹</td>
<td>0.072 ng mL⁻¹ᵇ</td>
<td>Tap water</td>
<td>Without any treatment.</td>
<td>Connelly et al. [41]</td>
</tr>
<tr>
<td>2,4-D</td>
<td>Direct competitive</td>
<td>0.1–10 ng mL⁻¹</td>
<td>0.09 ng mL⁻¹ᵃ</td>
<td>Barley</td>
<td>Extraction with 85% CH₃OH in PBS and dilution in buffer (1:1, v/v).</td>
<td>Deng et al. [33]</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>Indirect competitive</td>
<td>0.05–2 ng mL⁻¹ᵇ</td>
<td>0.03 ng mL⁻¹ᵇ</td>
<td>Corn</td>
<td>Extraction with 85% CH₃OH in PBS and dilution in buffer (1.5, v/v).</td>
<td>Piermarini et al. [35]</td>
</tr>
<tr>
<td>Aflatoxin M1</td>
<td>Direct competitive</td>
<td>0.03–0.22 ng mL⁻¹ᵇ</td>
<td>0.025 ng mL⁻¹ᵇ</td>
<td>Milk</td>
<td>Dilution in buffer (1:5, v/v).</td>
<td>Badea et al. [56]</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Direct competitive</td>
<td>0.01–100 µg mL⁻¹ᶜ</td>
<td>nr</td>
<td>Sheep milk bovine adipose tissue bovine muscle</td>
<td>Extraction with petroleum in according to EC 93/256.</td>
<td>Alaschi et al. [60]</td>
</tr>
<tr>
<td>Atrazine</td>
<td>Direct competitive</td>
<td>0.016–1.2 ng mL⁻¹ᵃ</td>
<td>0.006 ng mL⁻¹ᵃ</td>
<td>Orange juice</td>
<td>Dilution in buffer (1.5, v/v).</td>
<td>Zacco et al. [62]</td>
</tr>
<tr>
<td>Boldenone</td>
<td>Indirect competitive</td>
<td>0.03 ng mL⁻¹ᵇ</td>
<td>0.03 ng mL⁻¹ᵇ</td>
<td>Bovine urine</td>
<td>Dilution in buffer (1.10, v/v).</td>
<td>Lu et al. [42]</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>Sandwich</td>
<td>50–500 CFU mL⁻¹ᵇ</td>
<td>50 CFU mL⁻¹ᵇ</td>
<td>Milk and liquid surrounding chicken</td>
<td>Dilution in buffer (1.10, v/v).</td>
<td>Chemburu et al. [46]</td>
</tr>
<tr>
<td>Cry 1Ab protein (GMO)</td>
<td>Sandwich</td>
<td>0.25–4 ng mL⁻¹ᵇ</td>
<td>0.1 ng mL⁻¹ᵇ</td>
<td>Corn</td>
<td>Extraction with an appropriate buffer.</td>
<td>Volpe et al. [59]</td>
</tr>
<tr>
<td>Domoic acid</td>
<td>Indirect competitive</td>
<td>5–70 ng mL⁻¹ᵇ</td>
<td>5 ng mL⁻¹ᵇ</td>
<td>Mussel</td>
<td>Extraction with CH₃OH:H₂O (3:1, v/v) and dilution in PBS-M (1:250, v/v).</td>
<td>Micheli et al. [48]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Sandwich</td>
<td>50–1000 CFU mL⁻¹ᵇ</td>
<td>50 CFU mL⁻¹ᵇ</td>
<td>Milk and liquid surrounding chicken</td>
<td>Dilution in buffer (1.10, v/v).</td>
<td>Chemburu et al. [46]</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Indirect competitive</td>
<td>nr</td>
<td>0.2 ng mL⁻¹ᵇ</td>
<td>Bovine muscle</td>
<td>Extraction with 20% CH₃OH in PBS and dilution in buffer (1:10, v/v).</td>
<td>Ammida et al. [44]</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>Sandwich</td>
<td>10–1500 CFU mL⁻¹ᵇ</td>
<td>10 CFU mL⁻¹ᵇ</td>
<td>Milk and liquid surrounding chicken</td>
<td>Dilution in buffer (1.10, v/v).</td>
<td>Chemburu et al. [46]</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>Indirect competitive</td>
<td>nr</td>
<td>0.12 ng mL⁻¹ᵇ</td>
<td>Bovine urine</td>
<td>Dilution in buffer (1.10, v/v).</td>
<td>Lu et al. [42]</td>
</tr>
<tr>
<td>Ochotaxin A</td>
<td>Direct competitive</td>
<td>0.06–2.5 ng mL⁻¹ᵇ</td>
<td>0.06 ng mL⁻¹ᵇ</td>
<td>Wheat</td>
<td>Extraction ACN:H₂O (6:4, v/v).</td>
<td>Alarcon et al. [57]</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Sandwich</td>
<td>5 x 10⁶–5 x 10⁸ CFU mL⁻¹ᵇ</td>
<td>2 x 10⁶ CFU mL⁻¹ᵇ</td>
<td>Meat</td>
<td>Extraction in BPW and pre-enrichment (24 h).</td>
<td>Delibato et al. [34]</td>
</tr>
<tr>
<td>Sulfamidine antibiotics</td>
<td>Direct competitive</td>
<td>1.28–91.8 ng mL⁻¹ᵇ</td>
<td>0.44 ng mL⁻¹ᵇ</td>
<td>Milk</td>
<td>Dilution in buffer (1:4, v/v) for raw full cream milk.</td>
<td>Zacco et al. [45]</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Indirect competitive</td>
<td>0.03–1.6 ng mL⁻¹ᵃ</td>
<td>0.0018 ng mL⁻¹ᵃ</td>
<td>Bovine urine</td>
<td>Dilution in buffer (1.20, v/v).</td>
<td>Connelly et al. [40]</td>
</tr>
<tr>
<td>Tylosin</td>
<td>Indirect competitive</td>
<td>nr</td>
<td>2 ng mL⁻¹ᵇ</td>
<td>Bovine muscle</td>
<td>Extraction with 20% CH₃OH in PBS.</td>
<td>Ammida et al. [44]</td>
</tr>
</tbody>
</table>

2,4-D = 2,4-dichlorophenoxyacetic acid. nr = value not reported.

* LOD and working range values obtained in blank extract fortified with the analyte after the extraction procedure.

* LOD and working range value obtained with standard solutions prepared in buffer.

* In this case the working range was not evaluated and this only represents the range of concentrations tested.

---

2,4-D = 2,4-dichlorophenoxyacetic acid. nr = value not reported.

* LOD and working range values obtained in blank extract fortified with the analyte after the extraction procedure.

* LOD and working range value obtained with standard solutions prepared in buffer.

* In this case the working range was not evaluated and this only represents the range of concentrations tested.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Assay format</th>
<th>Working Range</th>
<th>LOD</th>
<th>Sample</th>
<th>Sample treatment</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Competitive</td>
<td>nr</td>
<td>15–60 ng mL(^{-1})(^{1b})</td>
<td>Milk</td>
<td>Dilution with buffer (1:10, v:v). Milk: vortex for homogenization; honey, prawn, muscle; extraction with PBS + centrifugation + dry + redissolution with buffer.</td>
<td>Haasnoot et al. [99]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02–0.07 ng g(^{-1}) (CC(\beta)) (depending on sample used)</td>
<td>Muscle honey prawn milk</td>
<td></td>
<td>Ferguson et al. [91]</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Competitive</td>
<td>nr</td>
<td>2.5–30 ng mL(^{-1})(^{1b})</td>
<td>Wheat</td>
<td></td>
<td>Tudos et al. [125]</td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td>Competitive</td>
<td>4.1 ng mL(^{-1}) (IC(50)) (^{b})</td>
<td>10 nmol L(^{-1})(^{c})</td>
<td>Clam extracts</td>
<td>Homogenization with MeOH + centrifugation + filtration + column purification.</td>
<td>Ferguson et al. [124]</td>
</tr>
<tr>
<td>Domoic acid</td>
<td>Competitive</td>
<td>13–200 nmol L(^{-1})(^{c})</td>
<td>10(^2)–10(^3) CFU mL(^{-1})(^{c})</td>
<td>Clam extracts</td>
<td></td>
<td>Stevens et al. [124]</td>
</tr>
<tr>
<td>E. coli O157:H7</td>
<td>Sandwich</td>
<td>nr</td>
<td>10(^2)–10(^3) CFU mL(^{-1})(^{c})</td>
<td>Milk apple juice ground beef</td>
<td></td>
<td>Waswa et al. [109]</td>
</tr>
<tr>
<td>Fenicol antibiotic residues</td>
<td>Competitive</td>
<td>nr</td>
<td>0.1 ng g(^{-1})</td>
<td>Shrimps</td>
<td>Extraction with ethyl acetate + wash with isooctane/chloroform + dry + redissolution with buffer.</td>
<td>Dumont et al. [94]</td>
</tr>
<tr>
<td>Nicarbazin</td>
<td>Competitive</td>
<td>nr</td>
<td>17–19 ng g(^{-1}) (depending on sample used)</td>
<td>Poultry liver eggs</td>
<td></td>
<td>Mc Carney et al. [93]</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Competitive</td>
<td>nr</td>
<td>7–20 ng mL(^{-1})(^{1c})</td>
<td>Chicken serum</td>
<td>Dilution with buffer (1:10, v.v.).</td>
<td>Haasnoot et al. [101]</td>
</tr>
<tr>
<td>Tylosin</td>
<td>Competitive</td>
<td>nr</td>
<td>0.5 ng g(^{-1})</td>
<td>Honey</td>
<td>Homogenization with phosphate buffer + SPEX cleanup.</td>
<td>Caldwell et al. [92]</td>
</tr>
<tr>
<td>β-Agonist</td>
<td>Competitive</td>
<td>nr</td>
<td>0.02–0.19 ng g(^{-1})</td>
<td>Bovine liver</td>
<td>Proteolytic digestion + enzymatic deconjugation + SPEX cleanup.</td>
<td>Traynor et al. [106]</td>
</tr>
<tr>
<td>β-Lactam antibiotic</td>
<td>Inhibition</td>
<td>nr</td>
<td>2 ng mL(^{-1})(^{1c})</td>
<td>Milk</td>
<td>Heating step + centrifugation + dilution with buffer.</td>
<td>Cacciatore et al. [96]</td>
</tr>
</tbody>
</table>

nr = value not reported. SPEX = solid phase extraction (this acronym has been proposed in order not to create confusion with screen-printed electrode (SPE) acronym).

\(^{a}\) In the case of SPR “competitive” assay format is always to be intended as a “direct competitive” assay.

\(^{b}\) LOD and working range value obtained with standard solutions prepared in buffer.

\(^{c}\) LOD and working range values obtained in blank extract fortified with the analyte after the extraction procedure.
immunochemical screening assays have been developed for chloramphenicol and chloramphenicol glucuronide residues using a chloramphenicol derivative coated sensor chip and a competitive assay [Fig. 1D] [91]. The devices were tested in poultry muscle, honey, prawn and cows’ milk showing acceptable mean recoveries for milk, poultry and honey spiked at 0.1 μg kg⁻¹ of chloramphenicol and also the comparison with both LC–MS/MS and GC–MS/MS as confirmatory methods gave good correlation with incurred samples of poultry muscle, honey and prawn. This example is quite important due to the use of different complex matrices and demonstrates the real applicability of the discussed technology for food analysis. The validation with confirmatory methods make this test even more important and the same comment could be valid for the detection of tyllosin reported below.

3.3.1.2. Tyllosin. In recent years there has been an increase in the use of tyllosin in apiculture as bacterial brood diseases become resistant to oxytetracycline. The development and validation of a screening method for the detection of tyllosin in honey samples using SPR immunosensor technology has been recently proposed [92]. The honey was first dissolved in a phosphate buffer and following solid-phase extraction (SPEX) cleanup was analysed using a Biacore instrument and a competitive assay (Fig. 1D). The assay showed 60% cross-reactivity toward spiramycin; however, no significant cross-reactivity to the other analytes tested was observed. Twenty confirmed blank honey samples and samples containing trace residue levels of tyllosin were then analysed by both the biosensor and LC–MS/MS. The false positive and negative rates for the assay were <5% and the results obtained compared well to those produced using the confirmatory technique, indicating the reliability of the developed biosensor. Although the use of a single matrix, the test is important to understand the potentiality of the SPR technique in food analysis; however, it would have been interesting to test the immunosensor performance in the absence of the cleanup step in order to have a more simple and rapid procedure. Moreover, the cross-reactivity with spiramycin seems to be the major drawback for the future application of this immunosensor.

3.3.1.3. Nicarbazin. A similar strategy was adopted to detect the nicarbazin marker residue DNC in poultry liver and eggs [93]. No significant antibody cross-reactivity (<0.6%) was recorded with the coccidiostats assessed (halofuginone, ponazuril, monensin or dimetridazole). Prior to analysis, nicarbazin was extracted from liver and egg samples using acetonitrile. Also in this case, for liver samples a cleanup of the extract and a hexane wash were needed before the measurement and a good correlation (r² = 0.88) between liquid chromatography (LC) and SPR biosensor results was observed.

3.3.1.4. Fenicol antibiotic residues. A qualitative screening method using a SPR biosensor and a competitive assay (Fig. 1D) was also developed for the detection of all fenicol antibiotic residues in shrimps [94]. This method requires ethyl acetate extraction followed by a single wash with isooctane/chloroform solution. After addition of buffer and centrifugation the sample extract is injected over the surfaces of two biosensor chip flow cells, one surface having the capability to detect florenicin amine (FF amine), florenicin (FF), and thiamphenicol (TAP) and the second surface for chloramphenicol (CAP) detection. The quick, simple test allowed the detection of CAP residues in shrimps at the minimum required performance limit (MRPL) of 0.1 μg kg⁻¹ for this compound and of FF, FF amine, and TAP below their maximum residue limits (MRLs).

3.3.1.5. β-Lactam antibiotic. β-Lactams constitute the major source of antibiotic residues in milk, as they are the most frequently prescribed drugs in the treatment of clinical mastitis of lactating dairy cows [95]. A SPR assay was also developed for the detection of residues of penicillins and cephalosporins [96] in raw milk based on the use of the high molecular mass penicillin-binding protein derivative of Streptococcus pneumoniae (PBP 2×) which possesses high affinities for penicillins. The principle of the immunosensor is original and is based on the inhibition of the binding of digoxigenin-labelled ampicillin (DIG-AMPI) to a soluble PBP derivative (PBP 2×) by other β-lactam antibiotics. Subsequently, the DIG-AMPI/PBP 2× complex was detected with a Biacore (BIA) instrument using antibodies against digoxigenin immobilised on the sensor chip (Fig. 5). It is also interesting to note that milk showed strong matrix effect probably due to non-specific binding which was decreased by heat treatment of the sample. Even if test with positive samples indicated the possible use of the method as screening assay, several problems still have to be solved to further assess the influence of milk matrix interferences and the assay requires more analyses of spiked and incurred samples and a validation with reference methods.

3.3.1.6. Aminoglycosides. Aminoglycosides are broad-spectrum antibiotics most commonly used in veterinary drug medicine in the treatment of infections caused by aerobic Gram-negative bacteria, such as mastitis [97]. The presence of residues of these drugs in food is considered a high risk to the consumer and MRLs have been established. In the EU the MRLs for gentamicin (Genta), kanamycin (Kana), streptomycin (Strep), dihydrostreptomycin (DHS) and neomycin (Neo) in milk are 100, 150, 200 and 500 ng mL⁻¹, respectively.

3.3.1.7. Streptomycin and dihydrostreptomycin. An interesting food analysis application of a SPR competitive immunoassay (Fig. 1D) regards the determination of streptomycin and dihydrostreptomycin residues in milk, meat, and honey [98]. In this case the direct analysis of bovine whole milk was possible showing low matrix effect. It is also important the fact that the results obtained by the biosensor with those generated using a commercially available kit and HPLC were in good agreement and only one false positive was found for kidney and, importantly for a screening test, no false negatives were found for either kidney or muscle.

A different approach was instead recently proposed based on the use of a mixture of four specific antibodies and four aminoglycosides immobilised onto a biosensor chip surface into a four serially connected flow channels (Fcs) (Fig. 1D) of a Biacore to simultaneously detect five relevant aminoglycosides in skimmed milk [99]. Milk (reconstituted from skimmed milk powder) was 10 times diluted with a mixture solution of the four specific antibodies and injected
through the four serially connected Fcs. Although limits of detection are far below the MRLs and the total analysis time was 7 min, an underestimation (especially for Kana) has been observed for the combined assay in milk samples and so additional experiments have to be performed to prove the suitability of the biosensor for the detection of the aminoglycosides in raw milk samples.

### 3.3.1.8. Sulfonamides

The sulfonamides are a group of antibacterial agents commonly given to food animals for prophylactic or therapeutic purposes. MRLs for sulfonamides have been established in many countries. In the European Union an MRL of 100 μg kg\(^{-1}\) for the total amount of sulfonamides is set for edible tissue [100]. A monoclonal antibody (MAb) raised against the sulfonamide sulfamethazine was applied to develop a SPR competitive immunoassay (Fig. 1D) for the detection of several sulfonamides in chicken serum [101]. The performance of this MAb was compared with two polyclonal antibodies (PAb) raised against sulfamethazine: the MAb-based assay resulted in a better sensitivity and was found suitable for the detection of eight sulfonamides in 10 times diluted chicken serum with comparable detection limits (between 7 and 20 ng mL\(^{-1}\)). An interesting result of this assay regards its high stability which allows the use of the chip for 1100 cycles without loss of performance.

### 3.3.1.9. \(\beta\)-Agonist

The \(\beta\)-agonists are used therapeutically to treat bronchial diseases in humans and animals. They are also extensively administrated in farm animals, where high doses give rise to a preferential muscle to fat ratio [102,103] resulting in financial gain for the farmer. This illicit use of \(\beta\)-agonists has resulted in a number of reports of human food poisoning [104,105]. A high-affinity, broad-spectrum \(\beta\)-agonist monoclonal antibody was applied in a SPR immuno-biosensor competitive assay (Fig. 1D) [106]. Also in this case, the sample treatment was quite complex and included proteolytic digestion, enzymatic deconjugation and solid-phase extraction. The detection capability (CCB) was determined by spiking 20 bovine livers with MBL, CBL and SBL in consecutive experiments. In each case, all the 20 samples were declared positive with low intra- and inter-assay variations. Authors declare a sensitivity similar to that obtained with the GC–MS method [107]. However, attention must be paid to the fact that the proposed SPR assay is not specific for a single analyte, giving highly different response towards several \(\beta\)-agonists. For this reason the proposed assay could only be adopted as a screening method.

#### 3.3.2. Pathogenic bacteria and related toxins

##### 3.3.2.1. Escherichia coli

A SPR technique has been widely tested for Escherichia coli determination. Fratamico et al. [108] developed an assay for detection of E. coli O157:H7 achieving a detection limit of 5–7 × 10\(^7\) CFU mL\(^{-1}\). More recently a sandwich SPR-based biosensor (Fig. 1B) was used to detect E. coli O157:H7 in different spiked food samples [109]. Milk, apple juice, and ground beef patties spiked with E. coli O157:H7, at varying concentrations, were injected onto the sensor surface on which were immobilised antibodies against the pathogen. Uninoculated samples were used as negative control. A significant change in the signal (RU) was observed for spiked samples versus the control and a LOD in the range of 10\(^2\)–10\(^3\) CFU mL\(^{-1}\) was calculated. However, from the data provided by the authors, it appears that different behavior is obtained with different samples, while there is no complete study of recovery and accuracy presented in the report. A specificity study was conducted demonstrating that response for non-target organisms, E. coli K12 or Shigella sp. at a concentration of 10\(^5\) CFU mL\(^{-1}\), was close to the response observed for a negative control. The experiments conducted demonstrates the potential of a SPR assay for direct monitoring of pathogens in food systems; however, real sample application appears to be still insufficient to demonstrate its applicability.

##### 3.3.2.2. Staphylococcal enterotoxin B (SEB)

SEB belongs to a family of 10 major serological types (SEA through SEK) of emetic enterotoxins (SEs) produced by *Staphylococcus aureus*. These 26–30 kDa toxins are monomeric, heat-stable, and potent gastrointestinal toxins [110]. A method is needed that allows detection of SEs in a quantity below the minimum intoxication level. For SEA, the most potent SE, this is about 2 ng g\(^{-1}\). Several approaches based on the use of SPR have appeared in the literature for the detection of SEB [111–113].

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**Fig. 5** – Schematic illustration of the principle of the \(\beta\)-lactam specific BIA for blank samples (negative samples) and samples containing \(\beta\)-lactam antibiotic residues (positive samples). Reproduced with permission from Cacciatore et al. [96]. See text for detailed description.
One of the examples of the application of SPR for the detection of this analyte in food samples was based on the use of a newly developed dual-channel SPR sensor [69] where two modes of operation of the SPR biosensor were described: direct detection (Fig. 1C) and sandwich assay (Fig. 1B). Due to the good reproducibility (13%) of the sensor response and successful elimination of the baseline drift, owing to the use of the referencing channel, the SPR biosensor detection limits are limited mainly by the noise of the sensor baseline. Several samples of milk spiked with SEB were tested, with no sample preparation, using a sandwich assay. Fortified milk was flowed over the measuring channel, while milk with no SEB was flowed over the referencing channel. Thanks to the use of the referencing channel, the detection limit for this SPR biosensor used on milk samples was estimated to be equal to that for standard solutions in buffer (0.5 ng mL$^{-1}$), thus also indicating that the employed polyclonal antibodies do not show substantial cross-reactivity to biomolecules adsorbed from milk on the sensor surface. These results suggest that crude samples with minimum sample preparation can be analysed by the reported biosensor technology and demonstrate that the use of a reference channel is useful for the attenuation of matrix effect.

Detection of E. coli O157:H7 in ground beef samples was also investigated by Geng et al. [89] by a sandwich fluorescent antibody-based FOBS (Fig. 1B) which was able to detect the pathogen at a concentration of 10$^3$ CFU mL$^{-1}$. In the case of real sample measurement, which were artificially inoculated at concentration of 1 CFU mL$^{-1}$, a pre-enrichment step of 4 h was always needed for the detection. Although this, the method demonstrated a good potentiality for food analysis.

3.3.2.3. Salmonella. Detection of Salmonella is of utmost importance in the food industry [114] and a rapid, simple, sensitive, specific, online and affordable technique for the detection of such pathogen is urgently needed. A SPR assay was developed as a sandwich model (Fig. 1B) using a polyclonal antibody against Salmonella as capture and detection antibody [115]. The authors also claim that the presence of milk fat and proteins did not affect the sensitivity of the assay and no negative effects due to the milk matrix were observed. For this reason, no sample preparation or clean-up steps were undertaken. The specificity of the assay was only assessed with an E. coli spiked milk sample (1.0 × 10$^6$ CFU mL$^{-1}$) which did not show any signal. The detection limit obtained in milk is comparable to those of commonly used and approved commercial Salmonella detection kits (ca. 1.25 × 10$^5$ CFU mL$^{-1}$). The authors claim an overall analysis time of only 1 h (including the antibody immobilization); however, they did not take in account any pre-enrichment step which, considering the sensitivity of the method, is absolutely necessary.

In another example, SPR [116] was applied with the use of a lipopolysaccharide antigen of Salmonella enterica serovar Enteritidis to detect egg yolk antibodies against S. Enteritidis. Results from this biosensor assay were compared to those from commercial ELISA kits based on LPS antigen and flagellar antigen. A very high number of egg yolks and combined egg white and yolk samples from chickens experimentally infected ($n=163$) with S. Enteritidis and from uninfected chickens ($n=90$) were analysed. Data showed a diagnostic sensitivity of 82% and a diagnostic specificity of 100% with a coefficient of variation for a positive internal-control egg yolk of 1%. The SPR biosensor assay was able to detect antibodies in a significantly higher percentage of known positive samples than the two commercially available ELISAs's used for comparison.

Kramer et al. proposed the use of the RAPTOR™, an evanescent fluorescence sensor developed by Research International, Monroe, Washington, for the detection of S. Typhimurium in sprout rinse water using a fluorescence sandwich method (Fig. 1B) [90]. The contaminated seeds were assayed with this method and concentration of S. Typhimurium as low as 50 CFU g$^{-1}$ was determined. Another interesting application in food analysis was proposed by Ko and Grant [117] and was based on the use of a FRET-based fiber optic biosensor for the detection of S. Typhimurium in pork samples. Authors claim that due to the high sensitivity of the method (limit of detection in homogenized pork sample was 10$^5$ CFU g$^{-1}$) and the fast response time (5 min) the portable immunosensor could be applied in the food industry with success.

An interesting approach has been also recently proposed based on the use of a multi-analyte array biosensor (MAAB) developed at the Naval Research Laboratory (NRL). This instrument is based on a fluorimunoassay format and is capable of detecting and identifying multiple analytes in multiple samples simultaneously [118–121]. A demonstration of the possibility of applying this instrument for food analysis was recently proposed by Taitt et al. [122] based on the detection of S. Typhimurium in chicken excreta spiked samples. Preparation of the spiked excreta samples consisted of vortexing, a 1-h room temperature incubation, and finally a filtration step. Negative controls (unspiked excreta) showed no significant difference in signal when compared to unspiked buffer blanks and a LOD in spiked samples of 4 × 10$^3$ CFU mL$^{-1}$ or 8 × 10$^3$ CFU g$^{-1}$ was observed.

3.3.3. Toxins

3.3.3.1. Domoic acid. DA levels in shellfish may be determined by several analytical methods. Some reports have described the use of SPR for detecting DA in buffer [75,123], and recently the use of a portable SPR biosensor system to detect DA in standard buffer solutions as well as in clam extracts, using competition and displacement-based assays, was proposed [124]. Standard curves for detection of domoic acid in phosphate buffered saline and in diluted clam extracts, analysed by the competition-based SPR assay, demonstrated a limit of detection of 3 ppb (10 nmol L$^{-1}$) and a quantifiable range from 4 to 60 ppb (13–200 nmol L$^{-1}$). A comparison with HPLC results gave a good correlation ($r^2 = 0.99$). Moreover, a high stability and reproducibility of the sensor chip coated with DA-conjugated BSA was demonstrated.

3.3.3.2. Deoxynivalenol. Deoxynivalenol (DON) belongs to a group of toxic fungal metabolites produced by the Fusarium species that may contaminate food and animal feed, mostly grains. A SPR-based immunoassay for the selective and quantitative determination of DON in naturally contaminated matrices was recently presented [125]. The assay is based on the competition for antibody binding between the immobilised DON (conjugate with the protein casein) on the
sensor surface and the free DON molecules in the test solution (Fig. 1D). The DON–casein sensor could be reused more than 500 times without significant loss of activity using 6 M guanidine chloride solution for regeneration. The assay had an optimal range between 2.5 and 30 ng mL\(^{-1}\) in the test solution. The analysis results of the optimised SPR assay and a validated LC-MS/MS method were compared for naturally contaminated wheat samples. The results indicate good agreement between the two methods for wet-ground wheat samples. In the case of the dry powdered samples, a different sample preparation procedure was used employing higher concentrations (80%) of acetonitrile, and problems due to the matrix or to the extraction solvent were observed. Also, some problems were encountered due to the cross-reactivity of the antibodies used with other trichothecenes (3-acetyldeoxynivalenol, 5-acetyl-deoxynivalenol, nivalenol, HT2-toxin, and T2-toxin).

4. Quartz crystal microbalance

QCM represents one of the most suitable alternatives to SPR as a label free method for the detection of immunological reactions [126–128]. Although many fundamental differences exist, there are nevertheless several similarities between the physical principles of QCM and SPR sensors and these have been very clearly outlined in a paper by Kösslinger et al. [127]. The quartz crystal microbalance (QCM) detection scheme is based on the measurement of the mass changes and physical properties of thin layers deposited on the crystal surfaces [129–131]. The quartz crystal is a highly precise and stable oscillator. In a fundamental work, Sauerbrey [132] described for the gaseous phase the relation between the mass of thin metal films deposited on quartz crystals and the corresponding change in resonant frequency of the crystal:

\[
\Delta m = -\left(\frac{A\sqrt{\mu\rho}}{2F_0}\right) \Delta F
\]

where \(F_0\) is the resonant frequency, \(A\) the area of coating, \(\rho\) the density and \(\mu\) the shear modulus of the quartz. Although the equation is derived for the gas phase, the applicability with liquid samples was also demonstrated. Even though this is still contested by several authors, the demonstration has opened to wide applications in the analytical field. QCM can then be used to monitor adsorption processes at solid/liquid interfaces in chemical and biological research, rendering the method an attractive low-cost alternative for bioanalytical applications. In recent years, methods based on the use of piezoelectric crystal devices have been developed for immunoassay applications. The QCM immunosensor is usually comprised of a quartz crystal with an antigen or antibody immobilised on its surface.

The applications of QCM immunosensors and future trends were surveyed in a review [133]. The first immunosensor based on QCM for detecting anti-BSA antibody by coating antigen was developed by Shons et al. [134]. Following this development, piezoelectric immunosensors have been widely used in liquid-phase analysis and have been adopted in the field of clinical analysis, environmental monitoring [135], veterinary diagnosis, and food analysis [136]. In the case of QCM, when compared with SPR, a much lower number of real applications in food analysis could be found in the literature. In fact, the examples showing the applicability of QCM immunosensor in food samples are few and practically no examples of comparison with reference methods could be found in the literature. As shown for the other methods considered in this review, we will only reference papers dealing with food samples and showing real applicability of QCM immunosensors in food analysis.

4.1. Applications in food analysis

4.1.1. Pathogenic bacteria

4.1.1.1. Escherichia coli. A flow-type immunosensor system (Fig. 1C) which uses a broad-spectrum anti-Escherichia coli antibody and quartz crystal microbalance was recently developed [137]. A linear sensor response was observed for the microbial suspensions ranging from 1.7 \times 10^6 to 8.7 \times 10^7 CFU mL\(^{-1}\). Sample measurements were done within 20–30 min after a homogenization treatment with the Stomacher apparatus followed by spiking with detectable concentrations of E. coli. Alternatively, the homogenised samples were spiked with a low concentration of E. coli \((10^9\text{ CFU mL}^{-1})\) and, before measurement, an enrichment step was performed. None of these samples contained endogenous E. coli when analysed by the viable cell count and the E. coli Petrifilm\textsuperscript{TM} identification. The conspicuous difference in the frequency shifts between the spiked or enriched samples and the negative controls clearly showed that the antibody sensor is capable of E. coli detection. Considering that the total time required for sample preparation is shorter than 24 h, enrichment and measurement, and the cheaper analysis price compared with the conventional culturing method for E. coli identification, the antibody sensor presented in this study seemed to provide a powerful screening tool for presumptive E. coli test. However, also in this case a real quantification of the analyte was very difficult to achieve and the authors only demonstrated the possibility of using such system as a screening tool.

4.1.1.2. Staphylococcal enterotoxins. Staphylococcal enterotoxins (SETs) are a significant cause of food poisoning. A paper describing a study of a direct (Fig. 1C) and label-free immunoassay for SEB, based on a PZ crystal immunosensor, recently appeared in the literature [138]. Responses were found to be linear up to 60 \mu\text{g mL}^{-1} SEB with a detection limit of 2.5 \mu\text{g mL}^{-1} and a correlation coefficient of 0.997. The feasibility of applying the proposed immunosensor to measure toxin levels in a complex matrix was also studied by spiking various levels of SEB into three milk samples. The responses measured were proportional to the amount of toxin in the sample with mean recovery of SEB of 111% (\(n=9\)). The proposed PZ immunosensor was thus judged feasible for SEB detection; however, sensitivity is still not optimal and a comparison with reference method needs to be performed.

4.1.1.3. Salmonella. QCM immunosensor was described for the detection of Salmonella Typhimurium with simultaneous measurements of the resonant frequency and motional resistance (\(\Delta F\) and \(\Delta R\)) [139]. In the direct detection of S. Typhimurium (Fig. 1C) in a chicken meat sample, \(\Delta F\) and \(\Delta R\) were proportional to the cell concentration in the range
of $10^5$–$10^8$ and $10^6$–$10^8$ CFU mL$^{-1}$, respectively. Using anti-Salmonella-magnetic beads as a separator/concentrator for sample pre-treatment, as well as a marker for signal amplification, the detection limit was lowered to $10^2$ CFU mL$^{-1}$ (for $\Delta R$ measurement). No interference was observed from E. coli K12 or the sample matrix. The QCM immunosensor was tested in a stop-flow mode for direct detection of S. Typhimurium in buffer as well as in the stomaching solution of chicken meat. However, in this occasion, authors did not use magnetic beads which have shown better detection limits. The net responses of $AF$ and $\Delta R$ caused by $10^2$ CFU mL$^{-1}$ of S. Typhimurium in buffer or in chicken meat sample were both significantly distinguishable from the negative controls. Because Salmonella must be absent in established amount of food samples authors should have test the samples spiked at very low levels of Salmonella using a pre-enrichment step.

5. Conclusion

This review has highlighted the applications of immunosensors in the food analysis. Examples of electrochemical, optical and QCM techniques have been described and for all the three methods only applications in real food samples appeared in literature in the last 5 years have been taken in consideration. Each technique has its own strengths and limitations as an immunoassay device; however, it has to be highlighted the fact that the most common and widespread technique based on the use of the immunoassay principle is the spectrophotometric ELISA which till now has undisputed advantages over the approaches discussed in this review. ELISA offers high sensitivity, easiness of use and is cost-effective and this makes this technique really difficult to be replaced at the moment.

SPR has probably better reproducibility and reliability over the ELISA approach; however, relatively complex and expensive instruments are needed. The success of SPR in recent years is surely due to the great potentialities of this technique but also to huge investments by multinational companies which have brought to the dissemination of the SPR approach; as a result, the associated technique has become successful probably beyond its real benefits.

Before a real routine application of SPR, several problems have in fact to be solved. The fact that a label is not needed is of course an advantage over the ELISA approach; however, it becomes a drawback when the sensitivity of the method is taken into consideration. An enzymatic label is in fact essential, for spectrophotometric immunoassays, to obtain detection limits which are far below those achieved with SPR.

Furthermore, non-specific binding interferences in complex matrix have still to be completely overcome and these represent one of the major problems of this detection system. Other concerns regard the high cost of the instrumentation, the low speed, and the difficulty with portability, miniaturization, or on-site analysis. All these factors have negatively affected the advancement of this type of immunosensors and for these reasons their use has been, up to now, limited only to research laboratories with the huge investment by private companies to make their use required in accepted analysis protocols.

The QCM setup is relatively simple and less expensive than SPR, but the frequency measurement is more environmentally sensitive and the results obtained up to now showed a lower sensitivity in comparison with other methods.

Electrochemical immunosensors, although requiring a label, offer higher sensitivities when compared with SPR and QCM and low-cost instrumentation and, as major advantages, they are miniaturizable and suitable for in situ applications. These advantages make the electrochemical approach more similar to the spectrophotometric immunoassay and its use could be proposed as alternative when ELISA is not applicable with certain matrices. The electrochemical detection is in fact insensitive to those interferences which sometimes affect spectrophotometric measurement. Also in this case, however, the examples based on this technique can be purely found in the research field and, to our knowledge, no commercial examples of electrochemical immunosensor kits are available at the moment. Compared with the spectrophotometric immunoassay, the electrochemical approach could provide shorter analysis times and similar sensitivity, while also requiring lower sample volumes.

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REFERENCES


