Screen-Printed Carbon Based Biosensors and their Applications in Agri-Food Safety

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Abstract

This review focuses on the ways in which screen-printed carbon electrodes have been tailored with different biorecognition elements, including enzymes, antibodies, and aptamers, often with other modifiers, such as mediators and nanoparticles, to produce electrochemical biosensors for a variety of analytes of importance in agri-food safety. Emphasis is placed on the strategies of biosensor fabrication and the performance characteristics of the devices. As well as biosensors for a range of analytes in different agri-food matrices, we have also included reports on novel devices that have potential in agri-food safety but as yet have not been applied in this area.

Keywords: screen-printed carbon, biosensor, enzyme, antibody, aptamer, amperometry, voltammetry, agri-food safety

1.0. Introduction

This review explores the fabrication and application of screen-printed biosensors for the analysis of selected species implicated in food safety in the agri-food sector. Screen-printing technology offers a number of advantages for the fabrication of electrochemical biosensors, including fabrication in a wide range of geometries, mass production at low cost, disposability and portability. These attributes are an important consideration in commercialising biosensors and the authors believe that the examples described in this review may be of particular interest to organisations wishing to market devices for agri-food safety.

A comprehensive review by Hughes *et al.* (2016) highlights the advantages of screen-printed carbon electrode (SPCE) biosensors for various applications including the agri-food area [1]. The advantages of carbon as an electrode material over other materials such as gold include affordability and versatility of fabrication and customizability with nanomaterials and biological elements due to its high surface area. Carbon is also non-toxic. Jewell *et al.* (2016) highlights important aspects of scaling up the production of SPCEs such as choice of solvent [2]. A review

by Wang *et al.* (1998) outlines differences in electrochemical properties of four commercially available carbon inks from different vendors. The author concludes that choice of ink should depend on the analyte and electrochemical technique used for measurement [3]. Trojanowicz (2016) also outlines the advantages of SPCE biosensors for a range of applications and reviews a large number of designs; the author states the biggest progression in this technology over the last decade is the inclusion of various nanomaterials [4], a statement which is supported by Yamanaka *et al.* (2016) [5]. Cinti *et al.* (2017) outlines the advantages of graphene as a nanomaterial, which are mostly in common with the advantages of carbon as an electrode material [6].

Bio-recognition elements are readily immobilised onto the surface of carbon electrodes using strategies including adsorption, entrapment, cross-linking and covalent bonding. The bio-recognition element is chosen depending on the target analyte which results in highly selective measurements. Simple analytical methods are used in conjunction with the biosensors; typical measurement techniques include amperometry in stirred solution, chronoamperometry and pulse voltammetry. This is an attractive feature for end users of this technology for application in agri-food safety.

This review is broadly divided into 4 sections based on the classes of analyte determined, these are: (i) toxins, antibiotics and microorganisms, (ii) naturally occurring compounds, (iii) pesticides and (iv) metals.

2.0. Toxins, antibiotics and microorganisms

A good insight into some of the most important factors and useful generic approaches to utilising antibodies in various sensor formats involving screen-printed carbon surfaces can be found in a recent article by Sharafeldin *et al*. [7]. The authors compared various immobilisation strategies for antibodies onto screen-printed carbon electrode (SPCE) arrays, for the resulting antibody coverage, and also antibody activity in capturing the enzyme horseradish peroxidase (HRP). Passive adsorption led to low limits of detection, but low stability compared to covalent immobilisation, whereas inclusion of a chitosan hydrogel, which has a large 3-diensional area, together with glutaraldehyde, was an effective way of increasing antibody coverage. Use of a graphene oxide coating (GO), or gold nanoparticles (AuNP) alone or with Protein A, also enhanced antibody coverage compared to use of a bare electrode. The activity of the immobilised antibodies was preserved well using Protein A, and in combination with AuNPs, resulted in the most active antibodies with good stability. The conclusion

was that a carbon electrode with enhanced surface area that is covered with covalently-conjugated antibody will protect the antibodies from denaturation and provide a highly sensitive response.

2.1. Toxins

Botulinum neurotoxins (BoNT) are metalloendoproteases produced by several species of *Clostridium* bacteria and are linked to food-borne botulism in man, animals and birds. They are found in a variety of foods including vegetables, fish, meat and dairy products. An impedimetric immunosensor for BoNT serotype A was reported [8], based on the principle that binding of the analyte (toxin) to surface-immobilised specific antibody will create changes in resistance and capacitance that can be measured (as impedance). The sensitivity of the device was enhanced by increasing the surface area upon which antibody (and therefore additional binding sites) could be placed. This was achieved by forming gold nanodendrites that were synthesised electrochemically on the surface of the 3 mm-diameter working SPCE surface, followed by a layer of self-assembled chitosan nanoparticles. The antibody was drop-coated onto this prepared surface and covalently bound using succinimide-carbodiimide and glutaraldehyde fixation. The final working surface was also then blocked with bovine serum albumin (BSA). The authors state that the preparation of biosensors using this approach can be carried out in around 14h, which is shorter than an earlier report by the same group using a similar approach, but involving the addition of a nanocomposite that included graphene [9]. Analysis by impedance measurement showed that the resulting device was capable of detecting BoTN serotype A, over the range 0.2 – 230 pg. mL^{-1}, down to a concentration of 0.15 pg. mL^{-1}. It was serotype-specific in that it did not detect the presence of E or B serotypes. The particular relevance to the agri-food area is that the device was tested in spiked milk and was shown to be capable of toxin detection with 101% recovery and an RSD of 2.5%.

Fumonisins are mycotoxins with carcinogenic properties that are of major concern as they can enter the food chain as a result of fungal contamination and end up in a wide variety of foodstuffs including various grains, raisins, figs, fruits and milk. Jodra *et al.* [10] reported on an electrochemical magnetoimmunosensor involving magnetic beads and disposable SPCEs for determination of fumonisins FB1, FB2 and FB3. Sample and HRP enzyme-labelled fumonisin competed for binding

to magnetic beads coated with specific monoclonal antibody. The beads were then brought into close proximity with the SPCE surface using a magnet. Finally, conversion of the enzyme substrate, hydroquinone, was quantified by amperometry at an E_{app} of -0.25V vs a Ag pseudo-reference electrode. The resulting dynamic range for fumonisin B1 was from 0.73 to 11.2 μg mL^{-1}, with a L.O.D. of 0.33 μg mL^{-1}. The sensor (using the same antibody) could be used to monitor any one of the three fumonisins individually, and was able to give an accurate average concentration when applied to beer samples spiked with the two fumonisins FB1 and FB2.

An impedimetric label-free immunosensor was reported by Malvano *et al.* [11] using an SPCE modified with gold nanoparticles. A capture layer of anti-OTA antibody was immobilised via a cysteamine layer, and determinations of OTA in samples was performed by electrochemical impedance spectroscopy (EIS). The response was linear over the range 0.3-20 ng mL^{-1}, with a L.O.D. of 0.37 ng mL^{-1}. Red wine samples spiked with OTA were tested and the results compared well with those from a competitive ELISA method; recoveries were between 95 and 103% for OTA concentrations ranging from 1.5 to 10.0 ng mL^{-1}.

A competitive electrochemical immunosensor for zearalonone (ZEA) in maize samples was fabricated using a layer-by-layer deposition of MWCNT/PEI dispersions onto SPCEs [12], followed by AuNPs and then anti-ZEA polyclonal antibody. Measurement was performed by competing ZEA in the sample with HRP-conjugated ZEA for antibody binding, followed by addition of H2O2 and amperometric measurement of the resulting reduction current. The method gave a L.O.D. of 0.15pg mL^{-1} which was below that of several earlier electrochemical immunoassay reports. This detection limit is well under the permitted maximum in the country of origin (Argentina), which is around 200 μg kg^{-1}.

An alternative approach to the use of antibodies for the development of selective affinity-based biosensors for the detection of toxins in the agri-food area, has been the development of aptasensors. A review of aptasensors by Rapini and Marrazza (2017) [13] included reports of several electrochemical devices. The authors emphasise the versatility of aptamers in their adaptability to recognise a wide variety of different analytes, as well as their potential advantages over antibody-

based devices, including higher stability, larger dynamic range, prolonged shelf-life, and lower cross-reactivity. Examples of aptasensors that utilise SPCE transducers include those for Ochratoxin A (OTA), Aflatoxin-B1 (AFB1) in alcoholic beverages [14], and Aflatoxin-M1 in milk [15]. The AFB1 and AFM1 aptasensors both used an EIS measurement step, performed in the absence (AFB1) or presence (AFM1) of ferri/ferrocyanide, to detect a change in electron transfer resistance/impedance when the analyte bound to the covalently-immobilised aptamer. L.O.Ds for these aptasensors were below those set by the EU for beer, wine, milk and dairy products for adults and infants. More recently updated reviews on aptasensors for mycotoxins include those by Goud *et al*. (2018 and 2020) [16,17], and within Li *et al*. (2019) [18]. Several SPCE-based aptasensors for OTA are reviewed, including an earlier flow system for OTA in beer, based on competitive analyte-ALP or aptamer-ALP capture onto magnetic beads over the SPCE surface, followed by introduction of a naphthyl phosphate substrate [19]. Enzymatic conversion of naphthyl phosphate to naphthol and its detection by amperometry gave a detection limit of 5.5 mg L^{-1}. Somewhat more recent examples of aptasensors using SPCEs include those for the detection of OTA in cocoa samples using impedimetric measurement [20] or DPV [21], giving detection limits of 0.15 ng mL^{-1} and 0.07 ng mL^{-1}, respectively. Rivas *et al.* [22] reported an SPCE-based label-free impedimetric aptasensor for OTA (in white wine) using iridium oxide nanoparticles to enhance the signal; the detection limit was very low at 14 nM. A recent discussion of these and related mycotoxin detection strategies including electrochemical SPCE-based biosensors can be found in Mishra *et al.* [23].

Algal toxins have been the subject of researchers developing electrochemical biosensors based on SPCEs as transducers. Catanante *et al.* [24] reported on a sensitive competitive biosensor for microcystin detection that employed recombinant protein phosphatase 1 (PPα) as the inhibitable recognition element and the generator of a dephosphorylated product (naphthol or paracetamol) that could be electrocatalytically oxidised at the surface of a PVA/CoPC-modified SPCE. A L.O.D. of 0.93 μg.L^{-1} was obtained. There have also been reports of electrochemical aptasensors for algal toxins based on SPCEs, including one for microcystin-LR which used a graphene-modified SPCE [25]. This achieved a L.O.D. of 1.9 pM in buffer, with no cross-reactivity to okadaic acid or microcystins-LA or -YR.

2.2. Antibiotics

The wide usage of antibiotics in farming for control of bacterial diseases, and as feed additives for growth promotion, means that there may be a risk of residues being present in animal-derived foodstuffs. One approach to developing a biosensor for the detection of antibiotic residues has been proposed by El-Moghazy *et al.* [26], for the detection of chloramphenicol (CAP). The device structure comprised a SPCE laminated with a layer of poly (vinyl alcohol-co-ethylene) nanofibrous membrane onto which an anti-chloramphenicol antibody was immobilised. Following incubation with samples containing chloramphenicol, the device was operated amperometrically at an E_{app} of -0.66V vs Ag/AgCl which reduced the nitro group. The current response was linear over the concentration range 0.01-10 ng mL^{-1}, with a limit of detection of 4.7 pg mL^{-1}. This immunosensor was tested for its ability to determine the presence of CAP in foodstuff using milk as an example; it was capable of quantifying the CAP in milk, with 92-95% recovery and RSDs ranging from 3.4 to 6.7%.

Another recent example of an electrochemical immunosensor being applied to foodstuffs, is an amperometric device which measured the growth-promoting drug Clenbuterol (CLB), again in milk [27]. The surface of an SPCE was modified with covalently linked PEDOT and GO. A competitive assay was then performed by mixing the sample with an HRP-CLB conjugate. Operation of the device was by amperometry at an E_{app} of – 0.1 V vs Ag/AgCl, which reduced the oxidised substrate, TMB. A linear range of 5 to 150 ng mL^{-1} was obtained, with a limit of detection of 0.196 ng mL^{-1}. The device was evaluated for its ability to determine CLB in milk spiked with 50 ng mL^{-1} CLB and gave values of between 44.6 and 53.8 ng mL^{-1}, with recovery between 89 and 108%. According to the FAO/WHO, the maximum residual limit for CLB is 0.05 μg/L [28]. Further work is required to improve the detection limit of biosensors to get down to the maximum residual limit, which could be an attractive avenue for researchers.

An interesting approach applied to the development of electrochemical biosensors for antibiotics in milk has been the use of magnetic beads (MBs) in conjunction with SPCEs to improve the sensitivity of the devices [29,30]. In this immunoassay approach, antibodies immobilised onto MBs capture the analyte, either in competition with HRP-conjugated analyte, or followed by a second HRP-conjugated

antibody. A magnet draws the beads close to the electrode surface where the applied voltage generates an amperometric response in the presence of H_2O_2 and the redox mediator, hydroquinone. This approach has more recently been applied to detection of β-lactoglobulin, an important allergen in dairy products, with a L.O.D. of 0.8 ng mL^{-1} [31].

2.3. Microorganisms

An interesting proposed immunosensor application for the wine industry is reported by Borisova *et al.* [32]. These authors have developed a disposable amperometric device for detection of the yeast *Saccharomyces cerevisiae*. SPCEs were modified with propionic functionalised graphene oxide, onto which a specific polyclonal antibody was immobilised. The yeast cells were quantified using a sandwich-type approach where, following their capture by the antibody, a Concanavalin A-HRP conjugate was added; Concanavalin A can recognise and bind to mannoproteins on the surface of the yeast cells, resulting in directly proportionate capture of HRP enzyme molecules. In the presence of added peroxide and hydroquinone (HQ), HRP catalyses the oxidation of the HQ, resulting in formation of p-benzoquinone which will yield an amperometric reduction current response at an E_{app} of -200 mV vs AgCl. Yeast cells were detectable in buffer over the range 10-10^7 CFU/mL, with a theoretical limit of detection of 6 CFU/mL. The devices gave an RSD of 16.3%, selectively recognised *S.cerevisiae*, and achieved a recovery of 95.5% for detection of 8.4 x 10^4 CFU/ml in spiked wine samples.

The poultry industry faces the risk of bird infection by *Salmonella* species *S. pullorum* and *S. gallinarum* which, although not a threat to human health *per se*, are the causes of chicken mortality and an economic threat to food producers. The disease-causing bacteria may end up in eggs and chicken meat. Fei *et al.* [33] reported a sandwich electrochemical immunosensor based on an SPCE coated with electrodeposited gold nanoparticles, polyclonal rabbit antibody, and ionic liquid. Following addition of the analyte solution containing the bacteria, the sandwich assay was completed by the addition of an HRP-conjugated antibody, followed by a mixture of thionine and peroxide. Measurement was by cyclic voltammetry and the magnitude of the current due to the reduction of thionine. The device gave a working range from 10^1 to 10^{10} CFU mL^{-1}, with a limit of detection of

3.0×10^3 CFU mL^{-1}; reproducibility was satisfactory, with a CV of 9%. The specificity of the device was good, as no significant responses were obtained for bacteria from other genera, and their presence did not interfere with the magnitude of the specific response. When the device was tested on real food samples (eggs, chicken meat) from market, good agreement was achieved with a standard culture method for determining bacterial numbers.

The Gram-positive bacterium *Streptococcus agalactiae* is a significant economic problem for the agricultural industry and in particular aquaculture, because it is the major cause of morbidity and mortality in Tilapia fish. In order to detect the presence of these pathogenic bacteria in environmental samples of interest to the fish industry, an amperometric biosensor has been developed based on an SPCE surface-modified with streptavidin [34]. Samples containing bacteria were incubated with a biotinylated polyclonal antibody, and the resulting cell-antibody conjugate was applied to the sensor surface. The biotin-avidin interaction trapped the bacteria on the sensor surface, then a streptavidin-HRP conjugate was added which bound to free biotin on (other) antibody molecules bound to the immobilised bacteria. Bound HRP activity, which was in direct proportion to numbers of immobilised bacteria, was then determined by the addition of the enzyme substrate TMB, plus peroxide. The amperometric signal was generated at an E_{app} of -200 mV vs a Ag pseudo-reference electrode, due to reduction of oxidised TMB. The device had a working range of $10^1 – 10^7$ CFU mL^{-1}, and a limit of detection of 10^1 CFU mL^{-1}. The device performed successfully when assessed using lake water samples spiked with *S. agalactiae* bacteria and was also applied to determination of contamination in pond and sludge samples. Some reduction in current magnitude in lake water compared to buffered standards was observed, suggesting some influence of the matrix, but the authors conclude that with some further development, their device shows future promise for application in the fish industry.

Screen-printed graphene electrodes have been modified by the addition of bacteriophages as the recognition element for detection of *Staphylococcus Arletta*, a potential human pathogenic bacterium found in poultry and goats. EIS was used as the electrochemical measurement technique, and the specific binding of bacteria was monitored in spiked samples of river water and apple juice, with a

detection limit of 2 cfu/mL [35]. A further discussion of electrode modification using carbon nanomaterials for foodborne bacterial detection, that includes SPCE examples, can be found in a recent review by Muniandy *et al.* (2018) [36].

Viruses pose a threat to human health as a contaminant of food and can lead to widespread infection. One virus for which an electrochemical biosensor utilising SPCEs has been developed is norovirus [37], utilising an aptasensor immobilised on a gold-nanoparticle-modified SPCE, and a square-wave voltammetry measurement step. The L.O.D. was 180 virus particles.

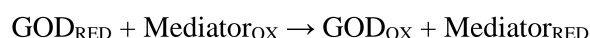
3.0. Naturally Occurring Compounds

3.1. Glucose

In this section, the fabrication methods are discussed in relation to the method of enzyme immobilization. This is summarised in Table 1.

The series of reactions leading to the generation of an amperometric response in the presence of glucose oxidase and a mediator, can generally be described by the following reactions.

$$\text{Glucose} + \text{GOD}_{OX} \rightarrow \text{Gluconolactone} + \text{GOD}_{RED}$$



$$\text{GOD}_{RED} + \text{Mediator}_{OX} \rightarrow \text{GOD}_{OX} + \text{Mediator}_{RED}$$

$$\text{Mediator}_{RED} \rightarrow \text{Mediator}_{OX} + ne^{-}$$

The method in which the enzyme, such as glucose oxidase, is bound to the surface of the electrode is an important consideration for several reasons. The process of enzyme binding should not compromise the structure of the enzyme active sites, which allows the enzyme to retain its activity and dictates its analytical performance [38]. Adsorptive enzyme carriers such as chitin, chitosan, silica, polyurethane and poly(oxyethylene glycol) have frequently been used in conjunction with SPCEs. The various methods of binding the enzyme to the surface of an electrode are demonstrated in Figure 1. Methods of enzyme immobilization are described in detail in Woodward (1985) [39].

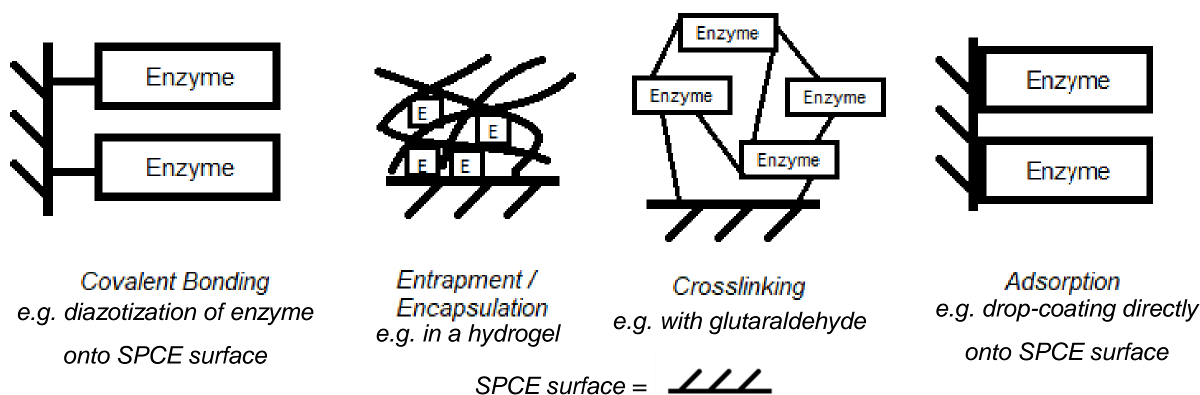


Figure 1. Methods for immobilizing enzymes to the surface of an electrode.

Glucose oxidase based sensors were the first commercially available biosensor and are the most commercially successful. Recent progress in the field of glucose based biosensors includes the use of nanomaterials to significantly increase the surface area of the biosensor, thereby increasing thereby increasing the number of biomolecules that can be immobilized to the transducer surface [40], thus improving sensitivity and detection limits.

Recent advances in glucose biosensors often focus on the non-enzymatic detection of glucose. However, these sensors frequently employ noble metals such as gold, platinum or metal composites resulting in a higher cost. In contrast, enzymatic biosensors often employ cheap materials such as carbon as their electrode material. Enzymes such as glucose oxidase are inexpensive and available to purchase in large volumes. As a result, the enzymatic based detection of glucose offers distinct advantages in terms of cost, which means the sensors can be considered disposable eliminating cross-contamination [41].

Agriculture and food applications frequently require fast, onsite detection of the analytes of interest. As a result biosensors offer significant advantages over traditional methods such as HPLC, capillary electrophoresis and mass spectrometry due to simplicity, ease of use and portability [42].

The determination of glucose is of great importance to the food industry and has implications in food safety. For example, at cooking temperatures, high concentrations of glucose can undergo the Maillard reaction which can lead to the formation of cancer-causing compounds such as acrylamide [43]. Acrylamide causes cancer in rats when administered in high doses, and as a result the monitoring of glucose concentrations is of particular interest to the food industry [44]. Food browning is an indication of food freshness or lack thereof, thus glucose biosensors can be utilised for this purpose. Additionally, glucose biosensors can be applied to the monitoring of fermentation processes in dairy, wine and beer [45].

Piermarini *et al.* [46] have previously reported a glucose biosensor for the monitoring of micro-alcoholic fermentations in red wine. A combination of glutaraldehyde and Nafion was successfully employed to immobilize the enzyme onto the surface of the electrode. A recovery study in diluted red wine has shown excellent recovery value with a coefficient of variation of <5%, however, the sensitivity of the biosensor is not given.

A device for measuring glucose in honey and blood using a simple fabrication technique was reported [47]. A mixture of horseradish peroxidase (HRP) and GOD was mixed, then drop coated onto the surface of a screen-printed ferrocyanide/carbon electrode.

Tian *et al.* [48] have developed a glucose biosensor by immobilizing glucose oxidase in a scaffold of 2-dimensional graphitic carbon nitride (g-C3N4) nanosheets. The nanosheets are synthesised from thiourea and urea via a hydrothermal method, which gives the nanosheets excellent biocompatibility. The sensor functions at a high operating potential of 1200mV, however interferences such as ascorbic acid and uric acid do not demonstrate a significant response, thereby demonstrating the biosensor's selectivity. The sensitivity improves upon previously discussed biosensors [46,47].

Entrapment is defined as the integration of an enzyme with a polymer matrix, whilst retaining the structure of the enzyme. The polymer matrix may also act as a barrier to interfering species which are likely to be present in food samples.

Gao *et al.* [49] constructed a glucose biosensor by electrodepositing alternating layers of GOx-SWCNTs and PVI-Os on the surface of an electrode, until a multi-layer structure was formed. An interference study demonstrated large currents in response to both uric acid and ascorbic acid. However, the biosensor possessed the highest sensitivity (32 $\mu A \cdot mM^{-1} \cdot cm^{-2}$) in comparison to other biosensors constructed by the adsorption of the enzymes onto the surface of SPCEs.

Subsequently, the addition of a Nafion membrane resulted in a change to the performance of the biosensor, resulting in a decrease in the sensitivity (from 32 $\mu AmM^{-1}cm^{-2}$ to 16.4 $\mu AmM^{-1}cm^{-2}$) and an increase in the linear range (from 500–800 µM to 200–6000 µM). This change in behaviour was likely due to the alteration of the conformational structure of the enzyme [50].

Chai *et al.* [51] have described the use of pure-graphene as an encapsulant for glucose oxidase. The graphene was washed with 1-pyrenebutyric acid N-hydroxysuccinimide ester (PSE), centrifuged, dispersed in ethanol and drop coated on the surface of a SPCE. The sensor exhibited an excellent sensitivity of 32.15 $\mu A \cdot mM$ which could be attributed to the increased surface area as a result of the graphene, and the direct electron transfer between the enzyme and the conductive mediator, PSE.

The biocompatibility of the encapsulation material is an important consideration in the construction of a biosensor. Barathi *et al.* [52] have demonstrated this phenomenon based on a mixture of chitosan, mesoporous carbon and glucose oxidase. The biosensor demonstrates a low applied potential of -450 mV vs. Ag/AgCl and a lower limit of detection of 4.1 μM. In comparison to an earlier paper by Gao *et al.* [49], the biosensor (Figure 2) demonstrates no significant changes in response in the presence of interfering biomolecules. The authors have successfully applied the biosensor to the analysis of saliva, human serum and urine samples, with a high recovery value (> 98%). Additionally, a recovery study was carried out by HPLC analysis, and was frequently found to be within 1% of the response of the biosensor, demonstrating the biosensors suitability for real sample analysis.

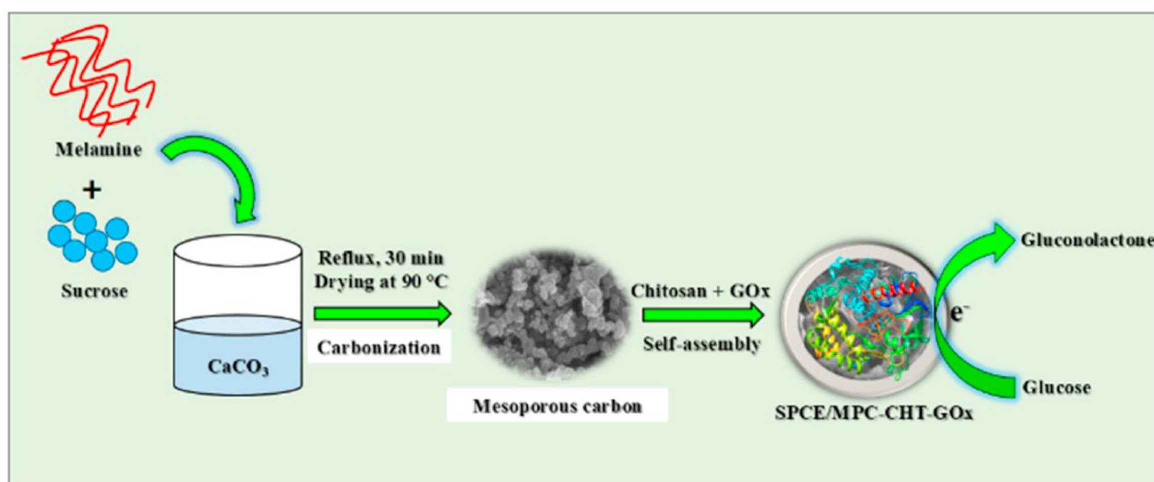


Figure 2. Figure describing the process of immobilization of the enzymatic components and the generation of the amperometric response. SPCE/MPC-CHT-GOx: screen-printed carbon electrode with mesoporous carbon, chitosan and glucose oxidase. Reproduced with permission from Ref. [52].

Entrapment of enzymes can be achieved by utilising conducting polymers such as polyaniline, which can also act as an electron mediator in enzymatic reactions, thereby leading to improvements in biosensor sensitivity. Zheng *et al.* [53] have demonstrated the feasibility of this approach by incorporating a mixture of platinum nanoparticles into a polyaniline-montmorillonite hybrid mixture. The biosensor improves upon the lower linear range (10 μM) in comparison to previously discussed articles [45–47]. However, given the high cost of platinum, the need to construct glucose biosensors with cheap, disposable and environmentally friendly materials is of great importance.

Pemberton *et al.* [54] have successfully demonstrated the integration of glucose oxidase into a water-based ink which was subsequently screen-printed. The electrode was employed for the determination of glucose in serum, the results of which compared favourably with a standard spectrophotometric assay. Subsequently published articles by Pemberton *et al.* successfully applied a microband biosensor based on an enzyme-containing water-based ink to the monitoring

of glucose metabolism of human hepatocyte carcinoma cells (HepG2) [55] and real time monitoring of cellular toxicity [56,57].

More complex fabrication techniques, despite their lack of feasibility for mass production, can result in improved analytical properties. For example, Chiu *et al*. [58] have immobilised glucose oxidase onto the surface of a SPCE by the sequential electrodeposition of poly(3,4-ethylenedioxythiophene), Prussian Blue and multi-walled carbon nanotubes, thereby entrapping the enzyme to the surface of the electrode. The presence of the entrapment mixture improved the analytical properties of the biosensor resulting in an extensive linear range of 1 to 10 mM.

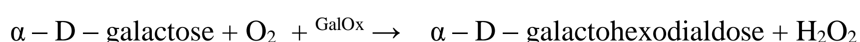
3.2. Galactose

The performance characteristics for the galactose sensors discussed in this section are summarized in Table 2.

The detection of galactose with a biosensor has great relevance for the determination galactosemia. Galactosemia is an autosomal recessive disorder which can cause an individual to experience side effects such as lethargy, vomiting and diarrhoea [59]. As such the detection of galactose in food could have implications for galactosemic individuals.

Kanyong *et al*. [60] have described a simple fabrication process for a galactose biosensor which consists of drop-coating 1% cellulose acetate (CA) followed by an aliquot of galactose oxidase onto the surface of the CA-CoPC-SPCE and left to dry.

The biosensor mechanism and the electrochemical response process can be described by the following equation. The enzymatically generated hydrogen peroxide can be detected by oxidation or reduction at the surface of an electrode.



$$\alpha - D - \text{galactose} + O_2 \xrightarrow{\text{GalOx}} \alpha - D - \text{galactohexodialdose} + H_2O_2$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

The biosensor was applied to the determination of galactose in fortified and unfortified bovine serum. A mean recovery value of 99.9% ($n = 6$) was attained, with a low coefficient of variation of 1.10%, implying its feasibility for potential application in food analysis.

In a subsequent report [61], a microband galactose biosensor demonstrated greater sensitivity (7.27 $\mu A \cdot mM^{-1} \cdot cm^{-2}$) to galactose in comparison to a conventionally sized biosensor (7.00 $\mu A \cdot mM^{-1} \cdot cm^{-2}$).

3.3. Glutamate

For the following reports on glutamate determination, the performance characteristics are summarized in Table 3. High levels of glutamate in certain foods cause Chinese

RestaurantSyndrome in susceptible individuals [62]. Therefore glutamate measurement is important in food safety. Several glutamate biosensors have been reported in the literature with demonstrated application to the determination of glutamate in food samples.

The mechanism of the enzymatic reaction occurring at the surface of the electrode can be described as follows. The formation of the NADH leads to the generation of the amperometric response.

$$\text{Glutamate} + NAD^+ + H_2O \leftrightarrow \text{2-oxoglutarate} + NADH + NH_4^+ + H^+$$

Firstly, Hughes *et al.* [62] have described the fabrication of an amperometric screen-printed glutamate biosensor based on the enzyme glutamate dehydrogenase (GLDH). The GLDH was immobilised to the surface of a Meldola's blue screen-printed biosensor (MB-SPCE) by chitosan. The biosensor was successfully applied to the determination of glutamate in a food sample. An unfiltered solution containing a beef OXO cube was analysed for monosodium glutamate (MSG) content. The endogenous content of MSG was 125.43 mg/g with a CV of 8.98%. The OXO cube solution was fortified with 0.935 g (100 mM) of glutamate, the resulting mean recovery was 91% with a CV of 6.39%.

Subsequently reports demonstrate the feasibility of integrating the co-enzyme and enzyme components onto the surface of the electrode by a layer-by-layer deposition approach. A combination of chitosan, multi-walled carbon nanotubes and Meldola's blue successfully encapsulate all the components [63]. The resulting biosensor response compared favourably to the previously discussed glutamate biosensor [62], whereby NAD^+ was present in free solution.

The detection of nanomolar concentrations of glutamate by utilizing screen-printed electrodes modified with carbon nanotubes has been described by Khan *et al.* [64]. The biosensors were prepared by drop-coating glutamate oxidase onto the surface of a carbon nanotube modified SPCE and left to dry overnight. The biosensor possesses a detection limit of 10 nM, which is the lowest detection limit reported for a glutamate biosensor to date.

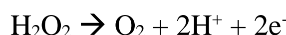
3.4. Lactate

For the following reports on biosensors for lactate determination, performance characteristics are summarized in Table 4.

The ability to detect lactate using a biosensor is of great importance to the food industry and personal safety. The detection of lactate can be also used to determine freshness. Microbial fermentation of milk can result in the increase in the concentration of lactate in milk samples. For example, typical concentrations of lactate in fresh milk are 1–2 mmol/L and following fermentation, this can increase to 10–20 mmol/L [65].

The amperometric biosensor response that occurs at the surface of the electrode can be described as follows.

L-lactate + O_2 + L-lactate oxidase → Pyruvate + H_2O_2



H_2O_2 → O_2 + $2H^+$ + $2e^-$

The development of an amperometric biosensor for the determination of lactic acid in probiotic yoghurts has been described by Radoi *et al*. [66]. This was fabricated by drop-coating a solution of lactate dehydrogenase mixed with neutralized Nafion, onto the surface of a variamine blue modified screen-printed electrode (VB-SPE). The performance of the biosensor compared favourably with a commercially available kit for the determination of lactic acid in foodstuffs.

Pereira *et al.* [67] have described the fabrication of a lactate biosensor by immobilizing lactate dehydrogenase and NAD^+ utilizing a mixture of multi-walled carbon nanotubes, glutaraldehyde and bovine serum albumin. The biosensor was successfully applied to the determination of lactate in blood diluted with PBS. Given that blood is a very complex media, it should be feasible to apply this sensor to food samples. Whilst this approach is based on a carbon-paste electrode, it has potential for modification into SPCEs. Similarly, Alizadeh *et al.* [68] have had success in applying a device employing multi-walled carbon nanotubes for the analysis of lactic acid in milk and yoghurt.

The detection of lactate in wines and ciders is also of great interest to the food industry as with other foodstuff is it is associated with presence of lactate-producing bacteria which can have an impact on the quality and taste of the beverage. As such, Loaiza *et al*. [69] have described the development of a lactate biosensor that utilises platinum nanoparticles deposited on the surface of graphitized nanofibers in combination with lactate oxidase in order to detect lactate. The mixture is then deposited on the surface of a screen printed electrode. The process covalently immoblizies the enzyme onto the surface and improves the electron transfer from enzyme to electrode. The biosensor demonstrated excellent stability (90% signal after 3 months at room temperature) and excellent selectivity towards lactate in complex samples such as ciders and other beverages.

3.5. Fructose

The performance characteristics for the following reports on fructose determination are summarized in Table 5. The detection of fructose is of considerable interest to many food companies. As such several biosensors have been recently reported which have been applied to the detection of fructose in foods such as wine and fruit juices. Additionally, fructose is often employed as a dietary sweetener in diabetic foods, thus it’s detection could be beneficial as a precaution for diabetics [70].

Antiochia *et al.* have described [71] a biosensor employed for the determination of fructose in honey, red wine and several other samples. The biosensor is constructed by wiring fructose dehydrogenase into an osmonium polymer hydrogel through a simple mixing process. The mixture was subsequently drop-coated onto the surface of the SPCE. The fabrication process is very simple and has demonstrated excellent specificity in the presence of interferants such as ascorbic acid and other sugars.

The development of a commercial, low-cost graphite-nanoparticle biosensor for fructose has been described by Nicholas *et al.* [72]. The biosensor was fabricated by depositing an enzyme containing solution on the SPCE surface, followed by ferricyanide and the fructose sample. Chronoamperometry was then employed to determine the response of the biosensor to fructose. The biosensor demonstrated a high sensitivity (58.56 µA mM-1 cm-2). The sensor was applied to the determination of fructose in commercial fruit juices following dilution, demonstrating a mean recovery of 97.12%,

Trivedi *et al.* [73] have reported a fructose biosensor which immobilizes fructose dehydrogenase to the surface of a screen-printed graphite electrode with a polymer matrix of polyethylenemine and poly(carabmoylsulphonate). Whilst the sensitivity is lower (0.62 ± 0.10 nA/µM) than previously discussed articles, the biosensor demonstrates excellent correlation with an enzymatic test kit for the analysis of several fructose containing liquids.

Table 1. Reports of screen-printed carbon electrodes incorporating glucose oxidase for glucose determination.

Immobilization Technique	Mediator	Assay Time (s)	Lower Linear Range (μM)	Upper Linear Range (μM)	Sensitivity	Applied Potential (mV)	Storage Stability (weeks)	Reference
Crosslinking with glutaraldehyde & Nafion	Prussian Blue	N/A	20	700	N/A	200	90% activity after 6 months	[46]
Glutaraldehyde & BSA	Os-polyvinyl pyridine wired HRP	60	0	700	28.24 nA/μM/cm	0	90% activity after 15 months	[47]
Carbon nanosheets mixed with Nafion	g-C3N4 nanosheets	2s	300	2000	21.7 μA/mM$^{-1\ cm-2}$	1200	90% after one month	[48]
Use of SWCNT	PVI	5	500	800	32 μA/mM/cm	300	90% activity after 1 month	[49]
Use of SWCNT	Osmium bipyridine-com PVI	5	200	6000	16.4 μA/mM/cm	300	90% activity after 1 month	[50]
Graphene cleaned with PSE	PSE	5	100	1000	32. μA/mM^{-1}	-400	2 weeks	[51]
Chitosan mixed with mesoporous carbon	Mediator Free	10s	250	3000	56.12 μA mM−1 cm−2	-450	N/A	[52]
PANI-montmorillonite hybrid mixture	PANI	20s	10	1940	35.56 μA mM−1 cm−2	750	91.7% after 2 months at 4°	[53]
Enzyme contained within water-based ink	CoPC	20	270	2000	16.4 nA/mM	400	N/A	[54]
Enzyme contained within water-based ink	CoPC	400s	*Buffer:* 450 *Culture Medium:* 2000	9000 13000	*Buffer:* 26 nA/mM *Culture Medium:* 13 nA/mM	400	N/A	[55]
Enzyme contained within water-based ink	CoPC	30	0	2000	7 nA/mM	400	N/A	[57]
Enzyme entrapped by electro-polymerization of PEDOT, MWCNT	Prussian Blue	N/A	1000	10000	2.67 μA/cm/mM	−100	82% activity after 1 month	[58]

SWCNT: Single walled carbon nanotube. **PEDOT**: Poly(3,4-ethylenedioxythiophene. **HRP**: Horseradish peroxidase. **PVI:** Poly(1-vinylimidazole). **CoPC**: Cobalt phthalocyanine.

Table 2. Reports of screen-printed carbon electrodes for galactose determination.

Immobilization Technique	Mediator	Assay Time (s)	Lower Linear Range (µM)	Upper Linear Range (µM)	Sensitivity	Applied Potential (mV)	Storage Stability (weeks)	Reference
Combination of cellulose acetate and polycarbonate	cobalt phthalocyanine	N/A	15	250	2.10 µA/mM	500	7 days, no loss of functionality	[60]
Cellulose acetate	cobalt phthalocyanine	2s	100	25000	7.00 µA mM^1 cm^2	500	14 days, no loss of functionality	[61]

Table 3. Reports of screen-printed carbon electrodes for glutamate determination.

Immobilization Technique	Mediator	Assay Time (s)	Lower Linear Range (µM)	Upper Linear Range (µM)	Sensitivity	Applied Potential (mV)	Storage Stability (weeks)	Reference
Chitosan	Meldola's Blue	2s	12.5	150	0.44 nA / µM	100	N/A	[62]
Chitosan mixed with Carbon Nanotubes	Meldola's Blue	20 – 30	7.5	105	0.39 nA / µM	100	2 weeks	[63]
Carbon nanotubes	N/A	N/A	0.01	10	0.72 ± 0.05 µA µM−1	20	24 days	[64]

Table 4. Reports of screen-printed carbon electrodes for lactate / lactic acid determination.

Immobilization Technique	Mediator	Assay Time (s)	Lower Linear Range (μM)	Upper Linear Range (μM)	Sensitivity	Applied Potential (mV)	Storage Stability (weeks)	Reference
Nafion	Variamine Blue	N/A	2000	10000	N/A	200	3 weeks	[66]
Glutaraldehyde	Meldola's Blue	5s	100	10000	3.46 A cm^{-2} mmol	0	N/A	[67]
MWCNTs (2%), dibutylphthalate (DBP) (65%), poly-vinyl chloride (PVC) (28.5%) and tetra phenyl phosphonium bromide (TPPB)	N/A	60s	10	1000000	N/A	N/A	2 months	[68]
Covalent bonding of the enzyme to graphite covered in platinum nanoparticles	PtNPs/GCNF	N/A	10	2000	41.3 mA / M cm^{-3}	300	90% after 12 weeks at RT	[69]

Table 5. Reports of screen-printed carbon electrodes for fructose determination.

Immobilization Technique	Mediator	Assay Time (s)	Lower Linear Range (μM)	Upper Linear Range (μM)	Sensitivity	Applied Potential (mV)	Storage Stability (weeks)	Reference
Dropcoating	Os-polymer		100	8000	2.1 (μAcm^{-2} mM)	150	10% decrease after 4 weeks	[71]
Dropcoating	Ferricyanide	N/A	100	1000	58.56 μA mM-1 cm-2;	800	N/A	[72]
Polyethylenemine (PEI) and poly(carbamoylsulphonate) (PCS)	Ferricyanide	N/A	300	3000	0.62 ± 0.10 nA/μM	400	N/A	[73]

3.6. Vitamins

Vitamins are important in food safety because they are essential in our diet, and a deficiency can cause a range of diseases. Dietary supplementation can be achieved through food fortification or pharmaceutical supplements. It is important to ensure supplements are providing sufficient amounts of vitamins and in the case of some vitamins it is important not to overdose. It is also essential to know the effect that processing and preparation of food has on vitamin levels. Most vitamins are electroactive and thus this property has been exploited with various electrochemical techniques using a variety of SPCEs [1]. The use of screen-printed carbon biosensors for analysis of vitamins has received less attention; however, the few examples reported show promise for food analysis and are given in Table 6.

Ho *et al*. [74] developed an immunosensor for biotin based on a SPCE which provided a very selective approach. The working electrode is constructed over four phases. In the first phase, a screen-printed carbon base layer is modified by the electrodeposition of a nano-structured gold network. Secondly, poly allylamine hydrochloride (PAH) is drop-coated onto the surface, which creates a 3D network for the addition of an anti-biotin antibody to be bound to. Following this the anti-biotin antibody/PAH/nano-Au/SPCE is immersed in a solution containing both biotin and biotin-tagged ferricyanide encapsulated liposomes with a short incubation period. In the final step, the addition of gold-nanoparticles was shown to significantly enhance electron transfer which resulted in increased sensitivity. The incorporation of a biological recognition element provides specificity for the voltammetric assay. The group reported further developments for a biotin immunosensor [75]; the two complex biosensors use different binding chemistries to enhance the orientation of antibodies on the SPCE surface. The most sensitive biosensor exploits the affinity of a sugar moiety on the anti-biotin antibody for the boronic acid-modified graphite surface.

Another antibody approach was developed by Martin-Yerga *et al.* [76]. Figure 3 shows construction of the biosensor and its competitive assay for biotin. The electrochemical method used for the detection of biotin was stripping voltammetry which resulted in a very low limit of detection (1 nM). This has been successfully used for measurements in dietary supplements. The other performance characteristics are shown in Table 6.

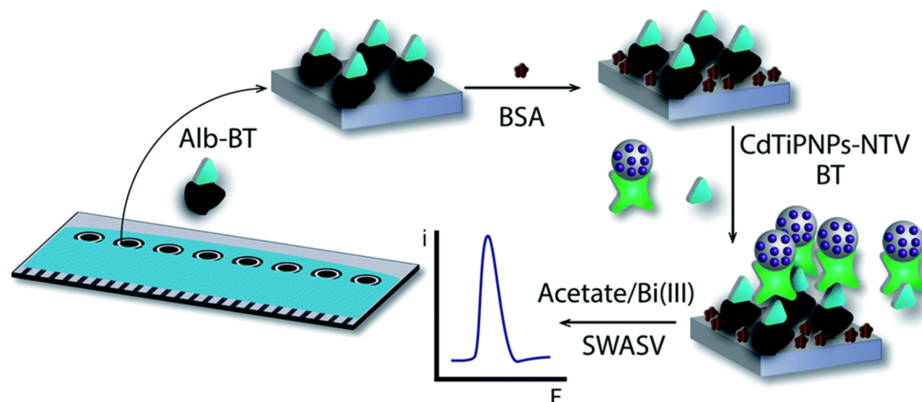


Figure 3. Schematic drawing of the competitive biosensor for the detection of biotin using CdTiPNPs as labels. Biotinylated albumin (Alb-BT) was used as the sensing element, bovine serum albumin (BSA) as the blocking agent, cadmium-modified titanium phosphate nanoparticles conjugated with neutravidin (CdTiPNPs-NTV) as labels, and biotin (BT) as the analyte. Square-wave anodic stripping voltammetry (SWASV) was employed as the detection technique. Reproduced with permission from Ref. [76].

As well as biotin, SPCE biosensors have been applied for the measurement of vitamin C in fruit juices and food supplements. Csiffáry *et al.* [77] immobilised ascorbate oxidase enzyme onto a SPCE using crosslinking agent poly(ethylene glycol) (400) diglycidyl ether. The principle of the measurement involves measuring vitamin C by direct oxidation at the electrode surface. Vitamin C is enzymatically oxidised at the surface of the biosensor, which results in a decrease of anodic current. This current is compared to the current obtained at a dummy biosensor (fabricated with an inert protein in place of the enzyme), and the current difference is proportional to the concentration of vitamin C in the sample.

Table 6. Reports of screen-printed biosensors for vitamin B_7 (Biotin) and vitamin C (L-ascorbic acid)

Vitamin	Electrode Components	Supporting Electrolyte	Measurement Technique	Detection Limit	Linear Range	Samples	Reference
B_7	W: PAH/nanoAu/SPCE R: Ag/AgCl C: Pt	0.1M PBS pH 7.2	SWV +0.6 V > -0.3 V Ep = +0.2 V	8.30 nM	0.01 nM – 0.01 M	None reported	[74]
B_7	W: Ab/APBA/SPGrE R: Ag C: Carbon	Phosphate buffer pH 7.2	Amperometry -0.2 V	0.16 nM	0.1 nM – 1.0 μM	None reported	[75]
B_7	W: MonoAb/nanoAu/SPGnE R: Ag C: Carbon	Phosphate buffer pH 7.2	Amperometry -0.2 V	14.00 nM	0.1 nM – 1.0 μM	None reported	[75]
B_7	W: Alb-BT/BSA/CdTiPNPs-NTV BT/SPCE R: Ag C: Carbon	0.1 M acetate buffer pH 5.0	SWASV -1.3 V deposition potential 300s deposition time 20 Hz frequency 30 mV amplitude 2 mV step potential	1 nM	2 – 40 nM	Two multivitamin tablets	[76]
C	W: AAO/PEGDGE/SPCE R: Ag C: Carbon	200 mM sodium acetate buffer pH 4.65 with 2 mM HPO_3 and 100 mM KCl	Amperometry +450 mV	3 μM	5 - 150 μM	Fruit juices and vitamin C effervescent tablets	[77]

PAH/nanoAu/SPCE: Poly allylamine hydrochloride nano-gold screen-printed carbon electrode. **Ab/APBA/SPGrE**: Anti-biotin antibody-aminophenylboronic acid-screen-printed graphite electrode. **MonoAb/nanoAu/SPGnE**: Monovalent half-antibody-gold nanoparticles-screenprinted graphite electrode. **Alb-BT/BSA/CdTiPNPs-NTV/SPCE**: Biotinylated albumin bovine serum albumin cadmium-modified titanium phosphate nanoparticles conjugated with neutravidin and biotin screen printed carbon electrode. **AAO/PEGDGE/SPCE**: Ascorbate oxidase enzyme poly(ethylene glycol) (400) diglycidyl ether screen printed carbon electrode. **W**: Working Electrode **R**: Reference Electrode **C**: Counter Electrode. **SWV**: square wave voltammetry. **SWASV**: square wave anodic stripping voltammetry.

4.0. Pesticides

The use of plain SPCEs in relation to the direct determination of organophosphates (OPs) has largely disappeared from publication [78], which has been dominated by the development of a variety of bio-recognition strategies. An exception to this has been described by Li *et al.* [79], who discussed the development of a photo-electrochemical assay using SPCEs with nano-sized titania surface modification with ultraviolet photocatalysis. By using differential pulse voltammetry, these non-selective sensors were able to detect 2 nM dichlofenthion in solvent vegetable extracts without the requirement for enzyme interactions. However, the direct electrochemical strategy remains uncommon and the majority of OP sensing devices under development remain enzyme or antibody-based, with the former predominant. Antibody-based strategies have been developed for the detection of specific organophosphates, for example parathion [80], with the use of impedimetric detection. They possess a singular advantage that they can detect the compound in the reduced and less toxic form as opposed to the electroactive -oxon form more associated with OP toxicity.

Enzyme-based biosensing for the detection of organophosphate and other pesticides has been the subject of considerable research since the early 1990s and has continued in the last 10 years (Table 7). The majority of enzyme-based biosensing strategies developed have focussed on two enzymes; organophosphate hydrolase (OPH) and acetylcholinesterase (AChE) [81]. However, butyrylcholinesterase (BChE) has also been utilised as a direct analog to AChE. The acetylcholinesterase-based system has been the most widely adopted, especially with respect to screen-printed electrodes. These biosensors have been repeatedly demonstrated as simple, rapid, and ultra-sensitive tools for pesticide analysis in food safety, although there is no commercial system currently available. When AChE or BChE is immobilized on the working electrode surface, its interaction with the substrate (for example, with acetylthiocholine) produces an electro-active species (thiocholine) and its corresponding carboxylic acid [82]:

$$\text{Acetylthiocholine} + H_2O + \text{AChE} \rightarrow \text{thiocholine (TCh)} + \text{acetic acid}$$

The subsequent anodic oxidation of the thiocholine at the working electrode gives rise to a current that constitutes a quantitative measurement of the enzymatic activity:

$$2\text{TCh (reduced)} \rightarrow \text{TCh (oxidised)} + 2H^{+} + 2e^{-}$$

The presence of pesticides in the sample inhibits enzymatic activity that leads to a drop in the current intensity, which is then measured. The sensitivity of these types of biosensors depends considerably on the chosen method of enzyme immobilization and incubation time of the biosensor in the presence of the OP [83]. Various strategies have been used to immobilise AChE onto the electrode surface, including adsorption [84], entrapment [85–87] and cross-linking [88,89] amongst others. In an

extensive study of immobilisation techniques Pohanka *et al.* [90] concluded that glutaraldehyde cross-linking was the preferred method and has proved to be a useful method for a range of electrode materials, including SPCEs. However, other strategies may be applicable depending on the composition of the sensor and surface chemistry. AChE-based biosensors have the potential to complement, rather than replace, standard classical analytical methods by simplifying or eliminating sample preparation and making field-testing easier and faster with a substantial decrease in cost per analysis [91].

In the past 10 years, there has been a high degree of diversity with respect to the composition and surface modification of screen-printed electrodes used in OP biosensors, as well as sensor design and morphology. Arduini *et al.* [92] took AChE biosensor design to new levels of practicality by screen printing onto paper such that the assay can be completed by folding the paper to introduce the sample to the biosensor surface (Figure 4). Carbon remains the most common electrode material (Table 7) and has been used in the detection of OPs in sub-ppb concentrations.

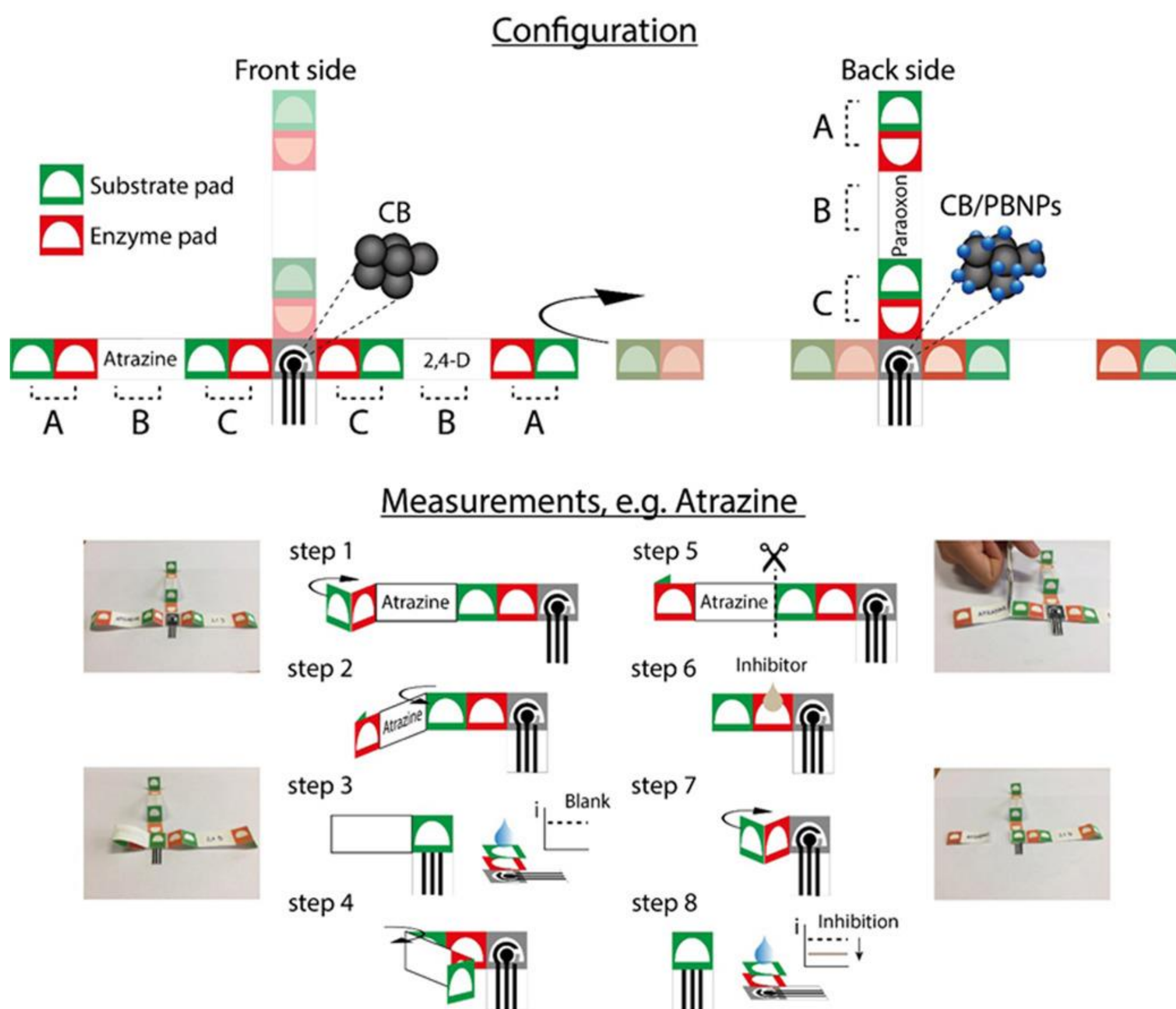


Figure 4. Schematic representation and photographs of the configuration of the paper-based platform and measurement procedure. Reproduced with permission from Ref. [92].

From the published research it is unclear that any of electrode materials have an inherent electrochemical advantage for use in OP biosensors and the selection of electrode material appears to rely on their practicality, cost, and the experience of the research group involved. A diverse range of electrode modifications have been made to the composition or the surface of SPCEs. These modifications have been to either entrap or cross-link the enzyme molecules to the sensor surface or commonly to improve the electrochemical properties of the working electrode. A variety of nano-particles have been tested for surface modification, including those made of titania [88], gold/platinum bimetallic [91], zinc oxide [93], manganese dioxide [94] and magnetic composite nanoparticles [95]. Multi-walled carbon nanotubes (MWCNT) [96] and single-walled carbon nanotubes (SWCNT) [97] have also been examined contributing to the detection of selected OPs at ppb levels.

Gan *et al*. [95] successfully detected dimethoate at ppt levels with the use of magnetic composite nanoparticles in buffer and vegetable extracts, however other strategies have consistently resulted in the detection of low ppb concentrations of OPs. An example of this can be observed in the development of electric eel AChE-based biosensors by Chen *et al.* [85] who incorporated both MWCNT and tin oxide onto the surface of SPCEs. The analysis of simple vegetable extracts using these sensors with cyclic voltammetry resulted in a detection of 50 µg/L chlorpyrifos.

One of the most common modifications for SPCEs in the past ten years has been the inclusion of the electron mediator cobalt phthalocyanine (CoPC) [89] within the carbon ink. The addition of CoPC allows the electron transfer from the reduction of the substrate to the electrode at lower potentials thereby removing potential interferences. Practical advantages to the inclusion of CoPC within the electrode ink have been shown in the use of CoPC-modified SPCEs to create array-based systems to allow some identification as well as quantification of OPs in a substrate.

Efficient electron-mediation combined with a SPCE designed for inexpensive manufacture can result in potentially commercially-viable reproducible and sensitive sensor arrays. This has been demonstrated in recent years by Alonso *et al.* [97], who used biosensors based on three separate AChE enzymes to differentiate chlorpyrifos and malaoxon in milk using chronoamperometry using an artificial neural network (ANN) to for signal interpretation.

Additionally, Crew *et al.* [89] refined previously developed AChE biosensor array systems for OP detection to develop a portable prototype instrument for the analysis of five organophosphates using a wildtype and five modified *Drosophila melanogaster* AChE enzymes in an array format (Figure 5). This prototype also used an ANN for signal interpretation and a simple three-minute inhibition step to allow rapid-analysis of food extracts or untreated environmental samples in the field. For the latter determination water samples were simply deposited into the wells of a 96 well plate and the biosensor array was automatically lowered into the well for the desired incubation time. The sequence was then continued automatically whereby the array was raised, and a wash step activated; the final measurement

step was performed in a separate row of wells containing the enzyme substrate acetylthiocholine; the product thiocholine was then quantified using chronoamperometry. The inclusion of ANN analysis with flexible SPCE array formats for OP analysis provides an optimistic future route for development for these biosensors for commercial applications.

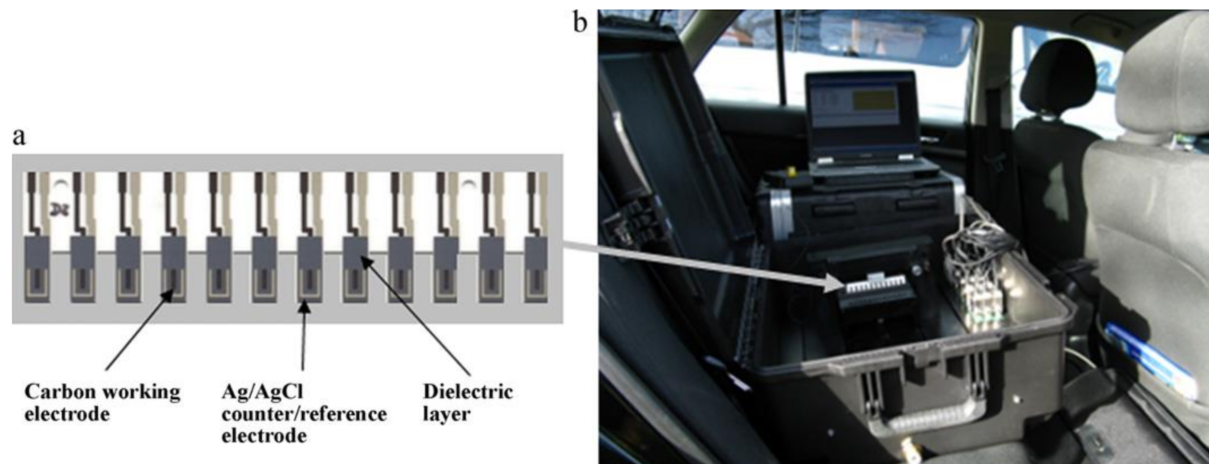


Figure 5. (a) Electrode array comprising 12 screen-printed carbon electrodes modified with CoPC and an Ag/AgCl counter/reference electrode printed on an alumina substrate; (b) array in the prototype biosensor system operating in the field powered from a car battery via the lighter socket. Reproduced with permission from Ref. [89].

Table 7. Reports of SPCEs for organophosphate determination

SPE modification	Immobilization method	Enzyme	Limit of detection	Real sample analysis	Analytical technique	Incubation time (mins)	Reference
CoPC	Entrapment	EE AChE	4 nM malaoxon	Olive oil	Chonoamp	10	[87]
Graphene	Cross-linking	Anti-parathion antibody	46 pg/L	n/a	Impedimetry	>15 minutes	[80]
Carbon black, CoPC	Entrapment	BChE	18nM paraoxon	Industrial waste water	Chronoamp	20	[98]
MnO_2	n/a	BChE	0.6nM diazinon	n/a	Chronoamp	15	[94]
PEDOT, PSS	Entrapment	EE AChE	4nM chlorpyrifos	n/a	Chronoamp	10	[86]
SWCNT, CoPC	Cross-linking	EE AChE	5ppb paraoxon, 2ppb malaoxon	Water	Chronoamp	15	[96]
Titania nanoparticles	n/a	n/a	2nM dichlofenthion	Vegetable extract	DPV/Photoelec	n/a	[88]
CoPC	Cross-linking	DmAChE	n/a	Lake water	Chronoamp	10	[89]
Ag/Pt bimetallic nanoparticles	Cross-linking	EE AChE/ChO	0.2µM paraoxon/carbofuran	n/a	Chronoamp	10	[95]
CoPC	Cross-linking	DmAChE	<1nM pirimiphos/chlorpyrifos/ malaoxon/omethoate/ dichlorvos	Food extracts, waste water, drinking water, river/lake water	Chronoamp	3	[97]
MWCNT, gold nanoparticles	Adsorption	AChE	30 ng/L	n/a	Chronoamp	n/a	[85]

According to EU regulations, maximum residual limits for all of the above is 0.01 mg/kg, except for parathion which is 0.05 mg/kg, malathion which is 2.0 mg/kg and chlorpyriphos which is 1.5mg/kg [99]. Table 7 shows the limit of detection (LOD) for a range of pesticide biosensors in a selection of real

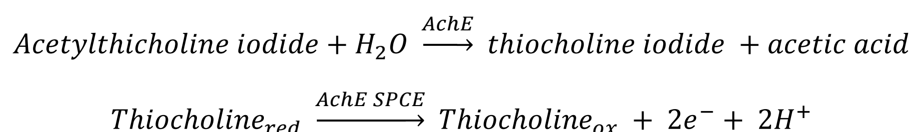
samples; for comparison with the MRLs, these has been converted into LOD mg/kg in the following explanation. Table 7 indicates that the pesticides: malaoxon in olive oil, (LOD 0.001 mg/kg) [87]; paraoxon and malaoxon in water (LOD 0.005 and 0.002 mg/kg) [96]; dichlofenthion in vegetable extract (LOD 0.0006 mg/kg) [88]; pirimiphos (LOD 0.0003 mg/kg), chlorpyrifos (LOD 0.0004 mg/kg, malaoxon (LOD 0.0003 mg/kg) omethoate (LOD 0.0002 mg/kg) and dichlorvos (LOD 0.0002 mg/kg) in food extracts and water [97], can all be detected at their MRLs using the described devices. It should also be mentioned that, where a real sample was not studied, the biosensor devices could have measured the pesticides in a real sample. The examples described above demonstrate the possibility that screen-printed carbon-based biosensors could have application for a range of pesticides in various matrices. This would seem to be a useful direction for commercial exploitation in the food safety area.

5.0. Metals

There is a pressing need for convenient, rapid, cost-effective analytic methods for the measurement of metals in the agri-food sector. This section will describe a selection of novel screen-printed carbon biosensors, demonstrating their advantages in challenging matrices. Table 8 summarizes the performance characteristics of screen-printed carbon biosensors for some important metal ion contaminants. Ingestion of metal ions can be toxic, whilst the literature does not always directly describe food safety as an application, these devices should still be applicable to metal ions in the agricultural environment and produce in the food chain.

Metal ions are known to inhibit enzyme activity [100-103] and this can be exploited using appropriate biosensors for their measurement. Alvarado-Gamez *et al.* [104] described a chronoamperometric method for the measurement of tungsten by employing screen printed carbon electrodes modified with gold nanoparticles. Alkaline phosphatase enzyme was immobilized onto the SPCE by depositing this species on top of a bovine serum albumin layer, followed by the cross linking agent glutaraldehyde. The substrate for the enzyme in this approach is 2-phospho-L-ascorbic acid, and this is enzymatically converted to L-ascorbic acid; the latter species undergoes oxidation at the modified screen-printed carbon electrode during the operation of the biosensor. Inhibition of the enzyme reaction occurs in the presence of tungsten, causing a decrease in the oxidation current; this decrease is proportional to the concentration of the metal species. The biosensor performance was validated using fortified bottled and tap water and was shown to be successful in monitoring down to 1µM.

The determination of arsenic at an acetylcholinesterase (AChE) modified SPCE has been reported [105]. The principle of the operation is shown below. The first equation shows the formation of thiocholine from acetylthiocholine. The second equation shows the electrochemical oxidation of thiocholine which produces the analytical response. In the presence of the metal ion inhibitor there is a reduction in the thiocholine generated at the electrode surface resulting in a decrease in the response; this decrease is proportional to the concentration of the metal ion.



$$Acetylthicholine\ iodide + H_2O \xrightarrow{AchE} thiocholine\ iodide\ + acetic\ acid$$

$$Thiocholine_{red} \xrightarrow{AchE\ SPCE} Thiocholine_{ox} + 2e^- + 2H^+$$

AChE was immobilised by covalent linkage onto the working electrode surface. To achieve this, N-cyclo-hexyl-N'-[2-(N-methylmorpholino) ethyl] carbodiimid 4 toluensulfonate solution was deposited. After activation at room temperature, buffer solution containing AChE was dropped onto the working electrode surface. During this activation step the reaction between carboxylic groups and carbodiimide gives rise to a more active substrate for its reaction with the amine groups of the enzyme. Then, the

electrode was kept at 30 °C for 2 h. Finally, the electrode surface was rinsed with buffer solution. The developed biosensor was shown to be able to successfully determine 1.0 μM As^{3+} concentrations in tap water. Further investigations were made on a certified As^{5+} water sample. It was shown possible to determine Arsenic concentrations in this sample following the addition of sodium thiosulphate to reduce the AChE inert As^{5+} to As^{3+}.

Guascito *et al.* [106] have utilised the widely used and commercially successful glucose oxidase enzyme system for the determination of a number of metal ions including: Hg^{2+}, Ag^{+}, Cu^{2+}, Cd^{2+}, Co^{2+} and Ni^{2+}. Detection limits in the low μg/mL levels were reported, with silver detection limits in the μg/L region as part of a flow injection system.

The possibility of utilising the inhibition of urease by mercury was investigated by Dominguez-Renedo *et al.* at a SPCE modified with gold nano-particles [107]. The gold nano-particles were deposited electrochemically onto the working electrode surface, followed by a mixture containing bovine serum albumin, urease and glutaraldehyde. Gold nano-particles were reported to enhance the sensitivity of the sensor. Using the biosensor, a steady-state current was obtained for urea. Additions of mercury were found to give a decrease in the urea current response proportional to concentration. Using the developed biosensor, it was found possible to determine mercury levels of 1.0 μM in fortified human plasma samples, which is a very complicated matrix and therefore this technology could also be applied to other matrices such as contaminated soil or food.

Llangovan *et al.* [108] also used a urease-based biosensor to measure different metals to those mentioned above (cadmium, lead and copper). The device consists of two interdigitated electrodes deposited on to an insulator base; the dimensions of the strip are 3.3 x 0.83 cm. The optimum quantity of urease was deposited onto of the electrodes followed by a sol-gel layer to produce the biosensor. For the measurement step the biosensor strip was deposited into a solution containing the metal ion to allow inhibition of the enzyme to occur. The biosensor was then removed and placed in a separate beaker containing urea. The resulting conductivity was measured and the decrease in response was related to the metal ion concentration.

Ogonczyk *et al.* [109] fabricated a biosensor to measure silver and copper, by depositing a thick film of silver on a polyester foil, then screen-printing on a graphite, rubidium oxide, and urease paste, and finally a protective dielectric film was deposited and UV-cured. Potentiometric measurements were performed using a 16-channel instrument; these were used in conjunction with Ag/AgCl reference electrode.

As well as enzymes, bacteria have been employed as a bio-recognition element in biosensors for metal ion determination. Prasad *et al.* [110] have shown the possibility of using *Shewanella sp*. as the electron

transfer material for electrochemical determination of arsenite. A *Shewanella sp*. bacterial suspension was drop-coated on an SPCE surface and allowed to settle. Surface characterization validated that this simple drop-coating procedure resulted in a well adsorbed bacterial layer on the surface of SPCE. Cyclic voltammetry was used to investigate the behavior of aresenite; the magnitude of the reduction peak increased in the presence of arsenite over the concentration range 50-500 µM. Whilst it did not include an example of an application, this study demonstrates the possibility of applying this biosensor to arsenite measurement in the agri-food sector.

A different strategy for the measurement of arsenite has been reported by Cui *et al.* [111]. An aptamer-based SPCE biosensor modified with gold nanoparticles was used for the indirect measurement of arsenite, using differential pulse voltammetry. An excellent limit of detection of 0.15 nM was achieved.

Biosensors for the detection of metals have the advantage of simplicity of operation and interpretation of responses compared to chemical sensors based on direct voltammetry. In the case of arsenite, Cui *et al*'s [111] aptamer-based biosensor had a much lower limit of detection than chemical sensors; a review by Hughes *et al.* (2016) [1] compares the performance of chemical sensors and biosensors for metals and other analytes in depth.

Whilst the above biosensors are very sensitive for particular metal ions, or mixtures of metal ions, the response may not be particularly selective. However, it may be feasible to greatly improve the selectivity using a similar approach to that described above by Crew *et al.* [89] (section 4.0 Pesticides). This used an artificial neural network to identify individual OPs in conjunction with an enzyme biosensor array which measured inhibition.

Table 8. Reports of screen-printed biosensors for the determination of metal ions.

Analyte	Recognition element	Supporting electrolyte	Measurement Technique	Linear Range	Detection Limit	Samples	Reference
W^{6+}	Alkaline phosphatase	28 mM Tris, 19 mM $MgCl^2$, 0.36 M Cl^- pH8.0	Chronoamperometry (+0.2V)	0.6–10 μM	0.29 μM	Tap water and bottled water	[104]
As^{3+}	Acetylcholinesterase	Britton-Robinson pH 7.0	Amperometry (+0.6V)	0.01 – 0.1 μM	0.011 μM	Tap water	[105]
Hg^{2+}, Ag^+, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}	Glucose oxidase	-	Amperometry	-	Ag^+ μg/L range, others μg/mL range	-	[106]
Hg^{2+}	Urease	0.1 M phosphate buffer pH 7.0	Chronoamperometry (+1.5V)	6.0 – 60.0 nM	5.6 nM	Fortified human plasma	[107]
Cu^{2+}, Cd^{2+} and Pb^{2+}	Urease	0.02 mM phosphate buffer pH 7.0	Conductometric	Cu^{2+} 0.1–10.0 μM	-	-	[108]
Ag^+ and Cu^{2+}	Urease	5.0 mM phosphate buffer pH 7.0	Potentiometric	-	0.1 μM	-	[109]
As^{3+}	*Shewanella sp.*	0.1M PBS pH 7.4	Cyclic voltammetry	50-500 μM (from voltammograms)	-	-	[110]
As^{3+}	Arsenite aptamer	10mM PBS 7.4	Differential pulse	0.2-100 nM	0.15 nM	Tap water and natural water	[111]

According to WHO guidelines, the maximum residual limits in drinking water are [112]: 10µg/L (0.133 µM) for As^{3+}; 6µg/L (0.0299 µM) for Hg^{2+}, 50µg/L (0.787 µM) for Cu^{2+}, 3µg/L (0.0267 µM) for Cd^{2+}, 70µg/L (1.192 µM) for Ni^{2+} and 10µg/L (0.0483 µM) for Pb^{2+}; no data is available for W^{6+} or Ag^+ [112]. Table 6 shows the detection limits for a range of metal ion biosensors which indicates that the metal ions As^{3+} ([105,111]), Hg^{2+} [107], Cu^{2+} [109] can all be detected at their MRLs using the described devices. There is an opportunity to further develop the biosensors for $Cd^{2+,}$ Ni^{2+} and Pb^{2+} to improve their detection limits for application for drinking water analysis.

6.0. Conclusion

This review has focused on the ways in which SPCEs have been biologically modified with enzymes, antibodies, aptamers and bacteria together with chemical modifications such as organic and organometallic mediators (electrocatalysts), nanoparticles and membranes to further enhance the performance of the biosensors for potential application to agri-food safety.

In this review, we have also described a wide variety of applications in which prototype biosensors, based on SPCEs, were successfully developed for different classes of target analytes in diverse matrices. In the case of toxins, a popular biosensor approach has involved immobilisation of an appropriate antibody on to the transducer to form an immunosensor. Such devices are very selective and sensitive as demonstrated by the measurement of BoTN serotype A, without interference from E or B serotypes, with a detection limit of 0,15 $pgml^{-1}$, which could be applied to milk samples [9]; the authors indicated that the combination of gold nanoparticles with graphene and chitosan, led to the achievement of this very low detection limit. This strategy may be useful to other workers wishing to improve detection limits of their immunosensors for toxins. An alternative platform to the use of antibodies, in the development of selective affinity-based biosensors for the measurement of toxins, involves the integration of aptamers with SPCEs. The potential advantages of these devices include higher stability, larger dynamic range and prolonged shelf life [13]. These devices have been successfully applied to the development of biosensors for a range of toxins, including ochratoxin, and aflatoxins.

The wide use of antibiotics in farming has led researchers to investigate antibody-based biosensors for their determination. As mentioned above this approach can result in highly sensitive and selective devices, and an example is for the measurement chloramphenicol in milk; a limit of detection of 4.7 $pgml^{-1}$, and recovery of 92-95% was achieved. It should be noted the measurement method was quite simple; following the incubation step, the captured antibiotic was subjected to a negative potential in order to reduce the nitro group and generate the analytical response. This strategy for other antibiotics containing a nitro group would be worth pursuing. The immunosensor approach has also been popular for the determination of microorganisms. The specificity of these devices is good as exemplified by an immunosensor for the determination of salmonella in eggs and chicken meat, where good agreement was achieved with a standard culture method for the detection of bacterial numbers [33].

Biosensor strategies, employing suitable immobilisation procedures for enzymes (usually including a mediator) appears to the most popular approach for the type of naturally occurring analytes discussed in this review. The four main approaches for immobilisation have been covalent bonding, entrapment/encapsulation, crosslinking and adsorption. For the determination of glucose a selection of these approaches have been investigated; of these a method of entrapment of glucose oxidase involving

incorporation of platinum nanoparticles into a polyaniline-montmorillonite hybrid composite resulted in the lowest detection reported (10 µM). However, this approach might be a more costly than others discussed, which have reasonable sensitivity, but do not contain platinum (see Table 1). Biosensor strategies for a range of other naturally occurring species, together with their performance characteristics are shown in Tables 2 to Table 6. From these, it is apparent that a wide selection of fabrication procedures exist, and the choice would be based on the attributes required by the end user. It is worth pointing out that for some types of food samples, sample preparation can be quite simple, as mentioned for the measurement of monosodium glutamate in a stock cube. A simple dissolution step where the stock cube was dissolved in buffer only was required. An aliquot of this could be added directly into buffer solution for amperometric measurement. The turbidity is of course not a problem in electrochemical biosensor measurements and the presence of the immobilised biological elements, and immobilisation reagents (as shown in Fig.1), together with membranes, produces barriers to potential interferents. This is an advantage over other techniques such as those based on spectrophotometry, and chromatographic methods, where time-consuming sample preparation procedures may be required

The main platform for the development of biosensors for the measurement of organophosphate pesticides (OPs) usually consists of the immobilisation of a suitable enzyme (either acetylcholinesterase or butylcholinesterase) onto a SPCE surface by crosslinking or entrapment methods (see Table 7). The electrochemical measurement is then based on the decrease in the signal produced in the presence of the pesticide due to inhibition of the enzyme. Low detection limits have been achieved for a variety of OPs as shown in Table 7. Importantly, these examples show that measurements down the MRLs can be achieved with all of the devices shown. Another important feature of several of these devices ([89][97]) is the ability to simultaneous measure, and discriminate, five different OPs in a sample; this was achieved with the aid of an artificial neural network. These features, should be attractive for commercial exploitation. It should also be mentioned that sample preparation for water samples is rather simple and the present authors [89] were able to simply deposit unfiltered samples into the wells of a 96 well plate; the measurement of OPs was then performed automatically, after a suitable inhibition period. The instrument was portable and operated from the cigarette lighter socket of car. This demonstrates the possibility for performing analyses off site, and could have much wider applications.

A similar approach has been used in the construction of biosensors for the majority of metal ion biosensors shown in Table 8; the responses were based on the inhibition of the enzymes, alkaline phosphatase, acetylcholinesterase (AChE), urease, and glucose oxidase. Two other recognition approaches are also mentioned in Table 8, namely, a bacterium (shewanella) and an aptamer. As discussed in the above section on metal ion analysis, three of these devices were able to achieve detection limits that would be able to determine the metal ions at their MRLs: As^{3+} based on AChE [105] and an aptamer [111]; Hg^{2+} based on urease [107]; Cu^{2+} based on urease [109]. These biosensors

should be of interest to end users involved in e.g. water quality monitoring. As mentioned above, the present authors have developed a portable instrument that was able to exploit the inhibition of enzymes biosensors for the simultaneous measurement and discrimination of different OPs remote from the laboratory. It would be readily feasible to adapt such an instrument for the measurement of different metal ions such as those discussed above and probably other species. The sample preparation for water and other liquid samples for metal ions could be simple and convenient requiring only transfer of the sample to a 96 well plate followed by the automated procedure, which could similar to that mentioned earlier. The ability to make multiple simultaneous measurements of analytes is now readily possible owing to the availability of electrochemical instruments based on multipotentiostats, such as the one used for OPs [89].

In summary, there is great potential for the use of screen-printed biosensors for a range of agri-food safety applications, and the attributes of screen-printing technology using carbon materials (mass production in a wide range of geometries at low cost, ease of use, disposability and portability) make this an attractive route to commercialisation.

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