Rapid electrochemical screening methods for food safety and quality

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ABSTRACT
This paper presents some examples of rapid, simple and cost effective screening methods that can be realized by the use of Screen Printed Electrodes (SPEs) coupled with portable and cheap instrumentation, for the monitoring of food safety and quality. When necessary, these SPEs have been modified with nanomaterials in order to improve their analytic performances. Arsenic detection, for example, has been obtained with SPEs modified with a composite of nanostructured Carbon Black and Au nanoparticles, while for the pesticide detection the SPEs were modified with Prussian Blue nanoparticles in addition to the enzyme Butyrylcholinesterase. In the case of immunosensors, a high sensitivity has been obtained making the entire immunological chain happen on the surface of magnetic beads (MBs), finally collected on the surface of screen printed arrays with the aid of magnets located just under the working electrodes. Application to real samples are presented, in order to demonstrate the effectiveness of such approaches.

1. INTRODUCTION
The potential impact of foods on human health is receiving increasing attention by public opinion, scientists and legislators. The interest of consumers, as well as of producers, for food safety and quality testing has increased over the last years. The food production, processing and distribution globalization further complicate the problem.

Thus, efficient and sensitive analytical methods able to detect food contamination would help to reduce social and health risks. Food control analyses require robust, sensitive, and selective detection methods. The most commonly used methods such as chromatography and mass spectrometry require expensive instrumentations and skilled technicians.

Therefore, the importance of new detection systems, which should be accurate and sensitive, cheap and preferably portable for on-site testing, is evident. Sensors and biosensors allow the development of fast, simple and cheap electrochemical screening methods, and some of them are presented in this paper. In particular, screening methods will concern the detection of chemical compounds and microbial contaminants such as Arsenic and Pesticides, Salmonella sp, marine toxins such as Palitoxin, and viruses such as the Hepatitis A virus (HAV).

In all the methods that will be presented, Screen Printed Electrodes (SPEs) and/or electrochemical arrays based on an 8-screen-printed electrode strips, connected to a cost effective and portable apparatus, have been adopted as electrochemical transducers or assembled as immunosensors. Some interesting and useful reviews on SPEs can be found in literature [1]-[3].

In order to obtain higher sensitivity, in some case SPEs have been modified with nanostructured materials such as carbon black (CB) or gold nanoparticles (AuNPs), while in the case of immunosensors the arrays have been coupled with magnetic beads (MB), where the immunological chain occurs.

In this paper, we summarize the results of some recent research carried out by our Analytical Chemistry group, and presented at the 1st IMEKOFOOD Congress held in Rome, Italy in October 2014. Experiments illustrating the optimization and analytical characterization of the developed screening methods and their application in real samples to evaluate matrix effect and recovery, will be presented.
2. ARSENIC DETECTION

Arsenic has an historical use for its toxic and medicinal properties. In more recent ages, Arsenic and its compounds have been widely used in pigments, as insecticides and herbicides, as an alloy in metals, and as chemical warfare agents. Due to its wide use, together with its presence in a wide range of minerals, large accumulations now exist in soils and sediments, up to contaminate waters, including drinking waters.

The problem of the presence of As in water is known since the 80’s, when high levels of As have been found in groundwater resources. Thus, contamination of natural water has been identified as a public health problem, due to the mutagenic and carcinogenic effects of this element, especially in the form of As(III) [4], prompting the World Health Organization (WHO) to set the maximum permitted level in drinking water at 10 μg/L [5].

At present, a lot of detection methods have been reported and reviewed [6]. Most of them obtain limits of detection below the WHO arsenic guideline value of 10 μg/L, but often they are only suitable for laboratory conditions. Therefore, a rapid and sensitive portable system for the screening of As in field could be really useful.

We have developed an electrochemical sensor based on SPEs modified with a nanocomposite of Carbon Black (CB) and Au nanoparticles (CB-AuNPs/SPE) for the detection of As(III) [7].

Actually, a wide variety of nanomaterials with different properties have found a broad application in several analytical methods. Between them, gold nanoparticles (AuNPs) have received large attention mainly due to their interesting electrocatalytic properties [8], [9]. Moreover, recently we demonstrated the advantages of using the CB modified screen-printed electrode (SPE) compared to the bare one, for its improved analytic characteristics [10], [11]. Further modification of SPEs with the composite CB-AuNPs still enhances these characteristics, due to a synergic effect of both nanomaterials. The SPEs were home produced with graphite-based conductive ink (Elettrodag 421) for working and counter electrode and silver-silver chloride conductive ink (Elettrodag 477 SS) for pseudo-reference electrode. The diameter of the working electrode was 0.3 cm, resulting in an apparent geometric area of 0.07 cm².

The sensors were prepared by modifying the SPE firstly with CB (6 μL of a 1 mg/mL solution in DMF/Water 1:1) and then with AuNPs, also homemade, by depositing 6 μL of the AuNPs solution on the CB-modified SPE.

The chosen electrochemical technique was the Linear Sweep Anodic Stripping Voltammetry (scan rate=0.8 V/s, tdeposition=300 s, Ecleaning= 0.2 V, tcleaning=10 s), applied with the portable potentiostat PalmSens.

After the electrochemical and analytic optimization, the As was measured with a high sensitivity (674 mA mM⁻¹cm⁻²) and a LOD of 0.4 μg/L.

Finally, CB-AuNPs/SPEs has been applied to measure As(III) traces in drinking water. The analysis requires only few minutes and this sensor provides the detection of As(III) with high values of percentage recovery (99.9 %) in a tap water sample spiked with the legal limit amount (10 μg/L).

The electrodes are easily fabricated at low cost, are disposable, and suitable for in situ analysis in real time.

3. PESTICIDES DETECTION

In agricultural or industrial production there is a growing exploitation of chemical compounds, but these substances may pose serious risks on humans, animals and environment. Among the various chemicals, pesticides are considered one of the most dangerous, due to their variable nature and to highly toxic effects on living organisms and wildlife. The toxicity of Organophosphorus Pesticides (OPs) mainly arises from their capability to inhibit the acetylcholinesterase enzyme (AChE), a crucial enzyme for the central nervous system processes [12], [13].

For these reasons, there is a general concern on pesticide contamination and on their detection in polluted sites. Traditional methods for the detection of organophosphorus pesticides employ chromatographic techniques, such as gas chromatography (GC) [14], [15].

Biosensors can represent suitable tools for rapid, reliable, cost-effective and in-situ analysis, for screening of environmental samples successively confirmed by traditional laboratory methods. In particular, several biosensors have been described in literature for organophosphorus pesticides based on their capacity to inhibit cholinesterase (ChE) enzymes [16]-[20].

In a recent work we developed an amperometric biosensor for the determination of paraaxon, based on the enzyme butyrylcholinesterase (BChE) immobilized on screen-printed electrodes modified with Prussian Blue Nanoparticles (PBPNs), and embedded in a flow system [21].

Prussian Blue Nanoparticles (PBPNs) modification of SPEs was accomplished by placing a drop (10 μL total volume) of “precursor solution” on the working electrode area. This solution was obtained by mixing 5 μL of 0.1 M potassium ferricyanide in 10 mM HCl with 5 μL of 0.1 M ferric chloride in 10 mM HCl directly on the surface of the working electrode. The solution was left on the electrode for 10 min and then rinsed with a few milliliters of 10 mM HCl. The electrodes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of PBPNs [22]. The PBPNs modified electrodes were stored dry at room temperature in dark up to one year.

To immobilize the BChE enzyme on the PBPNs modified electrode surface, 2 μL of 0.25 % glutaraldehyde was applied with a pipette exclusively on the PBPNs modified working electrode. The solution was left to evaporate; then, 2 μL of a mixture of BSA, enzyme and Nafion® was dropped on the working electrode.

The mixture was obtained by adding 25 μL of 3 % (w/v) BSA, 25 μL of 0.1 % (v/v) Nafion® and 25 μL of a stock enzyme solution (40 U/mL). All solutions were prepared in distilled water. The biosensor was embebbed in a flow system shown in Figure 1, and, in order to improve the working stability of the reference electrode, the Ag/AgCl reference electrode was covered with an acetate cellulose layer by applying 2 μL on its surface. The so prepared amperometric measurements were performed in a carrier solution consisting of 0.05 M phosphate buffer, 0.1 M KCl pH 7.4 at an applied potential of +200 mV vs. Ag/AgCl. Firstly, the carrier buffer was passed through the electrochemical cell for 5 min to register an intensity current (control). Then, a carrier buffer containing 5 mM butyrylthiocholine was passed through the flow cell where the biosensor was located. The substrate butyrylthiocholine was hydrolyzed by BChE immobilized on the SPE-PBPNs producing thiocoline, which is electroactive.
The resulted current signals were continuously recorded and the steady state current values were measured. Stabilization of the current in flow conditions was reached in 10 min.

The intensity currents were proportional to thiocholine produced, giving information about the enzymatic activity of the immobilized BChE. The inhibitory effect of organophosphates (i.e., paraoxon) on the BChE biosensor was evaluated by determining the decrease in the current obtained for the oxidation of a lesser amount of thiocholine produced by the enzyme when the sample contaminated with paraoxon was passed through the flow cell for a selected time. Thus, the biosensor was exposed to paraoxon, followed by a washing step with distilled water. The enzymatic residual activity was finally determined in a flow buffer, in the presence of the enzymatic substrate. The resulting currents were measured as described above and the degree of inhibition was calculated as a relative decay of the biosensor response through the (1):

\[ I\% = \left( \frac{I_0 - I}{I_0} \right) \times 100 \]  

(1)

where \( I_0 \) and \( I \) represent the biosensor responses before and after the incubation procedure, respectively.

The introduction of a washing step allowed to avoid electrochemical interferences such as ascorbic acid or phenolic compounds, since the enzymatic activity was always quantified in the phosphate buffer in the absence of any electroactive interfering species eventually present in the sample.

The integration in a flow system enables the biosensor to be applied for continuous automatic monitoring of paraoxon in environmental samples. In addition, in a continuous flow-system biosensor, manual procedures are minimized and analyses can be programmed and remotely delivered, minimizing the operator intervention. In order to optimize the biosensing system, a series of parameters was evaluated and optimized, such as different types of electrochemical cells, the flow rate during the enzymatic measurement and the incubation time. In optimized conditions, storage stability lasted up to 60 days at room temperature in dry conditions, demonstrating an excellent storage stability and making this system highly attractive for commercial use. Furthermore, the analytical system was characterized by satisfactory analytical performances in the detection of organophosphate tested (paraoxon) reaching a linear range of concentration between 2 and 10 ppb with a detection limit (LOD) of 1 ppb in standard solutions. This system was also challenged in drinking, river and lake water samples with satisfactory recovery values using a dilution step of 1:4 v/v.

The advantage of this integrated system is the possibility to measure irreversible inhibitors of cholinesterase enzyme (i.e., organophosphorus and carbamimic pesticides) in samples without extended pre-treatments and to automate the analyses reducing costs and time. This analytical system can be used as an alarm system for this class of compounds, followed, in the case of alarm, by the HPLC or GC-MS analyses to exactly detect the pesticides present in the sample.

4. PALLYTOXIN DETECTION

Palytoxin (PlTX) is one of the most potent marine toxin known to date [23]. Recently blooms of ostreopsis spp. have been reported along the Mediterranean coasts, posing serious risks to human health. Occurrence of ostreopsis spp may result in palytoxin contamination of seafood (250 μg/kg proposed regulatory limit) and, in order to prevent sanitary risks, there is the need to develop rapid and sensitive monitoring methods of PlTX-like compounds in seafood, coupled with an efficient extraction procedure.

Currently, there are no regulations on PlTX-group toxins in shellfish, either in the European Union or in other regions of the world, nor there is an official method for their determination. The method most commonly used for detection of PlTXs is the mouse bioassay [24] but for reasons of animal welfare, of poor sensitivity and specificity, and long analysis times, other methods detection are required by EFSA [25], [26].

We developed an electrochemical sensor, based on an 8-screen-printed electrode strip connected to a cost effective and portable apparatus, for palytoxin (PlTX) detection [27]. Sheep erythrocytes were used to test palytoxin and the degree of haemolysis caused by this toxin was evaluated by measuring the release of the cytosolic lactate dehydrogenase (LDH). The percentage of haemolysis, and therefore the amount of LDH measured, using NADH/pyruvate and appropriate electrochemical mediators, is correlated to the concentration of this toxin.

Two different electrochemical approaches were investigated for evaluating LDH release, but only one based on the use of a binary redox mediator sequence (phenazine methosulphate in conjugation with hexacyanoferate (III)) has proven to be useful for our purpose. The approach involves three sequential reactions:

\[ \text{LDH} \]
\[ \text{Pyruvate} + \text{NADH} \leftrightarrow \text{lactate} + \text{NAD}^+ \]  

(2)

\[ \text{NADH} + \text{PMS}^+ \leftrightarrow \text{NAD}^+ + \text{PMSH} \]  

(3)

\[ \text{PMSH} + 2 \text{Fe(CN)}_6^{3-} \rightarrow \text{PMS}^+ (\text{which cycles via the reaction 3}) + 2 \text{Fe(CN)}_6^{4-} + \text{H}^+ + 2e^- \]  

(4)

After incubation of the LDH (released into the medium) and its substrates for 30 minutes, untreated NADH (the amount depending on the concentration of LDH) spontaneously interacts with PMS+ forming PMSH.

This latter, produced by reaction (3) interacts with the second mediator, present in large excess, forming Fe(CN)₆⁴⁻. In
this approach, when the residual NADH is completely consumed, the reactions stop and reoxidation of \( \text{Fe(CN)}_6^{3-} \) on the SPE surface, at an applied potential of +260 mV, gives a current signal inversely proportional to the LDH concentration and therefore to the PITX concentration. No reduction of \( \text{Fe(CN)}_6^{3-} \) and PMS occurs at this potential.

After an analytical/biochemical characterization, the sensor strip was used to measure palytoxin. Sheep blood and standard solutions of PITX were let to react using two different incubation times (24 h or 4 h) obtaining working ranges of 7×10^{-3}-0.02 μg/l and 0.16-1.3 μg/l, respectively. The specificity of the test for palytoxin was evaluated using ouabain which acts as PLTX on the Na+/K+-ATPase pump. A cross-reactivity study, using high concentrations of other marine biotoxins was also carried out. Experiments to evaluate the matrix effect and recovery on mussel samples were also carried out, and results showed that the matrix effect was dependent on the PITX concentration, and thus it is necessary to use a matrix standard calibration curve for accurate analysis of PITX in experimentally and naturally contaminated samples. This was the first time that a biomolecular method for analysis of PITX in mussels has been evaluated for matrix and recovery effects.

Compared with the conventional haemolytic–spectrophotometric assay, the method proposed here proved to be faster (4 h blood/PITX incubation instead of 24 h) and uses a cost effective and portable apparatus.

5. SALMONELLA DETECTION

The increased consumption of fresh and ready to use vegetables has recently caused several outbreaks and illness due to their contamination by pathogenic microorganisms, such as Salmonella [28], [29]. In Italy, the production and processing of fresh vegetables are concentrated in the Campania region, and especially in the plain of Sele. Recently, fresh and ready to eat vegetables from this area have been the cause of repeated food alert in the EC. This problem is mainly due to cultivation practices, manipulation and transformation. Since the standard culture method for detecting Salmonella [30, ISO 6579:2002] requires up to 5 days to produce results, the need to develop rapid methods represents an important issue for the authorities and the producers. The purpose of this study was the development and evaluation of an ELIME (Enzyme-Linked-Immuno-Magnetic-Electrochemical) assay [31], [32] to detect Salmonella in vegetables of I and IV gamma.

The proposed ELIME assay is based on the use of magnetic beads (MBs) as support of a sandwich immunological chain, coupled with a strip of 8-magnetized screen-printed electrodes (localized at the bottom of 8 wells). The product of the enzymatic reaction is quickly measured by chronoamperometry at an applied potential of −100 mV for 60 seconds.

Four different kinds of MBs anti-Salmonella were tested:
- Dyntalbeads anti-Salmonella (ready to use)
- Pathatrix anti-Salmonella (ready to use)
- Pathatrix Same Day anti-Salmonella (ready to use)
- Pan Mouse IgG MBs coated with a broad reactivity MAb anti-Salmonella

An optimized dilution of PAb-HRP (1:100) has been used to complete the sandwich, and the couple TMB + H₂O₂ as enzyme substrate.

After verifying the ability of the system based on the use of Dynabeads anti-Salmonella to interact with different Salmonella serotypes, we focused our attention on S. Napoli and S. Thompson, recently isolated from vegetables grown in Italy. Being Salmonella, and in general pathogens, a small fraction of a large population of non-target (NT) organisms, present in food, able to adhere to various surfaces including MBs, we also tested several NT bacteria. In order to have the better sensitivity and specificity towards Salmonella, different PAb-HRP dilutions and several blocking agents (gelatin bovine, t-carrageenan, BSA, PVA, dry milk) were investigated. The best results were obtained using PAb-HRP=1:100 and dry milk, because of better selectivity (Figure 2). Among the 20 NT bacteria tested, only E. cloacae, C. freundii, E. aerogenes, E. coli, gave back current signals greater than the zero point.

Similar results were also obtained using Pathatrix MBs. For this reason, different Salmonella serotypes were also tested using Pan Mouse IgG MBs coated with a broad reactivity MAb anti-Salmonella (dry milk as blocking agent and PAb-HRP=1:100). In Figure 2, calibration curves for S. Napoli and S. Thompson are reported, while Figure 3 shows the selectivity study using dry milk and PVA as blocking agent.

From the results obtained, we can state that these particles are less sensitive but more specific than those ready to use. However, experiments on I and IV gamma vegetables, experimentally contaminated with salmonella, will be performed with both types of particles to evaluate their real capability to distinguish Salmonella from endogenous NTs.
6. HAV DETECTION

Hepatitis A virus (HAV) causes an acute hepatitis associated with a significant morbidity and occasional mortality. Outbreaks of waterborne diseases are certainly underestimated due to the lack of adequate programs for the epidemiological surveillance. Current legislation for water, shellfish (EC 2073/2005 EC B53/2004) and plant (EC 2073/2005) does not provide for any limitation due to the presence of HAV and other enteric viruses in the irrigation and housing waters [33]. In addition, there is no official method for the detection of these viruses. Actually, the presence of viruses in water and/or foods can be just indirectly deduced by the patient’s symptoms and confirmed through the search for anti-HAV IgM and/or anti-HAV IgG antibodies in patient’s blood.

Immunological approaches are reported as methods to determine HAV in drinking water before its use, thus avoiding the infectious disease.

In this work, still in progress, two electrochemical, competitive and sandwich, ELIME assays were developed. These systems are based on the use of new polydopamine-modified magnetic nanobeads as solid support for the immunochemical chain, and screen printed electrodes as a sensing platform. This rapid and low-cost analysis method involves the use of a portable instrument, able to perform measurements directly in the field.

These ELIME assays showed detection limits equal to $1 \times 10^{-8}$ IU/mL and $8 \times 10^{-7}$ IU/mL for sandwich and competitive formats, respectively.

In order to investigate the applicability of the proposed immunosensors for practical analysis, the immunosensors were used to evaluate the recoveries of different concentrations of HAV spiked in different tap water samples. Indeed, different HAV concentrations from $10^{-10}$ to $10^{-2}$ IU/mL spiked in tap water samples were analyzed.

Preliminary results obtained on real samples were compared with those obtained by the qRT-PCR analysis that is a routine technique used to evaluate the HAV contamination levels in samples. At moment, electrochemical values do not perfectly match the PCR results, being underestimated. Experiments are in progress to elucidate and overcome this problem.

7. CONCLUSIONS

New screening methods for the control of food safety have been presented. The use of SPEs in conjunction of portable and quite inexpensive instrumentation, could allow these methods to become popular because quite simple to be carried out also by not specialized operators.

Examples of determination of Arsenic, pesticides and toxins (PITX) at ppb levels have been illustrated, with application to real samples in order to demonstrate the feasibility and effectiveness of these determinations.

Where necessary, improved analytical characteristics have been obtained modifying the SPEs with nanostructured materials able to enhance their performances. Examples of immunosensors for the detection of bacteria such as Salmonella and viruses such as Hepatitis A have been also reported. In this case, a large enhancement of the sensitivity of the assay has been obtained using Magnetic Beads of micrometer dimensions as support for the immunological chain, and arrays of magnetized SPEs as electrochemical transducers.

All the methods presented are intended to be adopted as screening methods, and not used in place of the confirmatory ones. In such way, only samples suspected to be contaminated can be subjected to the required confirmatory analytical methods for residues in foods.

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REFERENCES


