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ELECTROCHEMICAL SENSORS FOR ASSESSMENT OF FOOD QUALITY AND SAFETY
Scientific field AGR/15

ALESSANDRO PELLICANO'

Tutor: Prof. Maria Stella Cosio
Co-tutor: Prof. Stefania Iametti

PhD Coordinator: Prof. Maria Grazia Fortina

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Abstract

ABSTRACT

The food industry is constantly challenged to meet consumer demands for new food products that are safe, convenient, affordable, pleasurable and healthy. Nanotechnology has begun to find potential applications in the area of functional food by engineering biological molecules toward functions very different from those they have in nature, opening up a whole new area of research and development. Of course, there seems to be no limit to what food technologists are prepared to do to our food and nanotechnology will give them a whole new set of tools to go to new extremes. The main objective of this research project was the development of analytical devices for the rapid and reliable determination of biological and chemical species (inorganic, and organic, including those of bacterial origin) present in foods of different types and origin for the assessment of food quality and safety. The project mainly focused on the development of micro- or nanosensor arrays to be used in electrochemical devices. For this purpose, it is planned to use conventional electrodes (glassy carbon, screen printed platinum, gold electrodes) and electrodes based on nanostructured materials suitably functionalized for application.

In fact, it is well known that these surfaces can be modified by using chemical or biological systems to facilitate both the electron transfer at the electrode surface and the specificity and selectivity of the electrochemical transducer. Such devices, especially in the form of a complex array, are an attractive alternative over the use of expensive laboratory equipment, since they can provide reliable and quantitative information simultaneously on multiple analytes.

The performance of these sensors was evaluated by using experimental and theoretical approaches based mainly on various electroanalytical techniques. Among the sensors developed in the project, those most appropriate for use as amperometric detectors in analytical systems (in flow or batch mode) will be subjected to validation studies and directly used on real samples. This phase of the project will require also the use of appropriate classical analytical instrumentation such as HPLC-UV, spectrophotometry, ELISA to assess the quality parameters of the methods developed.

Nanomaterials have high surface-to-volume ratios, unique surface activities, and high electron-transfer rates at relatively low overpotentials. These characteristics make them ideal platforms for the design of electrochemical sensors. In addition, they can be chemically activated to act as immobilization surfaces that improve the stability of the attached biomaterials. Based on these considerations, the main objective of this research project is to improve the existing methodology through the development of simple analytical methods based on electrochemical nanosensors, whether single or in arrays, enabling them multi-elementary and rapid analysis of food of animal and vegetable origin and reliable determination of potentially harmful species (inorganic, and organic, including those of bacterial origin) present at trace levels in food.

In research work, it is demonstrate the potentialities of nanosensors in two spheres of food-related applications: (i) nanosensors aimed at food quality control, (ii) nanosensors used for safety control in food. The present research work is thus structured in four chapters: C*hapter 1 were* the applicability of modified commercial sensors (screen-printed) in complex foodstuffs is demonstrated; *Chapter 2 is* devoted to the development and use of a nano-biosensor and modified electrode for determination of fish freshness (purine derivatives); *Chapter 3* describe the use of nanostructure (carbon nanotubes such as multi-walled) for the rapid evaluation of contaminant (bisphenol A) in water bottles and baby bottles. Finally, *chapter 4* is focused on the determination of the majors allergens present in peanuts (Ara h 1 and Ara h 2) using amperometric magnetoimmunosensor.

RIASSUNTO

L'industria alimentare è costantemente stimolata allo sviluppo di nuovi prodotti che non solo soddisfino le esigenze del consumatore, ma che al contempo siano sicuri, convenienti, piacevoli e salutari. Recentemente, la nanotecnologia ha trovato applicazione nel settore alimentare con l'introduzione di nuovi prodotti aprendo prospettive del tutto nuove per il consumatore. Si stima che più di 600 aziende nel mondo siano operative con prodotti alimentari sviluppati con l'uso di tecniche nano-tecnologiche. Tuttavia, il controllo della qualità e sicurezza alimentare può trarre certamente beneficio dall'uso delle nanotecnologie in quanto è possibile sviluppare nano-sensori rapidi e sensibili, che possono essere utilizzati, online e offline della catena alimentare.

L'obiettivo di questo progetto di ricerca è stato lo sviluppo di micro-nano-sensori per la determinazione rapida e affidabile di analiti di interesse alimentare utili nel controllo della qualità e della sicurezza alimentare.

In particolare, l'attenzione è stata rivolta alla messa a punto di micro-nanosensori elettrochimici in quanto si prestano, a differenza per esempio di quelli ottici, molto più facilmente alla miniaturizzazione e alle misure in situ.

A tale scopo sono stati utilizzati sia elettrodi convenzionali quali carbone vetroso, screen printed di carbonio, platino, oro che elettrodi basati su materiali nanostrutturati opportunamente funzionalizzati per applicazioni anche in fase gassosa. La funzionalizzazione è stata ottenuta utilizzando sistemi chimici o biologici per favorire sia il trasferimento elettronico a livello superficiale (elettrodo/sistema redox) che la specificità e selettività al trasduttore elettrochimico.

Tali nano-sensori, nella disposizione ad array si pongono come alternativa all'uso di costosa strumentazione da laboratorio, in quanto possono fornire in maniera affidabile informazioni quali e quantitative anche simultanee su più analiti presenti nella matrice di interesse.

Fra i sensori messi a punto verranno selezionati quelli più adeguati ad essere utilizzati come rivelatori amperometrici in sistemi analitici in flusso o in batch e sottoposti a studi di validazione per il loro immediato utilizzo sui campioni reali. Questa fase richiederà l'utilizzo di strumentazioni analitiche sofisticate quali HPLC-UV, spettrofotometria, ELISA che permettano di verificare i parametri di qualità delle metodiche sviluppate.

I nanomateriali hanno un elevato rapporto superficie-volume, superfici attive e alta velocità di trasferimento di elettroni. Queste caratteristiche rendono le piattaforme ideali per la progettazione di sensori elettrochimici. In aggiunte, possono essere attivati chimicamente per agire come superfici di immobilizzazione che migliorano la stabilità di biomateriali allegati. Sulla base di queste considerazioni, l'obiettivo principale di questo progetto di ricerca è quello di migliorare la metodologia esistente attraverso lo sviluppo di metodi analitici semplici sulla base di nanosensori elettrochimici, sia singolo o in combinazione, consentendo loro una rapida analisi dei prodotti alimentari di origine animale e vegetale e per la determinazione affidabile di specie potenzialmente nocive (inorganiche e organiche, comprese quelle di origine batterica) presenti a livello di tracce negli alimenti. In questa tesi, viene dimostrata le potenzialità di nanosensori in due sfere di applicazioni legate al cibo: (i) nanosensori rivolti al controllo qualità dei prodotti alimentari, (ii) nanosensori utilizzati per il controllo della sicurezza nei prodotti alimentari. La strategia metodologica seguita ha utilizzato un approccio ready-to-use e non proof-of-concept, con campioni, materiali e condizioni operative, il più possibile reali. Di conseguenza la tesi è stata strutturata in quattro capitoli: il capitolo 1 descrive l'applicazione di sensori commerciali modificati (screen-printed SP) usati per l'analisi di matrici alimentari anche complesse. Il capitolo 2 esplora l'utilizzo di nano-biosensori modificati con specie chimiche per la determinazione della freschezza del pesce (derivati purinici). Il capitolo 3 descrive l'uso di nanostrutture, come nanotubi di carbonio, multi-walled e single-walled, per la valutazione rapida del bisfenolo A presente nelle bottiglie d'acqua e nei biberon. Infine, il capitolo 4 è focalizzato sulla determinazione dei maggiori allergeni presenti nelle arachidi (Ara h 1 e Ara h 2) utilizzando un magnetoimmunosensore amperometrico.

PREFACE

Since nanotechnology was introduced by Nobel laureate Richard P. Feynman, there have been many revolutionary developments in physics, chemistry, and biology that have demonstrated Feynman's ideas of manipulating matter at an extremely small scale, the level of molecules and atoms, i.e., the nanoscale. While the meaning of "nanotechnology" varies from field to field and country to country and is widely used as a "catch all" description for anything very small, nanotechnology is commonly defined as the understanding, control, and restructuring of matter on the order of nanometers (i.e., less than 100 nm) to create materials with fundamentally new properties and functions. Nanotechnology encompasses two main approaches: (i) the "top-down" approach, in which larger structures are reduced in size to the nanoscale while maintaining their original properties without atomic-level control (e.g., miniaturization in the domain of electronics) or deconstructed from larger structures into their smaller, composite parts and (ii) the "bottom-up" approach, also called "molecular nanotechnology" or "molecular manufacturing," introduced by Drexler et al., in which materials are engineered from atoms or molecular components through a process of assembly or self-assembly. While most contemporary technologies rely on the "top-down" approach, molecular nanotechnology holds great promise for breakthroughs in materials and manufacturing, electronics, medicine and healthcare, energy, biotechnology, information technology, and national security. Nanotechnology developments using nanodevices/nanomaterials opens up potential novel applications in agriculture and food sector. Smart delivery systems, biosensors, and nanoarrays are being designed to solve the problems faced in agriculture sector. Similarly, food sector is also benefited through the use of smart biosensors, packaging materials, and nanonutraceuticals. Despite the great potential of nanotechnology in agri-food sector, people are ambiguous about use in food applications because of suspected potential health risks and environmental concerns. Among the nanomaterials, offer new directions in the design of novel, analytically useful, nano-sensing systems as materials that have physical and chemical characteristics. Use of these materials can improve the analytical performance of sensors, aiding in their establishment as reliable analytical instruments.

In chapter 1 nanofibrous membranes are investigated in electrochemical sensing of foodstuffs as selective barriers against interfering compounds and as recognition systems nano-biosensors. In the first case-study (section 1.1) nylon-6 nanofibrous membranes are used as coating for transducers (carbon electrodes) enabling the direct determination of ascorbic acid in real samples (fruits and fruit juices). In chapter 2 development of electrochemical sensing for the estimation of fish freshness. In the first case-study (section 2.1) the same kind of electrospun material is used for the covalent immobilization of xanthine oxidase and the subsequent (preliminary) development of an amperometric sensor for xanthine and hypoxanthine determination in foodstuffs. In the second case-study (section 2.2) The system report the immobilization of copper onto the surface of glassy carbon electrode that reduce the anodic peak potential, therefore increasing the sensitivity of the technique with the formation of cupric or couprous complex with purine derivatives in the fish meat samples. In chapter 3 modification and functionalization of conventional electrochemical sensors for determination of contaminants in foodstuffs. In the first case-study (section 3.1) was aimed at developing a new sensitive electrochemical sensor for BPA determination. For this purpose, a conventional electrode was modified with nanostructure functionalized by different approaches and the optimal working conditions and pH effect on overall sensors performance were investigated. Sensitivity, linear range, limit of detection and quantification and stability were evaluated in flow injection analyses (FIA). Finally in chapter 4 the applicability of the developed disposable magnetoimmunosensor was evaluated by determination of the mayor allergens content in different foods demonstrating its potential role in food safety and consumer protection. In the first case-study (section 4.1) development of the first amperometric magnetoimmunosensor based on a sandwich configuration, using two monoclonal mouse IgG antibodies against Ara h 1, one of them biotin-labeled, as capture and detector antibodies, respectively, onto carboxylic acid-modified magnetic beads (HOOC-MBs) for the selective and sensitive determination of the endogenous Ara h 1 in food samples and the evaluation of its analytical performance. In the second case-study (section 4.2) the

develop of disposable magnetoimmunosensor was evaluated by determination of the endogenous Ara h 2 content in food extracts and spiked samples at trace level (5.0 mg kg^{-1}) thus demonstrating its potential applicability in the implementation into portable and multiplexed electrochemical platforms for food safety. Each chapter (and sub-chapter) is accompanied by a specific introduction with its own state-of-the art review that will serve to the reader for better understanding the advances that each case-study represents to the particular technological sphere of the food sector where it has place.

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QUALITY ASSESSMENT OF FOOD SYSTEMS

Quality control is essential in the food industry and efficient quality assurance is becoming increasingly important. Consumers expect adequate quality at a given price, good shelf-life and high safety while food inspections require good manufacturing practices, safety, labelling and compliance with the regulations. Further, food producers are increasingly asking for efficient control methods, in particular through on-line or at-line quality sensors, firstly to satisfy the consumer and regulatory requirements and secondly to improve the production feasibility, quality sorting, automation and reduction of production cost and production time (increased throughputs).

Thus, all three drivers consumers, authorities and food producers, have great interest in the development of new sensing systems which are beyond the existing on-line technologies, like control of weight, volume, temperature, pH, viscosity, colour and appearance. It is evident the need of new technologies allowing food control on line for what concern production and fast devices for food control once on the market.

A food quality sensor can be defines as a device that can respond to some property of the food under analysis and transform the responses into a signal easily readable. This signal may provide direct information about the quality factors to be measured or may have a known relation to this quality factor. Sensors can be classified according to their mode of use: on-line and at-line.

On-line sensors operate directly in the process, giving a real-time signal which relates to some property of the sample and giving an immediate quality measurement. At-line sensors are device to be used for instance in split-flow measurements, requiring reagent additions or reaction times. They often have short response times (minutes or seconds) and can allow process corrections.

The new on-line or at-line food quality sensors may be classified in different ways, but generally, this is done according to the physical nature of the sensor: optical, chemical or electrical. So we have:

Biosensors, incorporating a biological material, like enzymes or antibodies; sensors based on an electric signal, for example potentiometric chemical sensors, metal oxide semiconductors, field effect transistors or conducting polymer sensors; sensors based on interaction by electromagnetic waves, in particular sensors using visible, ultraviolet and infrared (NIR, NIT, FTIR); sensors involving selective agents, such as molecular films or complexing films.

The great challenge is indeed to focus on the real-time and on-line sensors and data system surveying processes and products, controlling the automated process and the raw material stream, sensing the final product quality and typing the product labels, nutritional and health information and much more.

1. THE APPLICATION OF NANOTECHNOLOGY IN FRUITS

1.1. DIRECT *IN-SITU* DETERMINATION OF ASCORBIC ACID IN FRUITS BY SCREEN-PRINTED CARBON ELECTRODES MODIFIED WITH NYLON-6 NANOFIBERS

1.1.1. Introduction

Fresh fruits are an excellent source of vitamins, antioxidants and other biologically relevant phytochemicals. Vitamin C, one of the principal antioxidants present in variable amount in fruits and vegetables, is important in the human diet to prevent scurvy and other diseases, and as additive in food processing. The main biologically active form of vitamin C is L-ascorbic acid (AA) or L-ascorbate.

Due to the importance of this analyte in human nutrition, its detection and quantification is of great significance. Titration, spectrophotometry, spectroscopy, and their combination with separation techniques have been widely used for its determination. In general this methods are costly and time consuming. Moreover, the accuracy in the measurement of AA in food products depends highly on the extraction procedure that must avoid its oxidation and photodecompomposition.

In-situ AA determination by conventional and available techniques is limited considering that they require an extraction protocol from the original matrix, followed by a proper sample dilution and the use of antioxidant agents (e.g., metaphosphoric acid).

Therefore, the assessment of AA with techniques that are rapid, straightforward and portable that require a minor preparation, remains an important challenge in food and agricultural industry. Electrochemical analysis in comparison with the above mentioned techniques may realize these necessities and would make feasible the *in-situ* evaluation, providing a valid alternative for producers, in particular in the fresh food produce industry, and regulatory agencies.

Application of electrochemical analysis with various sensors to food-related substances has been widely studied. For what concern the electrochemical determination of AA with metal or carbon electrodes, especially in complex matrices such as food products, the main drawbacks are: (a) the relatively high working potentials required for its electrochemical oxidation that might lead to poor selectivity and (b) the fouling caused mostly by oxidation byproducts that usually leads to poor reproducibility. Several attempts to overcome these issues by using active mediators or by modifying carbon or metal electrodes have been described, but its determination in real food matrices has been not so widely investigated.

In the particular case of screen-printed (SP) sensors (modified or not), even though their effectiveness for quantification of AA and other vitamins in standard solutions or purified biological samples has been extensively studied and demonstrated, they have rarely been applied to the evaluation of AA in real food samples.

Recently nanofibrous membranes (NFM) prepared by polymer electrospinning, have attracted broad attention for electrochemical sensors modification due to their high porosities and specific surface areas. Large surfaces of non-woven nanofibers can be electrospun from a broad range of synthetic or bio-polymers, collected as mats of variable desired thickness and directly applied as a coating to the electrode. Their manufacturing by electrospinning is simple, fast and advantageous from the point of view of cost, production rates and reagent consumption. The membranes are of easy manipulation and functionalization. Furthermore, in contrast to thick film technology, electrospun nanofibrous membranes have a low barrier to diffusion.

In this work we report a rapid and simple system for estimating the content of AA of fruits directly in the sample (*in-situ*) without any extraction, nor the use of antioxidant reagents, by coating a SP carbon electrode with electrospun nylon-6 nanofibrous membranes (SP-NFM). The method is rapid, leads to accurate results and does not necessitate expensive equipment nor skilled operator.

1.1.2. Materials and methods

1.1.2.1. Chemicals

All chemicals and solvents were of analytical reagent grade and were used without any further purification. Solutions were prepared with ultra-pure MilliQ water (Millipore, Inc.; $\Omega = 18$ M$\Omega\cdot$cm^{-1}). Sulfuric acid (97%), metaphosphoric acid, nylon-6 and standards of L-ascorbic acid, caffeic acid, epicatechin, ferulic acid, p-coumaric acid, quercetin, naringenin and tannic acid, were purchased from Sigma-Aldrich (Milan, Italy). Formic acid (98%) was purchased from Fluka, Sigma-Aldrich (Steinham, Germany). Standard solutions of AA were prepared in buffer citrate (0.1 M; pH from 2.5 to 4.0) from daily-prepared and degassed stock solutions.

1.1.2.2. Samples

The samples (apple, pear, lemon, kiwi, orange, tangerine and strawberry) were purchased from a local retailer and selected to show the wider applicability of the methodology.

1.1.2.3. Fabrication of the nanofibrous membranes by electrospinning

Nylon-6 membranes were prepared as described with some modifications. Briefly, a 23 % (w/w) solution of nylon-6 was prepared in formic acid. Plastic syringes (10 mL) fitted with a metallic needle (Hamilton) were filled with the polymeric solution and placed in a KDS100 syringe pump (KD-Scientific, New Hope, PA) at a flow rate of 0.15 mL h^{-1}. The needle of the syringe was linked to a Spellman SL150 high voltage power supply by an alligator clip. A foil-covered copper tray, positioned at 11 cm in front of the needle, was used as collector and grounded. For the electrospinning, the electrical potential was set at 25 kV. At the end of the electrospinning runs, the membranes were peeled-off. Membranes with different thicknesses were obtained by stopping the collection after different times (from 5 min to 60 min). The characteristics of the nylon-6 nanofibrous membranes obtained are discussed; at electrospinning conditions described above, membranes thickened at a rate of 2.3 μm min^{-1} ($r^2 = 0.98$), resulting in thickness between approximately 10 to 125 μm. These fibers exhibit randomly oriented and interconnected arrangement with diameters of 95 ± 25 nm, and are free of beads (Figure 1.2). The average density and porosity of the membranes are 75 kg m^{-3} and 94%, respectively. Work at break varies for the different thicknesses from 1.3 to 4.1 N m^{-1}. The resistance to flow of distilled water varies from 2 to 5.4 x 10^{10} m^{-1}, confirming the membranes good permeability.
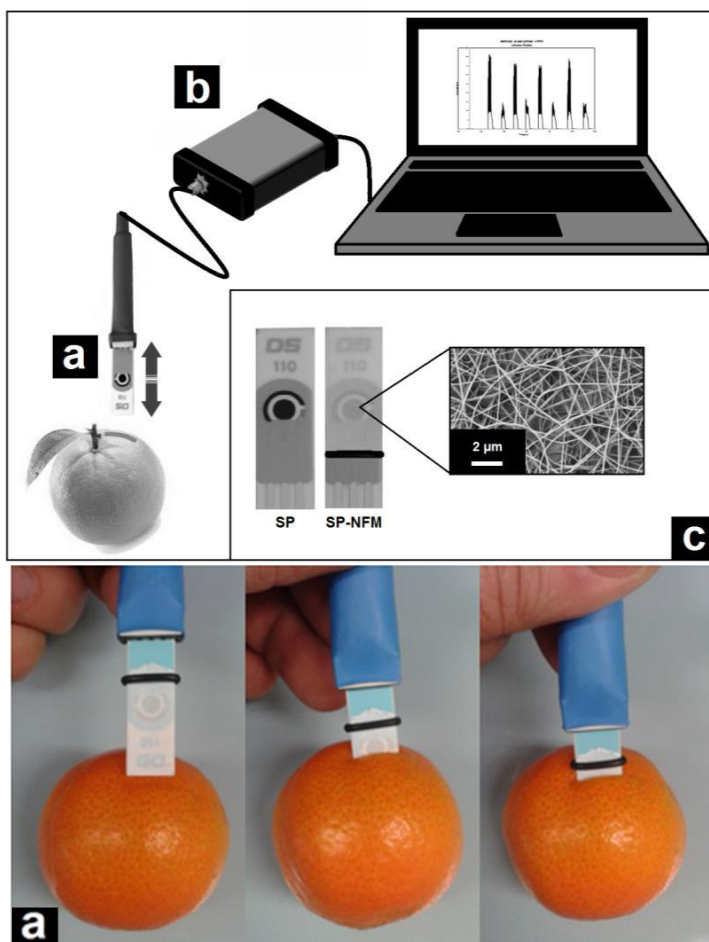
Figure 1.2.. *Schematic representation of the in-situ amperometric sensing unit: the sensor that is repeatedly taken in and out the fruit (a) is connected to a bi-potentiostat (b); in the box, a picture of the SP and SP-NFM electrodes with a SEM micrograph of the nylon-6 nanofibrous membrane (c).*

1.1.2.4. Electrochemical apparatus

Portable Bipotentiostat µstat 200 (DropSens, Spain) was used for the amperometric measurements. The system controlled the electrodes used in this work: SP electrodes were disposable screen-printed DS 110 (DropSens, Spain) formed by graphite electrodes as working and counter electrodes, and a silver electrode as pseudo-reference electrode.

1.1.2.5. Sensor modification

Modification of the electrode was done by coating its surface with a wet NFM followed by drying at room temperature (~15 min). Membranes were held to the electrodes with a rubber o-ring (Figure 1.2.a). It must be noticed that for SP sensors, the coated surface included the working, counter and reference electrodes.

1.1.2.6. Amperometric experiments

Amperometric batch measurements of standard solutions of AA and interfering compounds were performed in 10 mL stirred buffers, prepared as described above. The sensors were calibrated using AA standard solutions (50 – 1800 µg mL^{-1}) at a fixed applied potential of 0.35 V. Amperometric measurements in real samples were performed according to the following four-steps sequence: (i) the electrode was washed with distilled water; (ii) the electrode was immersed ~6 s in an AA standard solution several times (4 to 10) and the amperometric signal was recorded; (iii) the electrode was thoroughly washed with distilled water; (iv) the electrode was introduced ~6 s for several times (4 to 10) in the real sample by directly pricking the fruit, and the amperometric signal was recorded (Figure 1.2.a and 1.2.b). In the case of fruits with hard peel or pulp (e.g., apple, orange, tangerine, lemon) a ~2 cm-deep knife cut was enough to facilitate the sensor penetration (Figure 1.2.b). The AA concentration of the standard solutions was selected on the basis of the expected content of vitamin C reported in the literature. This sequence was repeated at least three times for each real sample analyzed.

1.1.2.7. Chromatographic apparatus

The HPLC system consisted of a Model 2080 plus PU pump and a UV-vis 2070 plus detector (Jasco, Japan). The chromatographic conditions were described briefly: column, Fruit Quality Analysis (100 x 7.8 mm id) (Bio-Rad, CA, USA); eluting solution, 0.001 M sulfuric acid at a 0.7 mL min^{-1} flow rate; detection at 254 nm. The sample injection volume was 20 µL. Integration of peak areas and retention time was performed with Borwin v. 1.2. software (Jmbs Developments, France).

1.1.3. Results and discussion

1.1.3.1. Sensor evaluation

To evaluate the performance of the sensor for *in-situ* determination, preliminary experiments were performed by measuring the amperometric response of the SP and SP-NFM sensors modified with membranes of various thickness, operating in AA standard solutions at the applied potential of 0.35 V. This potential was chosen on the basis of preliminary experiments of cyclic voltammetry (data not shown): the oxidation peak was found between 0.3 and 0.5 V with the maximum intensity at ~0.43 V. The potential of 0.35 V was considered low enough for favoring an appropriate selectivity, yet offering a good signal.

A dynamic range of AA concentrations from 10 to 1800 µg mL^{-1} was selected considering that the AA concentration of fruit pulps and extracts varies from less than 10 µg mL^{-1} to more than 1500 µg mL^{-1}.

As expected the sensitivity changed with membrane thickness. Figure 1.3 shows the sensitivity of the SP-NFM when using different membrane thicknesses. The thinnest membranes (11 ± 2 µm) allowed higher values of sensitivity whereas no influence of membrane thickness was observed when it varied from 34 ± 3 µm to 50 ± 3 µm. On the other hand, thicker membranes (108 ± 4 µm) caused a significant signal reduction. This is due to the electrical resistance of the membrane that adds to the ohmic drop of the sensor, contributing to a slower electron transfer. However, it must be noticed that membranes are prone to breakage when the thickness is less than 30 µm. In order to have reasonable resistance and sensitivity for the samples under study, membranes of 34 µm (obtained after 15 min of electrospinning) were used in all subsequent analyses.
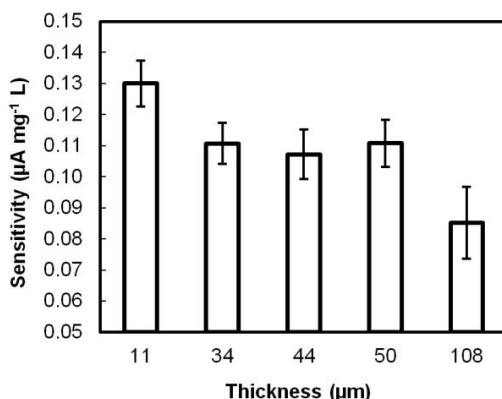
Figure 1.3. *Effect of the membrane thickness on the sensitivity of SP-NFM electrode (AA from 10–1800 µg mL^{-1}; 0.1 M citrate buffer at pH 4.0). Vertical error bars correspond to standard deviations of n = 3 repetitions.*

Figure 1.4 shows the amperometric responses obtained by using SP and SP-NFM sensors at increasing AA concentrations from 10 to 1800 µg mL^{-1} at pH 4.0. Each peak array corresponds to repetitive measurements of the solutions at the same concentration. Coating of the SP sensor with the nanofibrous membrane entailed a widening of the linearity range and a decrease of the sensitivity. Namely, linearity range of the SP electrode (Figure 1.4a) was 10-400 µg mL^{-1} whereas that of the SP-NFM (Figure 1.4b) was 10-1300 µg mL^{-1}. Sensitivity of the SP-NFM sensor was nearly 25% that of the SP sensor. These results are in agreement with the presence of a partial barrier-to-diffusion effect of the membrane. Since vitamin C can be present in high and different amount in fruits, and dilution in the proposed assay is not possible, the wider linearity range extends the applicability of this technique to a larger variety of fruits. Although the SP-NFM electrode was covered with the membrane, its response was rapid (<1 s). The shape of the peak arrays obtained with the SP-NFM electrode appears different (Figure 1.4.b). This is due to (i) the diffusion phenomenon, which causes a slight signal delay with respect to the SP electrode (Figure 1.4.a), and (ii) the effect of membrane impregnation. The latter refers to the elapsed time until the membrane is saturated with the solution, which results in a delay in the signal stabilization; in fact, the signal became stable after the third or fourth immersion in the solution, when the saturation condition is reached. In addition, until the membrane is thoroughly washed with water, it remains soaked with AA solution, which causes a noticeable background signal when the sensor is out of the liquid medium. As long as the measurement is repeated several times (at least 4 to 5), to guarantee a stable response, and the electrode is carefully washed between two different solutions (or samples), neither the repeatability nor the overall signal stability are compromised.
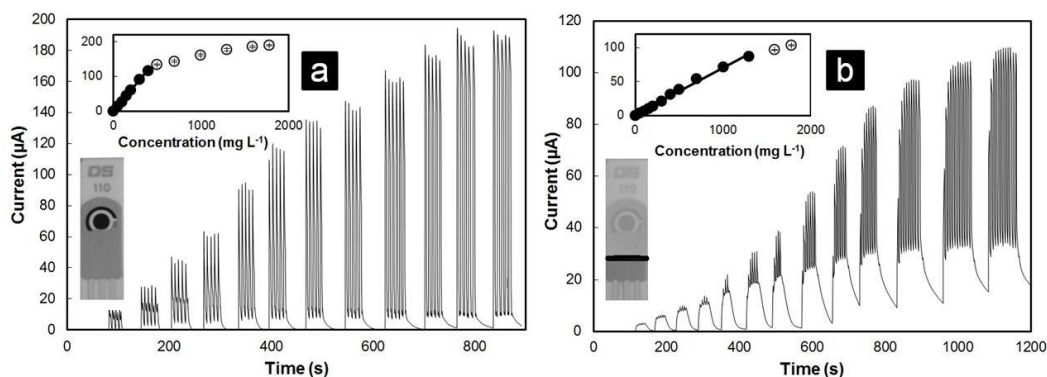
Figure 1.4. *Examples of in-batch amperometric responses of SP (a) and SP-NFM (b) electrodes in 0.1 M citrate*

buffer at pH 4.0 to increasing concentrations of AA (10 – 1800 mg L^{-1}) with the corresponding current versus concentration plots (in the box).

The time that the SP-NFM electrode remained immersed in the liquid medium, here named contact time, affected its sensitivity and repeatability. This effect was investigated and results are shown in Figure 4. As can be seen from the figure, contact times of 15 s or larger caused more than 20% of sensitivity loss, whereas no significant differences were found for contact times between 3 and 10 s. With regard to repeatability, contact times between 4 and 10 s were found optimal, with $RSD < 2\%$ ($n = 30$). Considering the high AA concentrations evaluated, the loss of sensitivity might be explained by an excessive accumulation/deposition of the oxidation product, namely dehydroascorbic acid, on the electrode surface, which is favored by large contact times, causing a passivation-like effect and consequently a decrease in the measured faradaic current.

The sensitivity of SP-NFM electrode was also evaluated at different pH's within the typical range of fruits. Figure 1.5. shows the current versus concentration plot from 50 to 1300 µg mL^{-1} at pH 2.5, 3.0 and 4.0. There was no correlation ($p < 0.05$) between pH and current for the range evaluated, meaning that, in these conditions pH does not exert a significant effect on the amperometric response.
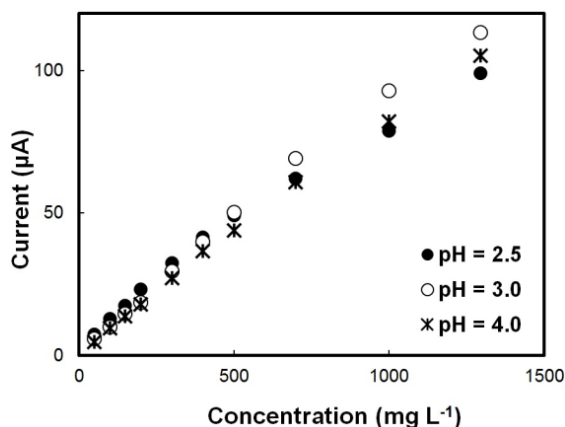
Figure 1.5. *Current versus concentration plots obtained with SP-NFM electrode at different values of pH (AA from 10 – 1300 mg L-1; 0.1 M citrate buffer)*

In order to evaluate the effect of potential interferences, the response of SP and SP-NFM electrodes to standard solutions of different phenolic compounds (caffeic acid, epicatechin, ferulic acid, p-coumaric acid, naringenin, quercetin and tannic acid) that can be naturally found in the fruits, was analyzed. Table 1 summarizes the amperometric response obtained at the maximum concentration evaluated (I_{max}) of both SP and SP-NFM electrodes and the I_{max} percentage reduction caused by the NFM-coating [100*(I_{max} of SP - I_{max} of SP-NFM)/ I_{max} of SP]. The coated sensor gave lower responses showing the efficiency of the membrane in reducing the effect of polyphenols present the fruits under study.

Table 1.1. *Sensitivity, current at the maximum concentration (Imax) and response linearity (r2) of SP and SP-NFM sensors to standard solutions (0.1 M citrate buffer at pH 4.0) of different phenolic compounds at a working potential of 0.35 V.*

Phenolic	Tested range mg L^{-1}	I_{max} (µA)		Sensitivity (µA mg L^{-1})	
		SP	SP-NFM	SP	SP-NFM
p-coumaric acid	5 - 20	4.485	0.395	0.081 (r^2 = 0.77)	0.014 (r^2 = 0.65)
caffeic acid	5 - 50	11.804	0.920	0.239 (r^2 = 0.98)	0.017 (r^2 = 0.50)
ferulic acid	0.2 - 1	0.332	0.013	0.313 (r^2 = 0.98)	0.015 (r^2 = 0.73)
naringenin	1 - 10	3.179	0.245	-	-
epicatechin	1 - 10	0.905	0.068	0.099 (r^2 = 0.99)	0.010 (r^2 = 0.92)
quercetin	1 - 10	4.485	0.395	-	-
tannic acid	1 - 10	0.427	0.029	0.043 (r^2 = 0.97)	0.004 (r^2 = 0.95)
ascorbic acid	10 - 1800	189.65	103.76	0.277 (r^2 = 0.99)	0.070 (r^2 = 1.00)

The decrease of the sensor sensitivity towards phenolic compounds cannot be fully explained by the decrease of diffusivity caused by the fibrous layer. As previously observed by Scampicchio *et al.*, nylon nanofibrous membranes act as selective barriers against polyphenol oxidation on carbon electrode surfaces (Figure 1.6.). In fact, the diminution of I_{max} varied from 85% for *p*-coumaric acid to 96% for ferulic acid. The membranes though, are more effective as barriers towards polyphenols that are highly undissociated (e.g. tannic acid and epicatechin) having more affinity to the NFM than to aqueous medium. Instead, the hydrophobicity of the polyamidic membrane limits the adsorption of smaller phenolic compound (e.g. *p*-coumaric and caffeic acids) that are partially dissociated at the pH of fruits. These results demonstrate that the NFM-coating help to preserve the sensors from the amperometric response distortion caused by phenolic compounds.
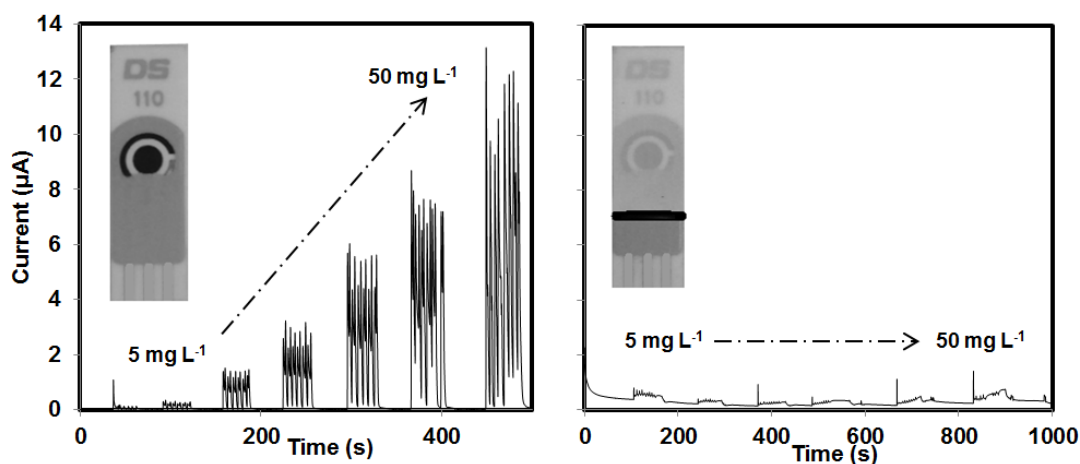
Figure 1.6. *Amperometric response of SP (left) and SP-NFM (right) to increasing concentrations of caffeic acid (5 – 50 mg L-1) at an operating applied potential of 0.35 V (0.1 M citrate buffer).*

The SP-NFM electrode showed very good operational stability at least after 22 measurements. However, considering the easiness of preparation and cost, it is recommendable to use a new membrane for each analytical session.

The proposed method was applied to different types of fruits containing between 30 and 600 mg of AA per kg of sample. Each sample was analyzed by directly "pricking" the fruits at least six times with the SP-NFM electrode in three different "slots" (i.e. in triplicate). The signal variability of the *in-situ* measurements, when the fruit was pricked in the same slot, was maximum 7.1% (*RSD*) whereas it was higher when considered the different slots (*RSD* up to 11%).

In Table 2 the results obtained by the *in-situ* amperometric method are compared to those obtained with a standard method (HPLC-UV). The regression line was obtained by linear ordinary least squares, provided that the concentrations obtained by the more precise standard method are reported in abscissa axis. There is a good correlation between the two methods ($r = 0.98$) showing that the membrane is effective in preventing any interference to reach the electrode. In fact, the regression line is $y = 1.046x + 5$ and the confidence interval ($p = 95\%$) for the slope and the intercept are ± 0.091 and ± 38, respectively. The slope is not significantly different from 1 and the intercept is not significantly different from zero, even if its confidence interval is quite large.

As can be seen in Table 1.2., the amperometric determinations gave higher values of standard deviation in all cases respect to the HPLC-UV method, due to the fact that the former does not entail any homogenization protocol and therefore the natural variability related to a complex matrix (in this case the fruit) adds to the intrinsic variability of the method.

It is expected that fruits whose AA concentration is not in the range of the proposed method can still be analyzed by tuning thickness of the membrane and/or the operating potential. Namely, thicker membranes allow an extended working range though with a reduced sensitivity, which in turn can be improved by increasing the working potential.

Table 1.2. *Determination of AA content in fresh fruits using SP-NFM electrode (in-situ) and a reference methodology (HPLC-UV) (n=3).*

Sample	mg of AA Kg^{-1}	
	amperometric detection SP-NFM	reference method (HPLC-UV)
apple	37 ± 8	32 ± 1
pear (1)	43 ± 5	39 ± 0
pear (2)	66 ± 6	56 ± 1
lemon	390 ± 22	432 ± 8
kiwi	324 ± 46	339 ± 9
orange (1)	542 ± 50	553 ± 43
orange (2)	428 ± 22	476 ± 12
orange (3)	521 ± 43	557 ± 4
tangerine (1)	366 ± 6	396 ± 3
tangerine (2)	448 ± 52	411 ± 8
tangerine (3)	501 ± 48	559 ± 1
strawberry (1)	545 ± 58	563 ± 6
strawberry (2)	547 ± 19	555 ± 19
strawberry (3)	470 ± 17	437 ± 8

1.1.3.1. Sensor lifetime

The SP-NFM electrode showed very good operational stability at least after 22 measurements (Figure 1.7-left). However, considering the easiness of preparation and cost, it is recommendable to use a new membrane for each analytical session. Furthermore, the protective effect of the NFM coating against fouling and detrition is evident in Figure 1.7. (right) that shows how the amperometric response at the fourth measurement of a real sample (in this case lemon juice) using uncoated electrode (SP) had decayed down to 20% the value of the first measurement, whereas for SP-NFM the response decay was much less dramatic.
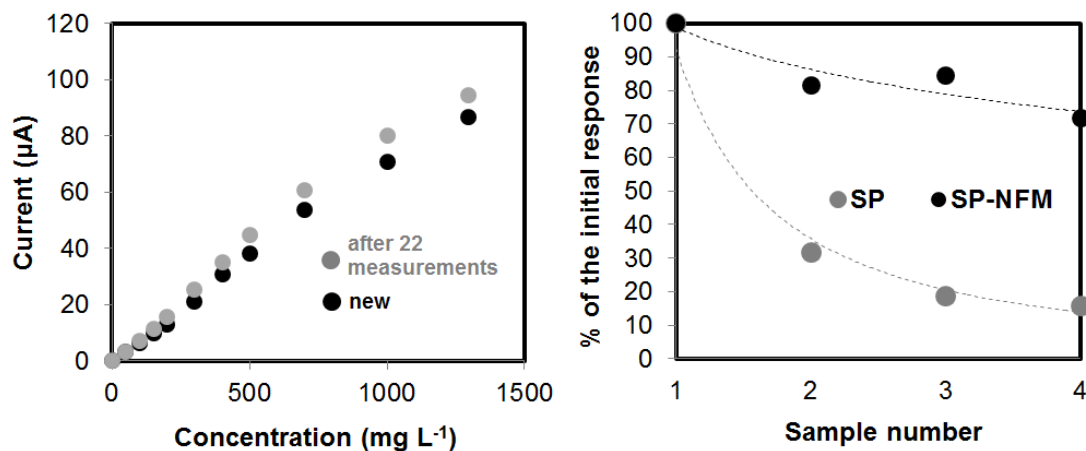
Figure 1.7. *Standard curves of AA built using a new SP-NFM sensor and the same SP-NFM sensor after 22 measurements (left); decay of the amperometric reponse of SP and SP-NFM to the same sample (right).*

In sum, the applicability of a novel sensing unit based on a disposable screen printed carbon electrode (SP) coated by a electrospun nylon-6 nanofibrous membrane was demonstrated for the in-situ determination of AA in both standard solution and real fruit samples. The sensor displays high sensitivity, reproducibility and selectivity towards AA with a good stability and a fast response. In short, the membrane in this sensing unit acts as a partial barrier-to-diffusion from the matrix to the electrode surface, especially towards phenolic compounds, protecting the electrochemical signal from the distortion caused by the oxidation of these chemical species. In addition, the membrane shields the electrode against the mechanical damage to which it is exposed when introduced in a complex solid matrix as a fruit pulp. Such kind of methodology is straightforward, rapid and inexpensive for the assessment, especially when compared to traditional analytical determinations that require a sample pre-treatment. Moreover, due to the simplicity of the apparatus, it could be used like a portable device to be applied in the field.

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2. NANOTECHNOLOGY AS A NOVEL APPROACH FOR FISH FRESHNESS EVALUATION

2.1. BIOSENSOR FOR DETECTION OF XANTHINE AND HYPOXANTHINE BASED ON XANTHINE OXIDASE IMMOBILIZED IN NYLON-6 MEMBRANES MODIFIED RHODIUM CARBON PASTE ELECTRODE

2.1.1. Introduction

Purines are heterocyclic aromatic organic compounds and consists of a pyrimidine ring fused to an imidazole ring. Purines best known for being nitrogenous bases of DNA and RNA are adenine and guanine; they are widespread in nature as derived nucleoside (adenosine and guanosine) phosphorylated (AMP, ADP, ATP, GMP, GDP, GTP). The oxidation products of these compounds play significant role in human metabolism, and hence they are of great biochemical and biomedical interest. Among them, xanthine, hypoxanthine and uric acid are intermediate metabolites of purine in human beings, which are useful biomarkers for several diseases such as perinatal asphyxia, hyperuricemia, cerebral ischemia and gout. Inosine is a purine nucleoside composed of hypoxanthine and D-ribose; it is a major degradation product of adenosine with potent immunomodulatory and neuroprotective effects and it has been used to relieve the symptoms of many disease. Inosine 5' monophosphate (IMP) is the ribonucleotide of hypoxanthine and the first nucleotide formed during the synthesis of purine.

Besides providing useful markers for clinical studies, purine derivatives are important in food quality control as index of the freshness of fish. In fish meat, a large number of post-mortem reactions are initiated during storage. These deteriorative changes consist on the formation of nucleotide and nucleoside metabolites resulting from the ATP degradation.

To evaluate the freshness and state of storage there are different methods; sensory (cadaveric rigidity, appearance and odor of skin, eyes, gills and meat), physical (electrical conductivity), biochemical (cytochrome oxidase, glutamate, aspartate, aminotransferase, lysosomal enzime), microbiological (determination of specific strains altering) and chemical (TVBN, TMA determination, K value).

The K-value is based on ATP breakdown and the subsequent formation of its by-products. The K-value measures how far ATP degradation has progressed within the tissue.

It is expressed as a percentage of the content of the last two final compounds of the ATP catabolic pathway (Ino and Hx) over the total content of ATP and its degradation by-products: ATP, ADP, AMP, IMP, Ino and Hx. Because ATP decomposes very quickly to IMP in most animals, a simplified K value (generally called Kindex (Ki)-or K-value) was proposed.

The K-value has been recognized for several decades as the most effective and objective indicator of the freshness of fish and seafood products, as well as of meat (beef, pork, lamb and poultry). The lower the K-value, the fresher the fish.

Many analytical methods were developed for their determination in solution or for monitoring of their concentration levels in biological fluids such as human blood, urine, or blood serum. Among them, a variety of methods namely HPLC, spectrophotometry, electrophoresis have been used for purines determination. These methods are found to be costly, time consuming and involve complex procedures. Most of these compounds are electrochemically active and can be determinate by electrochemical techniques using different conventional and nanostructured electrodes. Electrochemical analysis involves various advantages namely less time, high specificity, simple procedure involved. In the recent years, oxidation of some biologically important xanthines was studied by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and stripping voltammetry (CSV), in connection with carbon paste electrode (CPE), boron-doped diamond electrodes or glassy carbon electrode (GC). Most reports use the xanthine oxidase (XOD) to convert xanthine and hypoxanthine into hydrogen peroxide and uric acid. However, the cost of XOD is very expensive and there is no commercially available enzyme to facilitate the determination of the other purines. The electrochemical determination of these purines in a fish meat samples might encounter several problems. Immobilization of enzyme plays a crucial role in development of an electrochemical biosensor. Several methods of enzyme immobilization have been considered for this purpose. These include immobilisation of XOD in graphite, carbon paste, nylon mesh with glutaraldehyde (GLA) and covalent attachment to cellulose acetate membrane, nylon mesh on

platinum and carbon paste electrodes. Cross-linking with bovine serum albumin (BSA) and GLA is the most commonly used method for the development of an amperometric and potentiometric Hx biosensor because of its ability to improve the stability of immobilization of any mediator. Nafion coating has also been used to immobilize Hx and improve the selectivity of the electrode. Also used glassy carbon paste electrode to immobilized XOD.

In the first paragraph of this chapter we preliminarily explore the capability of XAO-immobilized on nylon-6 nanofibrous membranes to serve as recognition systems of xanthine, in a sensor configuration in which xanthine concentration is related to the faradaic current of its enzyme-catalized oxidation product (i.e., H_2O_2) with an appropriate transducer.

In the second praragraph, a high anodic potential and low sensibility were obtained using (GC) electrode and the extreme operating potential means a large number of antioxidants would influence the analytical results. The system reported here, presents the immobilization of copper onto the surface of glassy carbon electrode that reduce the anodic peak potential, therefore increasing the sensitivity of the technique with the formation of cupric or couprous complex with purine derivatives in the fish meat samples.

2.1.2. Materials and methods

2.1.2.1. Chemicals

Rodhium, xanthine, hydrogen peroxide, xanthine oxidase (XAO), bovine serum albumin (BSA), glutaraldehyde (GlA) and nylon-6 were obtained from Sigma Aldrich. Formic acid (98%) was purchased from Fluka. Stock solution was prepared by dissolving 0.01g in 10 mL of standard in buffer phosphate 0.1M pH 7.0.

2.1.2.2. Electrochemical apparatus and transducer preparation

Amperometric measurements were made with a portable Potentiostat (DS-Drop Sens µSTAT 100). A three-electrode system, including working electrode (transducer), saturated Ag/AgCl reference electrode and a Pt counter electrode. As transducer rodhium-carbon paste (5%) was used, based on a serious o previous experiments as it will be discussed below. The transducer was prepared in the dry state by hand-mixing (with a spatula) using powders of rodhium and carbon paste with paraffin wax for 15 min. A portion of the resulting composite was packed firmly into a Teflon electrode cavity in which the electrical contact was established via a copper wire. The composite surface was smoothed on a weighing paper and rinsed carefully with double-distilled water prior to each measurement

2.1.2.3. Fabrication of nanofibrous membranes

As in section 1.2.1.3.

2.1.2.4. Enzyme immobilization

The enzyme xanthine oxidase (XAO) was immobilized on the nylon nanofibers by a drop coating procedure, following the approach given by Scampicchio et al. (2010). An enzyme solution was prepared containing 10 mg of XAO and 40 mg of BSA in 1 mL of buffer phosphate. To this solution, 10 µL of the cross-linking agent glutaraldehyde (GlA) (2,5 % v/v in water) was added. Briefly, 5 x 10-6 L of a 20 g/L xanthine oxidase solution (in PB 0.1 M, pH 7.0) was dropped into the coated electrode. The biosensor was left to dry for 10 min at RT. Next, this dropping procedure was repeated twice. Finally, the biosensor was immersed in a PB 0.1 M solution at pH 7.0.

2.1.3. Results and discussion

2.1.3.1. Selection of the transducer

A series of experiments were carried out in order to evaluate the electrocatalytic action of different modified and conventional electrodes towards hydrogen peroxide, product of reaction carried out by xanthine oxidase:

$$\text{Xanthine } + \text{ O}_2 + \text{ H}_2\text{O} \xrightarrow{\text{XO}} \text{ Uric acid } + \text{ H}_2\text{O}_2$$
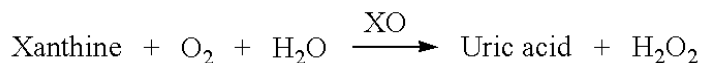
Optimization of working conditions was accomplished by batch amperometric detection using hydrogen peroxide as a substrate. The selection of rhodium-carbon 5% as the transducer was done on the basis of the comparison of several metal-based electrodes. In order to compare modified electrode, amperometric tests were performed at different fixed potential in buffer phosphate (0.1 M, pH 7.0) on the different electrodes, namely rhodium-carbon 5%, cobalt oxide, platinum-carbon 5% and ruthenium oxide 10%. Results are shown in Figure 2.1.

At higher operating potentials (> 0.4 V), the best sensitivity (i.e., higher peak current) was obtained with ruthenium oxide working electrodes. It must be noticed that such high operating potentials though are not ideal, since at these conditions there is a high tendency to reveal interfering compounds, especially if the analyte must be detected in a food-derived matrix. Conversely, at lower operating potentials (< 0.0 V), hydrogen peroxide produced a reduction signal, for which the highest sensitivity is provided by the rhodium-carbon as working electrode. Therefore, the rhodium-carbon electrode was selected as the transducer for the biosensor device, operating at a potential of -200 mV, in order to minimize possible interferences.

2.1.3.2. Effect of electrode nanofibers-coating on H_2O_2 sensitivity

The second step of this work consisted in comparing the response of hydrogen peroxide using (i) nude rhodium-carbon electrode, (ii) rhodium-carbon electrode coated with the nanofibrous membranes and (iii) rhodium-carbon electrode coated with the nanofibrous membranes, adding onto the membrane BSA and GlA. With the proposed approach, in the first place the analytes (in this case the purine) must be able to migrate from the bulk to the nanofibers surface (onto which the enzyme-oxidation occurs) and in the second place, the H_2O_2 produced must be able to "trespass" the fibrous network towards the electrode surface, where it is finally detected. Thus this comparison permitted to establish at which extent the membrane limits the H_2O_2 detection detecting a possible barrier-to-diffusion effect. The results of in-batch amperometric detection of H_2O_2 ($12 - 15$ µg mL^{-1}) experiments are showed in figure 2.1. The results show that the electrochemical behavior of the three electrodes coated with membrane, BSA and GlA and uncoated are comparable.
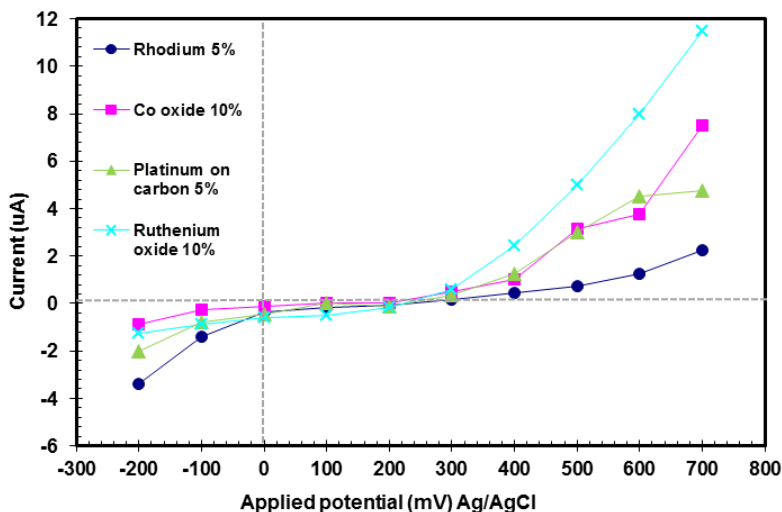
Figure 2.1. *Amperometric response of various metallic electrochemical sensors to 50 µg mL^{-1} solutions of H$_2$O$_2$ at variable applied potentials (phosphate buffer 0.1 M; pH 7.0).*

Figure 2.2 (insert) shows also the corresponding calibration curves of the three electrodes (by plotting the concentration against the plateau current obtained after the addition of the analyte subtracting the baseline signal). The sensitivity of the uncoated electrode was 0.10 µA mg^{-1} L in the linear tract, 0.08 µA mg^{-1} L for the membrane-coated electrode and 0.09 µA mg^{-1} L for the electrode coated with membrane, BSA and GlA. This indicates that the redox characteristics of rodhium-carbon electrode are minimally affected by the nanofibrous membranes. The comparison of the electrode with and without coating reveals only a slight change due to a decrease in the mass transport of hydrogen peroxide towards the electrode surface.
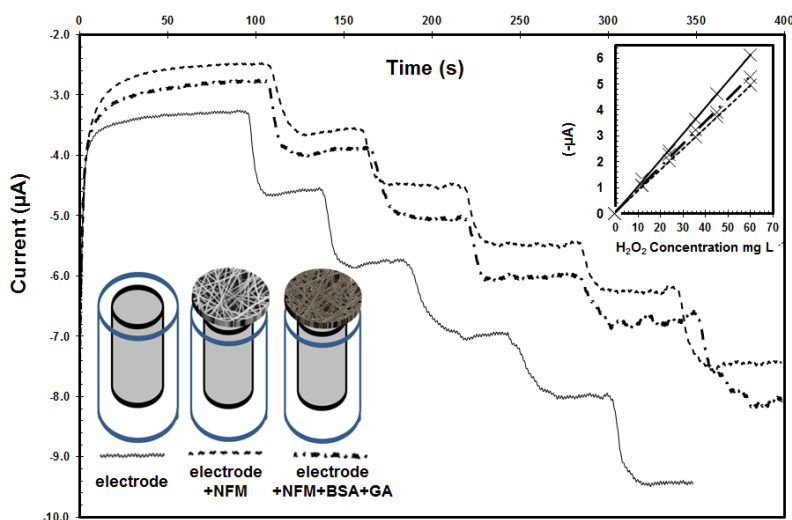
Figure 2.2. *In-batch amperometric detection of H2O2 (12 – 15 µg mL^{-1}) with rhodium-carbon electrodes (uncoated, coated with a nylon nanofibrous membrane and with a nylon nanofibrous membrane with BSA and GlA) at a constant -0.2 V fixed potential (phosphate buffer 0.1 M; pH 7.0). In the insert, the corresponding current-vs-concentration plots.*

2.1.3.3. Xanthine biosensing

Finally, the immobilization of xanthine oxidase on the nylon-6 nanofibers and the response of the sensor coated with the recognition system towards the presence of different concentrations of xanthine was studied.

The immobilization technique consisted in a simple procedure. BSA works as a carrier protein and the presence of amine groups, which are bound to the carbonyl group of the glutaraldehyde, leave the active centres of the enzyme at liberty to interact with the substrate (Scampicchio *et al.*, 2010). The amperometric current, at an applied potential of -0.2 V, of XAO-coated sensors in solutions containing 0, 5, 8 and 10 µg mL^{-1} was recorded for 2 hours. The resulting signal was xanthine concentration-dependent, showing a noisy-yet-stable trend after ca. 1 h. subtracting the baseline current allowed to correlate the signal with the xanthine concentration: Figure 2.3. shows the current-vs-time plot for the further 1 h after the stabilization time. The increase in current is attributed to the reduction of hydrogen peroxide on the electrode surface, which is constantly regenerated by the enzyme through the oxidation of xanthine to uric acid. Under this consideration, the reduction current increase rate (-µA s^{-1}) can be considered as a measurement of the reaction rate. Albeit there were too few points for making a statistically-valid quantitative correlation, this variable was positively associated to the xanthine concentration (Figure 2.3-insert), as can be expected for an enzyme-catalysed reaction.
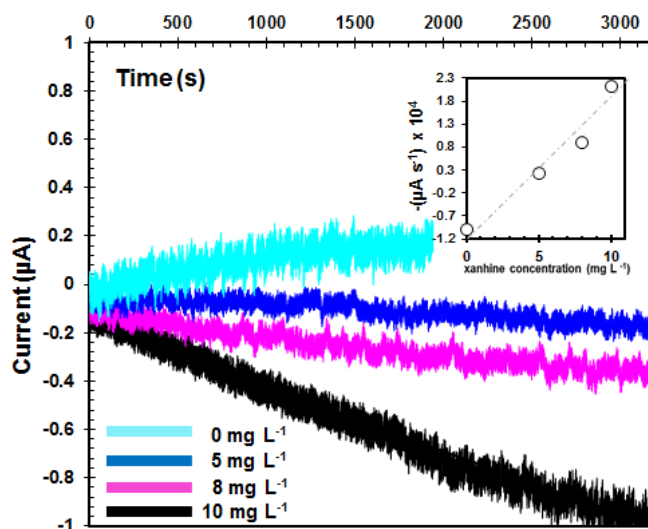
Figure 2.3. *Amperometric responses of the XAO/nylon-6/rhodium carbon biosensor, after 1 h of stabilization at a fixed potential of -0.2 V, to different concentrations of xanthine (phosphate buffer 0.1 M; pH 7.0).*

In order to produce a more ready-to-use system for xanthine quantitative assessment this kind of sensor can be prepared by using higher loads of enzyme, or enzymes with enhanced catalytic activity which would cope with the relatively long stabilization periods and further improve the overall sensitivity. Nevertheless the results indicate that this device can be a valid alternative for the selective biosensing of xanthine or other purines.

2.1.4. References

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2.2. DETERMINATION OF PURINES USING Cu (I) OXIDE MODIFIED GLASSY CARBON ELECTRODE IN FISH

2.2.1. Materials and Methods

2.2.1.1. Materials and Reagents

Xanthine (≥99,5%), hypoxanthine (≥99%), uric acid (≥99%), inosine (≥ 99%) inosine 5' monophosphate (≥ 99%) were obtained from Sigma Aldrich (Steinham, Germany), sodium hydroxide (≥97%) and potassium hydroxide (≥90%) Sigma Aldrich, dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4), cupric sulphate (≥99,99%) and tri-sodium citrate (≥ 99%) were obtained from AnalaR Normapur (United Kingdom). Perchloric acid (70%) was obtained from Merck, Italy. All chemicals were of analytical grade and used without any further purification.

Stock standard solution of purines were prepared by dissolving 0.05g in 50 mL of buffer phosphate 0.1 M pH 7.0. pH measurements were carried out using an Orion STAR A2115 digital pH meter (Thermo Scientific, Waltham, MA, USA).

2.2.1.2. Electrochemical Apparatus

A CHI1010 Potentiostat (CH Instruments INC., USA was used for voltammetric measurements. A three electrode configuration consisting of a glassy carbon electrode (0.3 mm, BAS, IN, USA) as working electrode, a platinum counter electrode and a Ag/AgCl (KCl) as reference electrode was used. An Ultra-Turrax homogenizer (IKA® T25 digital) was used for the fish samples treatment.

All measurements were carried out at room temperature

2.2.1.3. Preparation of copper-GCE

The copper-GCE was obtained by electrochemical deposition of copper. Before the deposition, Glassy carbon electrodes (GCEs) were mirror polished with 0.5 and 0.05 µm alumina slurries and the residual polishing material was removed by ultrasonication in a water bath. Then, GCE was transferred into 0.1 M CuSO4 / 0.1 M Na3C6H5O7 as reducing agent and a potential of -0.2 V for 2500s was applied.

2.2.1.4. Preparation of water-soluble fraction extracts

2.5g of muscle was separated from seabream fish (*sparus aurata*), minced and homogenized by means an ultra-turrax homogenizer with 8 mL of perchloric acid (7%) for 3 min. The mixture was shaken for 20 min at 400 rpm and then centrifuged at 4° C for 10 min at 4000 rpm. Supernatants were recovered, filtered and titrated using potassium hydroxide 9M until a value of pH 7.5 and centrifuged at 4° C for 20 min at 6000 rpm. After filtration the extracts were maintained at − 20° C, the electrochemical analysis (CV) were carried out.

2.2.1.5. Chromatographic Apparatus

The HPLC system consisted of a Model 2080 plus PU pump and a UV-vis 2070 plus detector (Jasco, Japan). A Hypersil ODS Thermo Scientific (50 x 4.6 mm, diameter 5 µm) column was used for separation with buffer phosphate pH 7.6 and methanol 1% as mobile phase. The flow-rate was 1 mL/min. The injection volume was 5 µL. The detection wavelength was 254 nm. Integration of peak areas was performed with 134 Borwin v. 1.2. Software (Jmbs Developments, France). Retention time of purines

were: Inosine-5- monophosphate 2.57 min, xanthine 4.6 min, hypoxanthine 6.30 min, uric acid 2.93 min, inosine 17.68 min.

2.2.2. Results and discussion

2.2.2.1. Voltammetric behavior of purine derivatives

The voltammetric behavior of different purine derivatives was studied using glassy carbon electrode. Purine derivatives gave a single oxidation peak at a different potential, with a stationary glassy carbon electrode, at pH 7.0. The adsorption on the electrode was very dependent on the pH, and especially at pH values of the neutrality 7.0, the anodic peaks were ill-defined. Figure 2.4. showed the oxidation peak potential of xanthine (0.80V), hypoxanthine (1.11V), uric acid (0.32V), inosine (1.41V) and inosine 5' monophosphate IMP (1.42V) on glassy carbon electrode. The high overpotential and the low oxidation peaks current of these purines is a major drawback for their detection in complex biological fluids because a large number of antioxidants would influence the analytical result. Secondly, the transient product would become a dimer and adsorb on the electrode surface, and a problem of low reproducibility in electroanalysis of these purines need to be overcome.
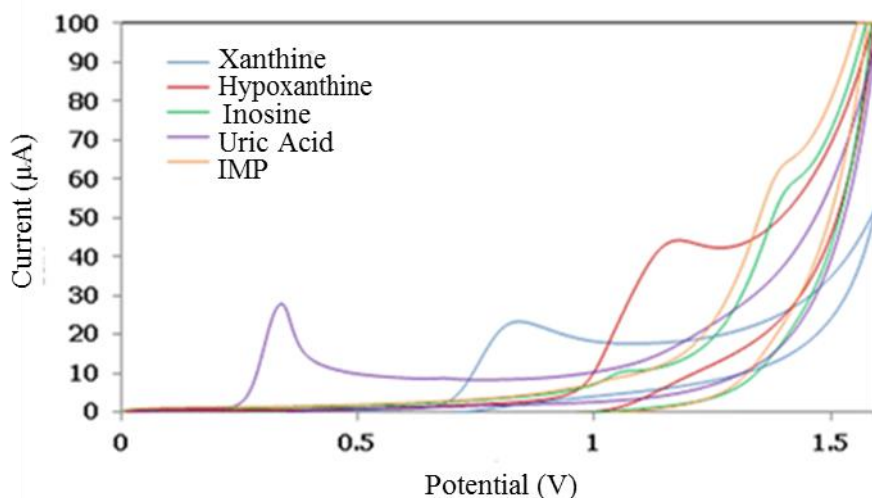
Figure 2.4. Cyclic *Voltammograms of 10 µg mL⁻¹ purines in phosphate buffer (pH 7.0) at GCE. Xanthine (0.80V) dashed/dotted line, Hypoxanthine (1.11V) marked line, Inosine (1.41V) dashed line, Uric acid (0.32V) dotted line and Inosine 5 monophosphate (1.42V) continuous line. Sweep from 0.0 to 1.5 V (Ag/AgCl); scan rate: 100mV s⁻¹*

A novel voltammetric scheme in the determination of purine derivatives by using a copper electrode was developed. This method measure a reductive current which is attributed to the electrochemical regeneration of the surface passive oxide layer when this oxide layer was dissolved by the analyte to form a cuprous complex. This special technique is based on the chelated property of the analyte rather than is redox behavior and provides to eliminate a surface fouling. In contrast to the inadequate operating potential of the glassy carbon electrode, copper-GC electrode shows a catalytic capability to these purine derivatives.

The immobilization of copper onto the surface of GC electrode reduced the peak potential, around −0.25V, which is believed to the self-redox of the copper electrode. After adding different concentration

of xanthines it is found that the intensivity of the reductive peak increases significantly.

This might be explained by assuming that the application of a potential at the copper glassy carbon electrode results in the production of a cuprous oxide on the surface. After addition of purine derivatives, this cuprous oxide would be converted into a cuprous-purine complex and dissolved in the buffer solution, therefore, an anodic current would be induced to regenerate the passive layer, and its intensity would be proportional to the concentration of purine compounds. The calibration curve of xanthine, hypoxanthine shrunk at lower concentration with respect to inosine, inosine 5' monophosphate. This might indicate that the adsorbabilities of the xanthine and hypoxanthine complex are higher than inosine and inosine 5' monophosphate complex as showed in figure 2.5.
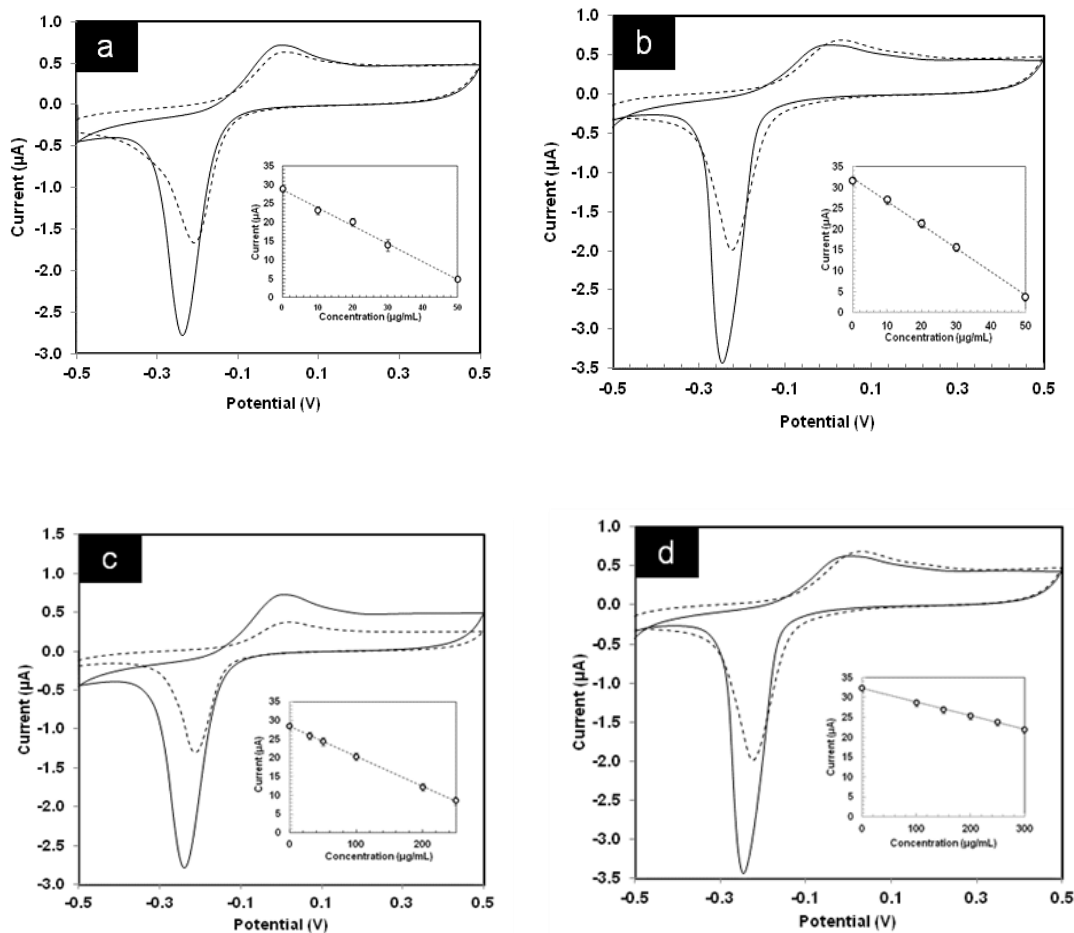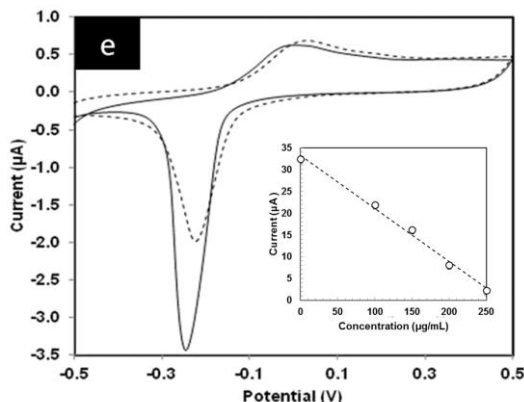
Figure 2.5. *Voltammetric behavior of purine derivatives on phosphate buffer pH 7.0 0.1M using Copper-GCE. (a) Main body: peak current of copper-GCE continuous line and peak current of Hypoxanthine 30 μg mL^{-1} dotted line. Insert: Calibration curve of Hypoxanthine (10-50 μg mL^{-1}). (b) Main body: peak current of copper-GC electrode continuous line and peak current of Xanthine 30 μg mL^{-1} dotted line. Calibration curve of Xanthine (10-50 μg mL^{-1}). (c) Main body: peak current of copper-GC electrode continuous line and peak current of Inosine 250 μg mL^{-1} dotted line. Insert: Calibration curve of Inosine (30-250 μg mL^{-1}). (d) Main body: peak current of copper-GCE continuous line and peak current of Inosine 5'monophosphate 300 μg mL^{-1} dotted line. Insert: Calibration curve of Inosine 5'monophosphate (100-300 μg mL^{-1}). (e) Main body: peak current of copper-GCE*

2.2.2.2. Kinetics of purines-complex

The different voltammetric behavior of purine derivatives was evaluated using Copper-GC electrode and it is showed in figure 2.6. From the results obtained it was found that the formation of cuprous-purines complex varies significantly in time adding fixed concentration. The intensity of the reductive peak current of xanthine increase significantly after 30 seconds and hypoxanthine increases significantly after 1 minute and uric acid after 2 minutes. Inosine and inosine 5' monophosphate (IMP) have very similar behavior due to the formation of complex after adding high concentration and waiting periods of at least 3 minutes for inosine and 4 minutes for inosine 5' monophosphate (IMP).
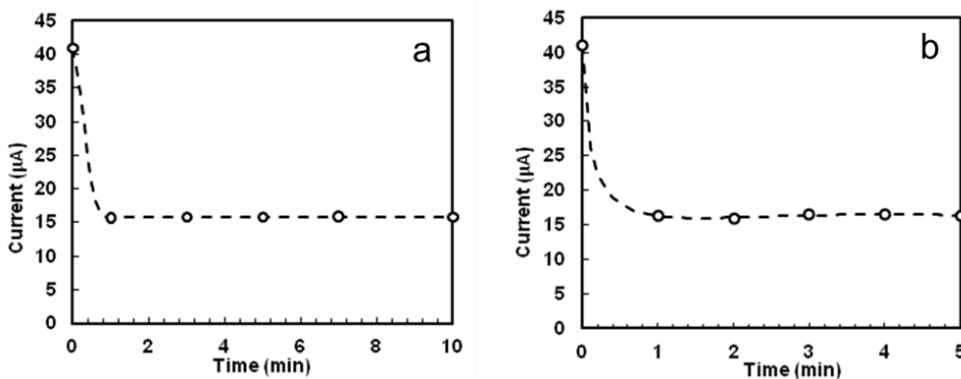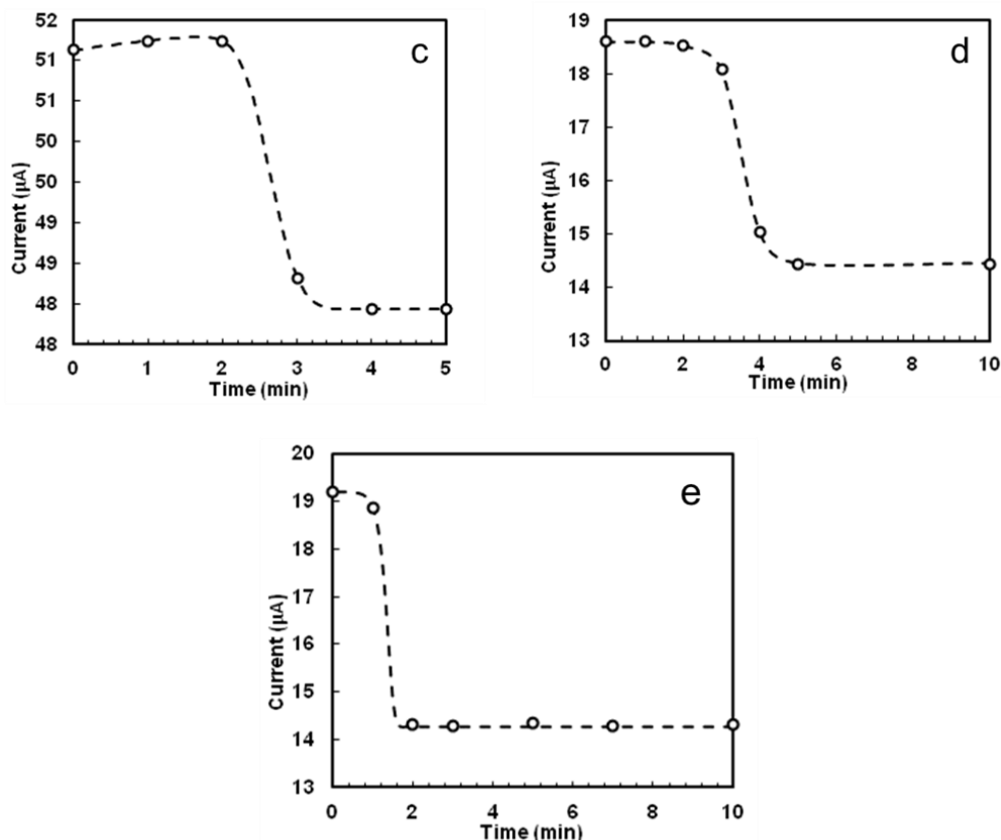
Figure 2.6. *Time dependent response of purine derivatives in cyclic voltammetry. (a) xanthine-complex formation after 30 seconds of 30 μg mL^{-1} fixed concentration. (b) hypoxanthine-complex formation after 1 minute of 30 μg mL^{-1} fixed concentration. (c) inosine-complex formation after 3 minutes of 200 μg mL^{-1} fixed concentration. (d) inosine 5'monophosphate-complex formation after 4 minutes of 300 μg mL^{-1} fixed concentration. (e) uric acid-complex formation after 2 min of 150 μg mL^{-1} fixed concentration.*

This behavior is due to the fact that copper form with purines a large number of complex at variable stoichiometry. It is clear that everything depends on the structure and stability of the various complexes. Therefore the times are dependent on the stability and the type of complex copper form with the various purines. To occur a chemical reaction it is necessary that the reagents have a minimum level of energy defined activation energy. If two states, a transition state and an unstable intermediate, occur consecutively during a reaction process and have nearly the same energy content, their interconversion will involve only a small reorganization of the molecular structures. Thus, if a bond which is being broken during the process is nearly covalent in the first state it will be stretched by an arbitrarily small amount in going to the second. If a bond is being made from two particles which are kinetically free in the first state there will be only a loose association between the two in the second. Furthermore, other changes in molecular geometry, such variations in bond angles, will also be limited to small changes. In these conditions, the bonds originating characterizing the molecules of the reagents have a cleavage with formation of new bonds to form an intermediate and metastable compound, characterized by high potential energy, and defined "activated complex". Therefore, new weak chemical bonds formed have a rearrangement to form the final reaction products. The equilibrium of a reaction is governed by redox processes between the oxidation stages of the metal complex. These are important parameters that control the kinetics of reaction. The electrochemical process behaves like a slow electron transfer,

involving a deep internal reorganization of the structure of the complex. In particular the higher the Cu (I) complex reducing power the higher the complexion velocity.

The reducing power increases with the number of N atoms of the ligand as a function of the carbonyl group present in the molecules. During the formation of xanthines-complex the ring structure involving C-6 and N-7 is essential for complexing with cupric ion. In particular C-6 lost keto group character and no hydrogen ion was released after complexing, therefore, the most probable mechanism is proposed and shown in Scheme I:
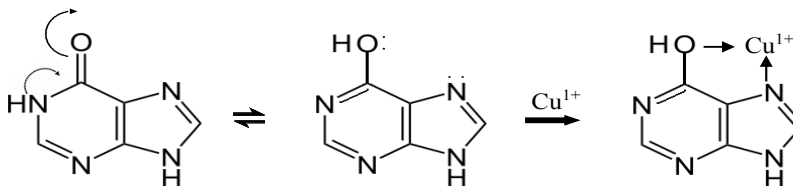
2.2.2.3. Analytical application and analytical figures of merit

For the characterization of the method were evaluated the coefficient of determination (R^2), the sensitivity, repeatability (RSD %) and the limit of detection (LOD) were calculated according Miller and Miller 1993.

Figure 2 shows a calibration curve relative to the standard of Hypoxanthine (a) with the equation: $y = -0.4776 x + 28.66$. The value of R^2 is 0.9947 which indicates the existence of a positive correlation with the concentration range. The sensor saturation occurred after 100 μg mL^{-1}. The sensitivity, given by the slope of the line is -0.4776. The RSD is 4 %. The detection limit was found to be 7.3 μg mL^{-1}.

A calibration curve relative to the standard of Xanthine (b) has equation: $y = -0.563 x + 32.234$. The value of R^2 is 0.9980 which indicates the existence of a positive correlation with the concentration range. The sensor saturation occurred after 100 μg mL^{-1}. The RSD and the detection limit (LOD) were found to be 2.6 % and 4.3 μg mL^{-1} respectively.

A linear relationship between the measured current of IMP and Inosine concentrations range were found between $100 - 300$ μg mL^{-1} and $25 - 250$ μg mL^{-1} respectively with a slope values (- 0.0251 ± 0.02) μA/mL μg^{-1} and (-0.4776 ± 0.03) μA/mL μg^{-1}, an intercept values included (32.776 ± 10) and (28.351 ± 12) μA. The RSD and the detection limit (LOD) of IMP, 1.5% and 60 μg mL^{-1} and for Inosine, 2.1% and 22 μg mL^{-1} respectively, were calculated according to Miller and Miller 1993.

2.2.2.4. Analytical determination of purines in real samples

The method developed was applied to real matrices of sea bream. The samples were store at 4°C and divided into groups and prepared immediately (24h) or after 4 and 8 days for the determination of xanthines (IMP, inosine, hypoxanthine, xanthine and uric acid). All the results obtained by the inquiry of the samples of gilthead bream with electrochemical method have been compared by the reference method through HPLC chromatography. Particularly, there have been at first compared the values of Ki determined with both methods, as showed in the table 1.

Table 1. *Comparison of Ki values between electrochemical method and HPLC – UV reference method*

Sample	Electrochemical Method	HPLC-UV Method
	K value (%)	K value (%)
sample 24h	5.49	5.19
sample 4 days	25.31	26.19
sample 8 days	40.2	40.6

It was considered a freshness indicator used as muscular content of ATP in a relationship to the total quantity of ATP. The K, K_i values were calculated according to Saito et al. (1959), Karube et al. (1984), Burns et al. (1985) and Luong et al. (1992), respectively.

K (%) = (HxR + Hx) / (ATP + ADP + AMP + IMP + HxR + Hx) * 100

K_i (%) = (HxR + Hx) / (IMP + HxR + Hx) * 100

K_i < 20 %   it is a fresh product

20 % < K_i < 40 %   it is advised to consume the product after cooking

K_i > 40 %   the product is not advised for the human alimentation

where: ATP, adenosine 5 -triphosphate; ADP, adenosine 5 -diphosphate; AMP, adenosine 5 - monophosphate; IMP, inosine 50 -monophosphate; Ino, inosine; Hx, hypoxanthine.
ATP degraded to IMP very soon after death and, consequently, a Ki value may be used which does not involve determination of ATP, ADP or AMP. Where the Ki value is used, with some species it increases very rapidly and then remains constant. This is due to accumulation of a large quantity of Ino relative to the quantity of Hx produced. The Ki value of the fish dead after 24h is 5.19%, indicating that the product can be considered as fresh. The Ki values of the fish dead after 4 days was found to be 25.31% suggesting these products to be consumed only after cooking, on the contrary, the Ki values of the fish dead after 8 days was found to be 40.2% it means that the product cannot used for the human alimentation..
The Ki values obtained from the electrochemical measurement are in good agreement with results obtained by HPLC. In a similar way purine concentrations determined by cyclic voltammetry agree very well with the concentrations obtained using the reference method, as showed in the table 2.

Table 2. *Determination of purine derivatives content ($mg\ g^{-1}$)in fish meat using Cu (I) oxide-GC electrode and a reference methodology (HPLC-UV)*

Purines	Electrochemical Method			HPLC- UV Method		
	Fresh fish	Stored fish (4 days)	Stored fish (8 days)	Fresh fish	Stored fish (4 days)	Stored fish (8 days)
Inosine 5MP	3.09 ± 0.03	1.59 ± 0.04	0.81± 0.03	3.10 ± 0.02	1.62 ± 0.04	0.75± 0.07
Uric acid	0.018±0.005	0.052 ± 0.004	0.11± 0.03	0.016 ±0.02	0.048 ± 0.011	0.08 ± 0.02
Xanthine	0.06 ± 0.01	0.20 ± 0.01	0.26± 0.02	0.05±0.01	0.16 ± 0.03	0.18 ± 0.02
Hypoxanthine	0.04 ± 0.01	0.18 ± 0.02	0.39± 0.02	*nd	0.16 ± 0.01	0.40 ± 0.02
Inosine	0.21 ± 0.05	0.22 ± 0.06	0.12± 0.02	0.18 ± 0.05	0.24± 0.03	0.10 ± 0.03

*nd: not detectable

A paired-t test applied to the detachable data has not shown significant differences between two methods at the level of probability of 95%, therefore it is possible to declare that the electrochemical method can be used by the routine analysis of the matrixes.

In order to evaluate the effect of potential interferences, the response of copper-glassy carbon electrodes to standard solutions of different biological compounds (urea, creatine, ascorbic acid, glucose and lactic acid) that can be naturally found in fish meat, was analyzed. Here, the advantage of the low operating potential is demonstrated in the interference study. At -0.25V peak potential, no complex was formed after the addition of increasing concentration of biological compounds.

These conventional biological compounds shows no-significant increase of reductive peak current.

2.2.3. References

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SAFETY ASSESSMENT OF FOOD SYSTEMS

Safety Assessment of food systems

Food safety is a scientific discipline describing handling, preparation, and storage of food in ways that prevent foodborne illness. This includes a number of routines that should be followed to avoid potentially severe health hazards. In this way Food Safety often overlaps with Food Defense to prevent harm to consumers. The tracks within this line of thought are safety between industry and the market and then between the market and the consumer. In considering industry to market practices, food safety considerations include the origins of food including the practices relating to food labeling, food hygiene, food additives and pesticide residues, as well as policies on biotechnology and food and guidelines for the management of governmental import and export inspection and certification systems for foods. In considering market to consumer practices, the usual thought is that food ought to be safe in the market and the concern is safe delivery and preparation of the food for the consumer.

We all consume many thousands of different chemicals in our food every day. Most of these chemicals are natural constituents of the food we eat. Some are present as a result of contamination from the environment, some arise during production processing and preparation, and some are intentionally added to food. All chemicals have one characteristic in common: the potential to cause toxicological harm to consumers. The purpose of risk analysis is to identify those chemicals in food which might cause harm, to analyze the potential consequences, to consider any possible benefits and to decide on any action necessary to protect consumers, whilst not unnecessarily impending trade.

Until recently, in all but a few countries, information about the presence, effects and likely exposures of consumers to chemicals in food was scarce. Little reliable scientific information was available, so food chemical standards tended to be based on what industry was prepared to bear and on the absence of any obvious cases of food poisoning from chemicals. Some authorities would allow no added chemicals in food at all and set their limits for contaminates at zero.

In Europe, food businesses are legally responsible for the safety of the food they produce, transport, store or sell. They are required to adopt a preventative approach by identifying and controlling hazards before they compromise the safety of food. Hazard Analysis and Critical Control Points (HACCP) is a system used by food businesses to ensure the safety of food. It was originally pioneered in the 1960s for the production of safe foods for the United States space program. It is a preventative, risk-based system which enables food businesses to identify critical control points (CCP) for physical (e.g. glass), chemical (e.g. pesticides) and/or microbiological (e.g. food poisoning bacteria) hazards before they compromise the safety of food. By law, all food businesses in Europe must implement and maintain procedures based on HACCP principles.

Before implementing HACCP, good hygiene practices (GHP) must be in place. These are known as prerequisite programs and are mostly specified in corresponding legislation.3 Examples include personnel hygiene and training; cleaning and sanitation; maintenance and services; pest control; plant and equipment; premises and structure; storage, distribution and transport and waste management. More extensive hygiene requirements apply for primary producers (e.g. farmers).

Although European legislation lays down minimum requirements regarding HACCP and GHP, it does not describe how these requirements should be implemented by the food industry. Standards often fill this gap by providing the necessary procedural information for industry.

Now much more information is available and we are facing a revolution in the traditional approaches to food safety with the importation of risk analysis techniques from other disciplines, particularly engineering. The aim of introducing such techniques is to adopt a more scientific approach to food safety wich will, in turn, result in more relevance, accuracy, reproducibility and transparency. Such improvements will bring benefits to both food consumers and food producers by ensuring safety whilst facilitating trade. The convergence of new technologies, including nanotechnology, biotechnology, and information technology, has opened new horizons in electrochemical biosensors. The integration of micro- and nanostructured materials within biosensing devices (graphite microparticles, bioreceptors, gold nanoparticles, and magnetic micro- or nanoparticles) has provided excellent analytical performances in the detection of food residues (pesticides, antibiotics), food additives (folic acid) and allergens (gliadin), and food-borne pathogens. One of the key contributions in the electrochemical

biosensing field relies on the design of novel transducers, not only with enhanced transducing features but also with improved immobilization of biomolecules while preserving their biological activity. The integration of magnetic particles provides further improvements in the detection of food residues in a complex matrix, achieving better electrochemical response due to the increased size of the transducing active area. Electrochemical immunosensors and genosensors systems recently developed are described in this contribution. These sensitive, hand-held, user-friendly devices provide new analytical approaches for the in-field detection of food contaminants, mainly food residues and pathogens. With food allergies on the increase in Western societies, and new legislation on allergen labeling in both Europe and the USA, the food industry is confronted with the issue of providing safe foods for the food-allergic consumer. While most food-allergic reactions occur after ingestion of non-packaged food products, the food industry has been subjected to increasing scrutiny of its allergen controls; the resulting impact on the industry has been remarkable.

In the past 15 years, the food industry has made significant investment, effort, and improvements in allergen control. In the past eight years, tests for some allergenic foods have been commercialized and have proven useful to the industry in controlling allergens, and also to regulatory agencies investigating food-allergic consumer complaints. There are many strategies food manufacturers can exercise in controlling allergens in their plants, from changing raw materials to improving cleaning procedures and using precautionary labeling indicating allergens that might be present. However, measuring the content of particular allergenic residues on processing equipment, in ingredients or in final products, provides information that can also be used for risk assessment, enabling the food industry to provide the food-allergic consumer with practical information. For individuals with food allergies and related illnesses, consuming certain foods can be a debilitating, and possibly even life-threatening, experience. Consequently, the joy of eating is diminished by the ever-present fear of consuming a food or food ingredient that will cause an adverse reaction. Food-allergic consumers must assiduously avoid the offending foods and/or food ingredients because strict avoidance diets are the only available preventive strategy. For such consumers, food selection often becomes a tedious task requiring meticulous reading of ingredient lists on labels, dependence on food manufacturers to maintain accurate labels, and a continual search for more knowledge about food composition. For these individuals, food preparation requires careful attention to detail, cooking 'from scratch', and seeking alternative recipes for many dishes. Because very small amounts of the offending food can elicit allergic reactions in some affected individuals, these consumers live in constant fear that, despite their caution, trace amounts of the offending food, sufficient to elicit an adverse reaction, might still exist in the foods that they consume. They are concerned about ingredients derived from the offending food because such ingredients might contain residual allergenic proteins from the source food. This fear is compounded by the fact that declaration of the source of ingredients used in foods is not always required on food labels.

3. NANOTECHNOLOGY IN BEVERAGE INDUSTRIES TO DETECT CONTAMINANTS

3.1. AMPEROMETRIC DETECTION OF BISPHENOL A BY MULTI-WALLED CARBON NANOTUBES AND HYDROXYL GROUPS MODIFIED GLASSY CARBON ELECTRODE.

3.1.1 Introduction

Bisphenol A (BPA) also referred to as [2, 2-bis(4-hydroxyphenyl)] propane is a synthetic compound widely used in the polymer industry, in particular as a monomer for producing polycarbonate plastics and epoxy resins. Indeed, this material can be commonly found in clear shatterproof plastics used in small kitchen accessories, metal food containers, baby bottles, rigid water bottles, in the lining of beverage cans and in food packages in general.

BPA has been proved to be an endocrine disruptor chemical and, as such, to be implicated in a wide number of illnesses, ranging from infertility, obesity, breast and prostate cancer to diabetes, thyroid malfunction, and even the attention deficit syndrome. The effect of BPA in the occurrence of these diseases has been observed even at very low exposures.

BPA has been detected in freshwater, seawater, landfill, sludges, air and dust particles. Moreover, it has been shown that it can migrate from packaging into a wide range of food matrices, from water storage tanks to drinking water.

Currently, there are no regulatory restrictions on the amount of BPA in most final plastic products, but the tendency of BPA to migrate in the food from the contact materials and its negative impact especially on children and infants and has been largely acknowledged in the European Union food law. In January 2011, the use of BPA in baby bottles was forbidden in all EU countries and the Canadian and Chinese government are considering similar restrictions.

In January 2015, the European Food Safety Authority (EFSA) indicated a lower tolerable daily intake (TDI) for BPA based on the results of specific health studies. The TDI has been set from 0.05 a 0.004 mg kg^{-1} body weight/day.

Therefore, the development of new and rapid methods with cheap instrument and real time detection for the determination of BPA has become one an attractive subjects of research in analytical chemistry because of the practical applications.

Various analytical methods, have been employed such as high performance liquid chromatography coupled to UV, fluorescence and electrochemical detection, gas chromatography, liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry. These methods can offer accurate and precise results, but they often require skilled operators and expensive equipment. A number of other methods, such as chemiluminescence immunoassay, enzyme-linked immunosorbent assay, and electrochemical methods, have been developed. Among them, the latter have attracted wide attention because they usually offer fast response, portability, simplicity for operators, low cost, high sensitivity and the possibility to perform on-site measurement.

The BPA electrochemical activity is due to the presence of phenolic hydroxyl groups that can be oxidized. It has been studied at different electrode materials, including carbon and metals. However, the relatively high potential required for the oxidation of phenolic compounds results in an increase of the background current and hence in a decreased sensitivity.

Different approaches have been proposed to minimize this drawback, including the use of enzymes and other catalysts like cobalt phtalocyanine and of the modification of the electrode surface. Some chemically modified electrodes, for example, decrease the redox potentials of BPA and weakly increase the selectivity of the determination. Despite the good results, it is still a challenge developing new electrochemical sensors based on common nanomaterials using a simple treatment. In particular, carbon nanotubes (CNTs) have attracted a large interest in BPA sensing, after being activated by grafting specific chemical functionalities at their surface, since they allowed improving the electrochemical reactivity of electrodes, promoting the electron-transfer reactions, minimizing surface fouling and modulating their responsiveness towards certain analytes (e.g., BPA) under certain conditions.

In this work, multi-walled carbon nanotubes (MWCNTs) modified glassy carbon electrode (MWCNTs/GCE) have been used to investigate the electroxidation of BPA. For this purpose MWCNTs were chemically modified by three simple approaches: by acid activation, either with or without activation with cobalt phthalocyanine functionalization (MWCNTs-COOH/CoPc and MWCNTs-COOH, respectively), and by basic activation (MWCNTs-OH). Based on the literature, MWCNTs treated with acid contains carboxyl groups with a small part of hydroxyl groups and in comparison MWCNTs-OH contains more hydroxyl groups. The optimal working conditions and pH effect on the overall sensors performance were investigated by cyclic voltammetry. A comparison with unmodified GCE was also considered. Among the modified GCEs, the MWCNTs-OH/GCE showed the best performances and was used for BPA measurement. To date, examples concerning hydroxyl and MWCNTs are very limited and moreover, there are no previous reports on it application regarding BPA analysis.

Sensitivity, stability, linear range, limit of detection and quantification of MWCNTs-OH/GCE were evaluated in flow injection analyses (FIA).

Finally, this method was applied for BPA quantification in real samples, i.e., in water from baby bottles and mineral water bottles. The results were compared with those obtained by an independent chromatographic method.

3.1.2. Materials and methods

3.1.2.1. Reagents and solutions

BPA (>99% purity), multi-walled carbon nanotubes (MWCNTs) (purity 95%, 0.7–1.2 nm diameter, 2–20 μm length), cobalt phtalocyanine (CoPc) (~97%), sodium hydroxide (50%), nitric acid (69%), sulphuric acid (96%), methanol (99%), ethanol (99%) and acetonitrile (≥99.9%) were purchased from Sigma-Aldrich (Steinham, Germany). All chemicals were of analytical grade and used without any further purification. Buffer solutions (0.1 M) were: sodium acetate (pH 4.5), sodium phosphate (pH 6.5) and sodium glycine (pH 8.0 and 10.0). The BPA stock solution (500 μg mL^{-1}) in ethanol was kept at 4°C in the darkness. Working solutions were freshly prepared before use by properly diluting the stock solution in the different buffer solutions. All the solutions were prepared with Millipore Milli-Q ultrapure (>18 MΩ·cm^{-1}) water.

3.1.2.2. Electrochemical Apparatus

Cyclic voltammetric experiments were performed with a multi-potentiostat CHI 1010 (CH Instruments Inc., USA). The three electrodes cell consisted of a modified or bare glassy carbon disk (GC) working electrode (0.3 mm, BAS, IN, USA), an Ag/AgCl (KCl 3 M) reference electrode and a Pt wire counter electrode.

The apparatus for amperometric flow injection analysis already described elsewhere, consisted in a carrier solution reservoir, a peristaltic pump (Ismatec, Switzerland), a portable Potentiostat (DS-Drop Sens μSTAT 100) and an injection valve with a 10 μL loop.

pH measurements were carried out using an Orion STAR A2115 digital pH meter (Thermo Scientific, Waltham, MA, USA). All measurements were carried out at room temperature.

For BPA analyses in samples, solid phase extraction (SPE) cartridges (Strata-X × 33 μm polymeric reverse phase sorbent, 200 mg/6 mL) from Phenomenex (Torrance, CA, USA) were used.

3.1.2.3. Preparation of the modified electrodes

Glassy carbon electrodes (GCEs) were mirror polished with 0.5 and 0.05 µm alumina slurries. The residual polishing material was removed by ultrasonication in a water bath. The modification was achieved by dropping 10 µL of the chosen functionalized MWCNTs suspension onto the surface of the GCE. After solvent evaporation at room temperature, the electrode was rinsed with deionized water and stored in a refrigerator at 4°C.

3.1.2.4. Preparation of MWCNTs

It is known that MWCNTs have a hydrophobic surface, which is prone to aggregation and precipitation in water in the absence of a surfactant. Up to now, many efforts have been made to prepare water-soluble MWCNTs and numerous procedures for their chemical functionalization have been proposed. The procedures chosen in this work were the following:

First procedure: MWCNTs were treated by an acid solution previously described. Briefly, 100 mg of MWCNTs were dispersed in 100 mL of sulfuric acid/nitric acid 3/1 v/v and then sonicated for 2 hours. According to previous results, by this treatment, acidic are produced on the nanotubes surface. MWCNTs obtained with this step are herein referred to as MWCNTs-COOH.

Second procedure: MWCNTs-COOH obtained with the first method were further treated with cobalt phthalocyanine (CoPc) by mixing a slurry of CoPc (4 mg) in 1 mL of ultra-pure water with a 1 mg mL-1 slurry of MWCNTs-COOH, followed by ultrasonication for 30 min. MWCNTs obtained with this procedure are herein referred to as MWCNTs-COOH/CoPc.

Third procedure: 100 mg of MWCNTs were added to 100 ml of potassium hydroxide (25% by weight) and ultrasonicated for two hours. Then, the solution was filtered and the filtrate washed with redistilled water until neutralization. According to literature results this treatment introduces hydroxyl groups onto the MWCNTs surface. MWCNTs obtained with this procedure are herein referred to as MWCNTs-OH. After each treatment, MWCNTs were separated from the solution by filtration and washed with redistilled water up to neutral pH, then dried overnight in an oven at 70°C.

3.1.2.5. Samples preparation and BPA extraction

Two different brands of baby bottles (one of which including the claim "BPA-free") and two brand of PET mineral water bottles were purchased from a local supermarket.

Water from the bottles was analyzed after heating at 50°C for 20 min in a water bath for favoring the migration of BPA from the plastics and shaking vigorously for 30 min. This treatment has been made using mild conditions, like in real life. In all cases, 100 mL aliquots of the water samples were treated by solid phase extraction (SPE) using Phenomenex protocol. To this aim, the cartridge was conditioned with 4 mL of methanol followed by 4 mL of ultrapure water. After loading 100 mL of the sample, at a flow-rate of ~1 mLmin^{-1}, under vacuum, the elution of BPA was obtained using 4 mL of methanol. The extract was evaporated to dryness under a gentle nitrogen stream and reconstituted with 1 ml of ultrapure water (concentrate 100-fold). The SPE procedure was initially evaluated using water ultrapure as blank to verify the absence of BPA in the final eluate. For the recovery test, 10 and 100 ng mL^{-1} amounts of BPA were added to mineral water.

3.1.2.6. Chromatographic method

The HPLC system consisted of a model 2080 plus PU pump and a UV-vis 2070 plus detector (Jasco, Japan). A Kinetex, C18 Phenomenex (250 x 4.6 mm, diameter 3µm) column was used. The injection volume and the detection wavelength were 5 µL and 228 nm, respectively. For the separation, a mobile phase consisting of water and acetonitrile (65:35 v/v) in isocratic at a flow rate of 0.8 mLmin^{-1} was used. Retention time of BPA was 6 min. Integration of peak areas was performed with Borwin v. 1.2 software (Jmbs Developments, France).

3.1.3. Results and discussion

3.1.3.1. Electrochemical behavior of BPA at the modified GC electrodes.

As known, MWCNTs can largely increase sensitivity and reduce the overpotential in the electrochemical detection of many species, BPA included. Moreover, it was also reported that MWCNTs modification by proper functional groups can modulate the electrostatic interactions with target molecules.

Preliminary cyclic voltammetric experiments were performed to study the BPA redox behavior at the three MWCNTs modified GCEs. The corresponding voltammograms were compared with that recorded at the unmodified GCE.

Figure 1 shows the cyclic voltammograms of 0.4 µg mL^{-1} BPA, recorded in 0.1 M sodium glycine buffer (pH 8) at the scan rate 100 mV s^{-1}. The oxidation system was characterized by an anodic peak in the positive-going step and by the absence of any cathodic peak on the reverse scan, indicating that the oxidation of BPA is irreversible in accordance with other reports
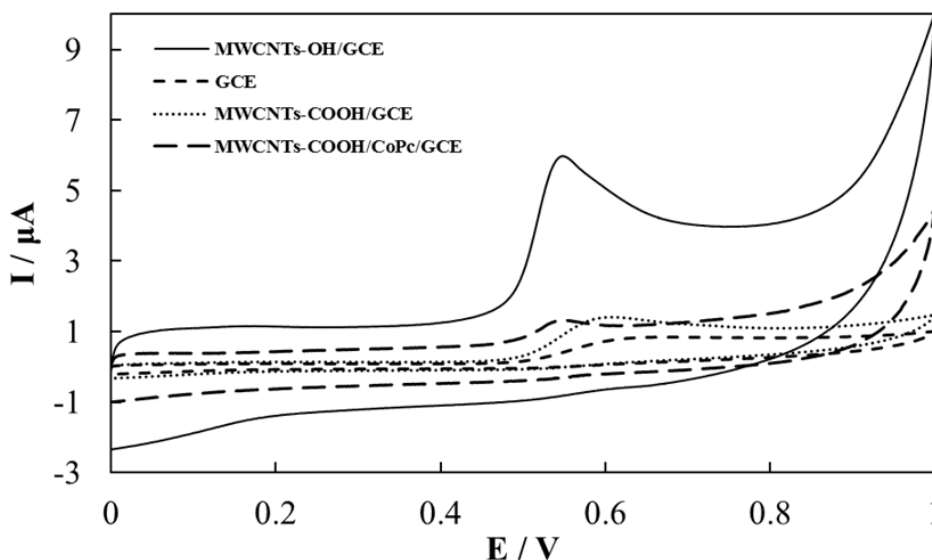
Figure 1. *Cyclic voltammograms of 0.4 µg mL^{-1} BPA at different electrodes: GCE, MWCNTs-COOH/GCE, MWCNTs-COOH/CoPc/GCE and MWCNTs-OH/GCE in 0.1 M sodium glycine buffer (pH 8); sweep from 0.0 to 1.0 V (Ag/AgCl); scan rate: 100 mV s^{-1}.*

As it can be seen from Figure 1, the best sensitivity was shown by the MWCNTs-OH/GCE, whose peak current (*Ip*) was about fifteen times higher than that recorded at the unmodified GCE. The peak potential (*Ep*) shifted from 0.64 V at the GCE to 0.55 V (Ag/AgCl), demonstrating the efficient catalytic ability of the activated MWCNTs-OH/GCE towards BPA oxidation and the promotion of the electron transfer. A similar behavior, with a lower sensitivity, even if still higher than at the GCE, was observed at the MWCNTs-COOH/GCE and MWCNTs-COOH/CoPc/GCE. For these two modified GCEs, the oxidation current of BPA resulted slightly higher than that at GCE, (accompanied by a decrease in *Ep*). Therefore, these effects were slightly larger at MWCNTs-COOH/CoPc/GCE, still indicating a synergetic activity produced by the use of MWCNTs functionalized with CoPc.

Considering that the mechanism governing the redox reaction of BPA is mediated by H+ ions the pH of the supporting electrolyte should have a great influence. Based on this information, experiments were carried out to evaluate the performances of all electrodes in different 0.1 M buffer solutions (sodium

acetate, sodium phosphate, sodium glycine) in the 4.5-10 pH range. The variations of *Ip* and *Ep* on changing the pH, are shown in Table 1.

Table 1. *Effect of pH value on the peak oxidation potential (Ep) and peak current (Ip) of BPA (0.4 µg mL^{-1}) at GCE, MWCNTs-COOH/GCE, MWCNTs-COOH/CoPc/GCE and MWCNTs-OH/GCE in different buffer electrolytes as reported in the text; scan rate: 100 mV s^{-1}.*

Electrodes	Electrolytes							
	Acetate pH 4.5		Phosphate pH 6.5		Glycine pH 8		Glycine pH 10	
	E_p	I_p	E_p	I_p	E_p	I_p	E_p	I_p
GCE	0.80	0.35	0.69	0.45	0.64	0.39	0.54	0.31
MWCNT-COOH/GCE	0.76	0.84	0.68	1.30	0.61	1.10	0.51	0.43
MWCNT-COOH/CoPc/GCE	0.73	0.99	0.64	1.53	0.56	1.35	0.48	0.65
MWCNT-OH/GCE	0.76	3.13	0.64	3.23	0.55	5.88	0.44	2.34

Interestingly, the MWCNTs-COOH/GCE shows the maximum peak current value at pH 6.5. As already reported, at higher pH values, the residual carboxylic groups on the surface of carbon nanotubes are deprotonated to anions, thus resulting in mutual repulsions with the BPA phenolic oxide anions. The results in decreased peak currents. Differently, the MWCNT-OH/GCE reached the maximum current at pH 8.0, lower than the pKa of BPA (pKa = 9.73) which suggested that the non-dissociated BPA could be adsorbed on the electrode surface better than the dissociated one. The current increase can be explained considering that hydroxyl groups of the MWCNTs do not change so much the charge of the surface, but they affect the absorption features of the electrode. These groups can interact with phenolic BPA, favoring the electron transfer rate and consequently the electrocatalytic property to the electrode. For what concern the peak potential (*Ep*) of all the electrodes (Table 1), it can be observed, that it shifted to lower values by increasing the pH. As already underlined, this could be explained by the fact that at lower pH, there is an excess of H+ from the supporting electrolyte, which tends to compete with those of BPA. As a consequence, BPA oxidation requires a driving force, shifting the peak potential at higher values. In the case of the electrode modified by MWCNT-OH/GCE, the linear relationship between Ep and pH was Ep = -0.059 pH + 1.0433 (r=0.9985). The slope of 0.059 V pH^{-1}, as showed in figure 2, is practically the same of the theoretical one, indicating that the numbers of protons and electrons involved in the oxidation mechanism is the same.
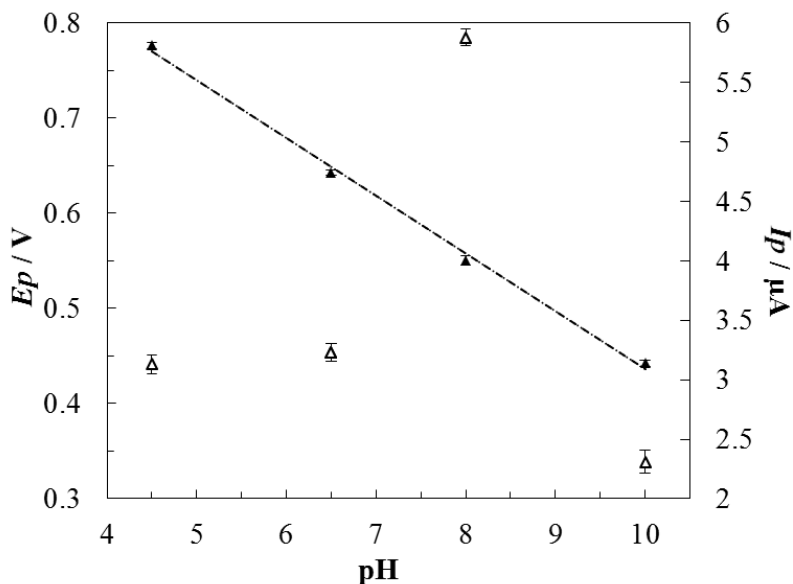
Figure 2. *Dependence of pH on the peak current Ip (Δ) and peak potential Ep (▲) for 0.4 μg mL BPA at MWCNTs-OH/GCE in 0.1 M supporting electrolyte under different pHs: 4.5 (sodium acetate), 6.5 (sodium phosphate), 8 and 10 (sodium glycine); scan rate: 100 mV s⁻¹.*

For this electrode, effect of scan rate (v) on the oxidation of BPA, was also investigated. Figure 3 shows the cyclic voltammograms of 1 μg mL^{-1} BPA with different scan rates. It can be seen that with the increase of the scan rate, the oxidation peak current (Ip) increase gradually. As shown in the insert of Figure 3, a linear relationship between Ip and v (in the 20 – 200 m V·s^{-1} range, r^2 = 0.999, n = 12) was obtained, demonstrating that the electrode reaction was a typical absortion-controlled process, which is favorable for quantitative applications. Although the peak current increased with the increase of scan rate above 150 mV·s^{-1}, the background current was enhanced too, hence in this work, the scan rate was chosen as 100 mV·s^{-1}. Hence in this work, the scan rate was chosen as 100 mV·s^{-1}.
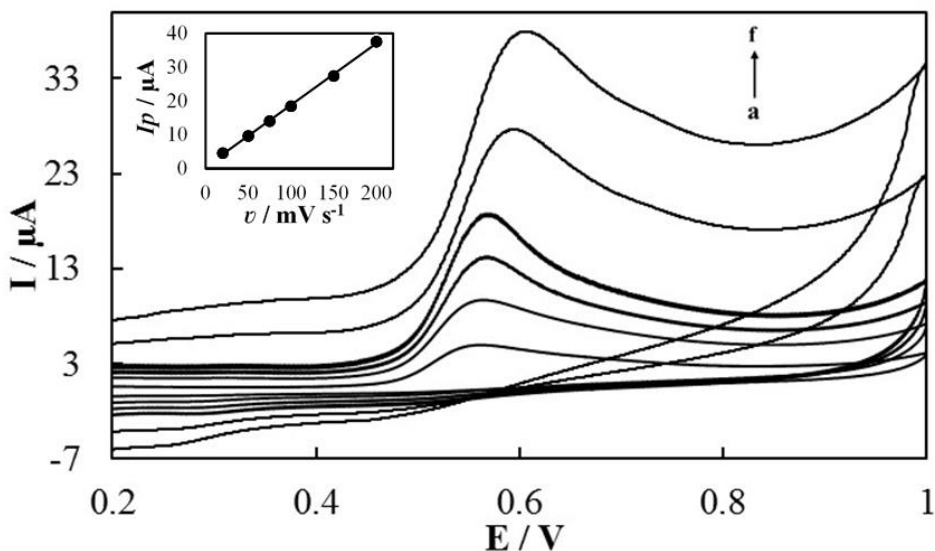
Figure 3. *Cyclic voltammograms of 1.0 μg mL^{-1} BPA in sodium glycine (pH 8) at different scan rates (v). Curve a-f is obtained at 20, 50, 75, 100, 150 and 200 mV s^{-1}, respectively. Insert: relationship between the peak current (Ip) and the scan rate (v).*

3.1.3.2. Flow injection quantitative analysis of BPA standard solutions

Considering that the best sensitivity was achieved by the MWCNTs-OH/GC electrode in sodium glycine buffer solution at pH 8.0, this electrode/supporting electrolyte combination was chosen for the quantification of BPA. Since amperometry has a much higher current sensitivity than voltammetry, it was used for this determination. Based on the data obtained from the cyclic voltammetric experiments, a potential value of + 0.50 V (vs Ag/AgCl) was chosen for the flow injection amperometric (FIA) experiments. The best results were obtained by using a sample loop of 10 μL at a flow rate of 0.5 ml min^{-1} which proved to be the best compromise between sensitivity, stability and time of analysis. As shown in Figure 4, the current response (peak height) relevant to BPA oxidation increases linearly with the analyte concentration within in the 0.1 - 2.4 μg mL^{-1} concentration range (R^2=0.9998, n=25) according to the equation: Y = (0.3859 ± 0.0011) X + (0.0016 ± 0.0067)
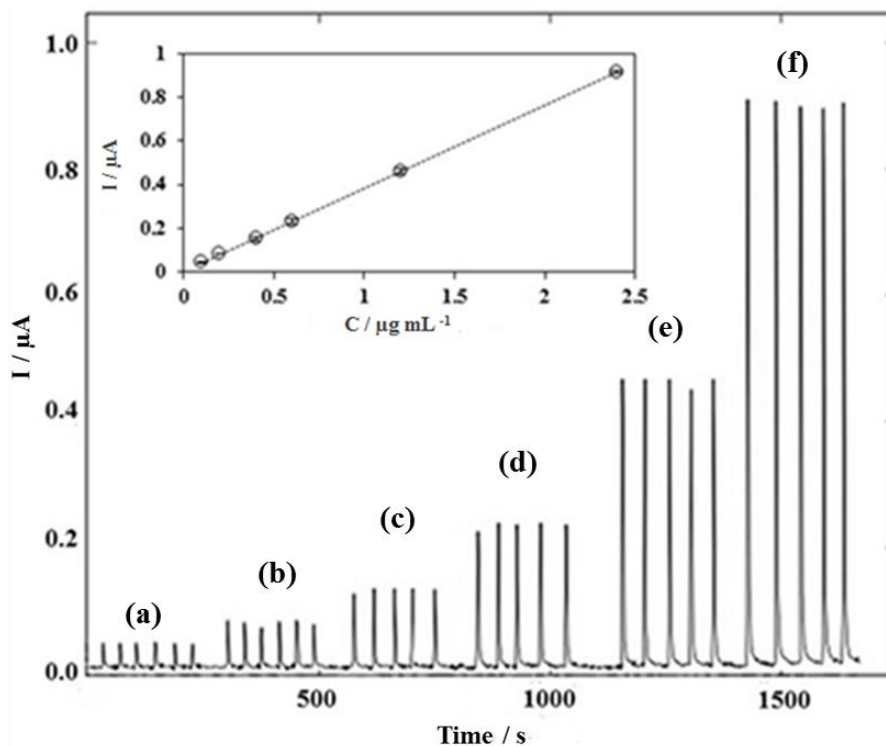
Figure 4. *Flow injection amperometric responses for increasing concentrations of BPA: concentrations of 0.1 (a), 0.2 (b), 0.4 (c), 0.8 (d), 1.2 (e), 2.4 (f) µg mL^{-1} at MWCNTs-OH/GCE; supporting electrolyte: 0.1 M sodium glycine buffer, pH 8; potential 0.5 V (Ag/AgCl); flow rate 0.5 ml min^{-1}; injection volume 10 µL. Insert: Calibration curve for the detection of BPA is obtained at the showed concentrations.*

The 0.11 µg mL^{-1} limit of detection was estimated according to the approach LOD =3.3·(sy/x/b), where sy/x is the residual standard deviation and b is the slope of the regression equation. This approach allows to control both false positive and false negative errors ($\alpha = \beta = 0.05$).

The limit of quantification (LOQ = 0.33 µg mL^{-1}) were estimated using the approach 10·(sy/x/b).

The sensor responded rapidly to the dynamic changes of BPA concentration, allowing about 40 determinations per hour. This indicates a rapid reaction mechanism which includes transport of the substrate to the reactive surface and conversion to a final product away from the electrode.

In order to evaluate the precision (repeatability) of the electrochemical method, subsequent injections (n=18) of BPA standard solutions at the same concentration of 1 µg mL^{-1} were analyzed. The resulting relative standard deviation (RSD = 1.81%) demonstrated an excellent repeatability of the electrode preparation procedure.

In addition, the electrode showed long term stability. As a matter of fact, testing the repeatability of the same electrode after two weeks, entailed a decrease of less than 5% in the recorded mean current (*Ip*).

Furthermore, to evaluate the selectivity of the proposed method, the influence of common interfering species such as ascorbic acid, catechol, phenol, p-nitrophenol, and hydroquinone was considered. These species were added at concentration levels similar and superior (10-fold) to those in which they are commonly present in real samples. The tolerance limit was estimated to be less than 10% of the relative standard deviation, therefore these compounds do not significantly influence the BPA analytical signal.

3.1.3.3. Real samples analysis

In order to demonstrate the potential application of the proposed method for the assessment of real samples, it was used to detect BPA in water samples contained in baby bottles and PET mineral water bottles. BPA was extracted from the samples as previously reported in the experimental section and the standard addition method was chosen for the analysis.

BPA, as expected, was not detectable in a blank sample consisting of ultrapure water.

To evaluate the efficacy of the method, recovery evaluation was also carried out by spiking known concentrations of BPA to a known volume of mineral water. The addition of $10 - 100$ ng mL^{-1} of BPA resulted, as reported in Table 2, in a recovery of 98 – 102%. The results, evaluated after waiting of one hour to allow the equilibration with the sample, match the expected values at the tested concentrations, as reported by AOAC.

Table 2. *The BPA content and recovery percent value determined by the proposed and reference HPLC/UV method in water samples contained in PET mineral water bottles and baby bottles.*

Bottles water samples	Added ng mL^{-1}	Measured* ng mL^{-1}		Recovery (%)	
		FIA	HPLC/UV	FIA	HPLC/UV
Mineral water (brand 1)	–	–	–	–	–
Mineral water (brand 2)	–	–	–	–	–
Mineral water (brand 2)	10	9.90±0.3	10.1±0.2	99.0±1.0	101±0.8
Mineral water (brand 2)	100	99.7±0.9	99.8±0.7	99.7±1.8	99.8±1.3
Water in baby bottle**	–	–	–	–	–
Water in baby bottle	–	20.2±0.4	19.8±0.3	–	–

* MWCNTs-OH/GCE in FIA and reference method, respectively n = 5 and n = 3.
**Baby bottle BPA free.

As reported in Table 2, the results obtained by the proposed method were compared with those obtained by an independent HPLC-UV method. In this case, the functional relationship (area of the peak vs concentration was: Y = (57465 x ± 1795) X + (− 4642 ± 412) (linear range 0.2 – 10 µg mL^{-1}, R^2 = 0.999, P 95%, n = 15), with a LOD of 0.36 µg mL^{-1} calculated as 3.3·(sy/x/b).

As can be seen in Table 2, the results obtained with the both methods are in good agreement. A t-paired test showed no statistical differences at the 95% confidence level of significance. These results demonstrate the applicability of the proposed method for the determination of BPA in the samples considered in this study.

3.1.4. References

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4. NANOTECHNOLOGY IN FOOD ALLERGY

4.1. SENSITIVE AND SELECTIVE DETERMINATION OF THE MAJOR PEANUT ALLERGEN, ARA H 1, IN FOOD EXTRACTS AND SALIVA SAMPLES USING A NOVEL MAGNETOIMMUNOSENSING PLATFORM

4.1.1. Introduction

Peanuts (*Arachis hypogaea*) are one of the foods most frequently associated with severe allergic reactions, including life-threatening, food-induced anaphylaxis. According to the Food Allergen Labelling and Consumer Protection Act of 2004 (FALCPA 2004, Public Law 108-282, Title II) in the United States, and the Directive 2000/13/EC, as amended by Directives 2003/89/EC and 2007/68/EC, in the European Union, the presence of peanut in a food product has to be declared on its label. The peanut allergies have occurred with such an increasing incidence that recently they have been called an epidemic and have become a major health issue in most developed countries. This type of allergy, which affects approximately 1% of the population worldwide, persists in 80% of sensitized individuals, and for many of them contact with even very small amounts of the allergens are sufficient to elicit mild reactions (e.g. anaphylaxis). No cure has been found for peanut allergy, and strict avoidance of peanuts is the only way to prevent severe symptoms. Due to the wide use of peanut-containing foods, improper labeling, or peanut contamination in raw materials or in production lines, traces of peanuts may exist in foods supposedly free of peanuts. This fact poses a potential risk for peanut allergic individuals. Thus, reliable methods for detection and quantification of peanut allergens are necessary for ensuring the compliance of food labeling and for improving consumer protection. Furthermore, an inadvertent route of oral exposure to this allergen is through passionate kissing of an allergic person with a partner who has consumed the allergen, or by sharing utensils, cups, and so forth and that the severity of the allergic reactions via this mode of exposure likely depends on the amount of allergen present in saliva. Then, for practical patient care advice, it is essential to know how much peanut protein is residual in saliva after ingestion of peanut and the time course over which it diminishes.

Eight *Arachis hypogaea* proteins (Ara h 1-8) are currently considered the most important peanut allergens. Sensitization in up to 95% of peanut-allergic patients has been attributed to Ara h 1, a 7S seed storage glycoprotein or vicilin with a molecular weight of 63.5 kDa which represents 12–16% of the total protein content in peanut extracts and is an established major food allergen. The highly stable nature (thermostable and resistant under a variety of manufacturing/food processing conditions and to digestion in the human gastrointestinal tract) of Ara h 1 prevents IgE binding epitopes from degradation, thereby preserving allergenicity of peanuts during food processing. Therefore, Ara h 1 is considered a suitable marker to identify the presence of peanut in food products and production lines.

Several analytical techniques have been used to detect peanuts allergens. They can be classified into 2 groups: protein-based or DNA-based assays. Protein-based assays detect either a specific peanut allergen (Ara h 1 or Ara h 2) or total soluble peanut proteins. DNA-based techniques detect the presence of allergens by amplifying a specific DNA fragment of a peanut allergen gene through polymerase chain reaction (PCR). Protein-based methods include qualitative or semiquantitative dot immunoblotting, dip stick assay, RIE, and LFIA and quantitative ELISA, RAST, RIA, and LC/MS/MS methods.

Electrochemical immunosensors have been emerging as powerful alternatives to these methods for the detection and quantification of allergens, but their application in this field is still scarce. Two electrochemical immunosensors have been reported for the specific detection of Ara h 1 but both involved time-consuming electrochemical transducers modification protocols. Nowadays, the use of magnetic beads (MBs) are known to be a powerful tool in the development of electrochemical immunosensors avoiding the need for applying laborious protocols for electrochemical substrate modification and improving their performance in terms of sensitivity, reduced assay time and minimization of matrix effects which is essential for the analysis in complex matrices such as food extracts.

The main objective of this work was the development of the first amperometric magnetoimmunosensor for the selective and sensitive determination of Ara h 1 in food samples and the evaluation of its analytical performance. The magnetoimmunosensor is based on a sandwich configuration, using two monoclonal mouse IgG antibodies against Ara h 1, one of them biotin-labeled, as capture and detector antibodies, respectively, onto carboxylic acid-modified magnetic beads (HOOC-MBs). The electrochemical detection of the enzyme reaction product was carried out at disposable screen-printed carbon electrodes (SPCEs) using hydroquinone (HQ) as electron transfer mediator and H_2O_2 as the enzyme substrate. The applicability of the developed disposable magnetoimmunosensor was evaluated by determination of the endogenous Ara h 1 content in different foods demonstrating its potential role in food safety and consumer protection.

4.1.2. Materials and methods

4.1.2.1. Apparatus and electrodes

Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments) controlled by software CHI812B. All measurements were carried out at room temperature. The electrochemical transducers were SPCEs (DRP-110, DropSens) consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode. Furthermore, a specific cable connector (ref. DRP-CAC also from DropSens, S.L.) acted as interface between the SPCEs and the potentiostat.

Homogenization of the solutions was facilitated by a Bunsen AGT-9 Vortex. A Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik) was also used. Magnetic separation steps for incubation/washing processes were performed using a Dynal MPC-S (product No. 120.20, Dynal Biotech ASA) magnetic particle concentrator. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified-MBs to the SPCE surface. Centrifuges Cencom (J.P. Selecta S.A.) and MPW-65R were used to perform the centrifugations.

4.1.2.2. Reagents and solutions

HOOC-MBs (2.7 µm, 10 mg mL^{-1}, Dynabeads® M-270 Carboxylic Acid) were purchased from Dynal Biotech ASA. 2-(N-morpholino) ethanesulfonic acid (MES), NaCl, KCl, Tween®20, sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, and Tris-HCl were purchased from Scharlab. EDC, sulfo-NHS, ethanolamine, HQ and hydrogen peroxide (30 %, w/v) were purchased from Sigma-Aldrich. The capture antibody (Mouse monoclonal IgG1, 2C12, clone 2C12 A11 A3), the biotin conjugated detector antibody (Mouse monoclonal IgG1, 2F7, clone 2F7 C12 D10) and the Ara h 1 standard components of the Ara h 1 ELISA Kit and the Ara h 2 standard, were purchased from Indoor Biotecnologies, Inc. This kit was also used for validation purposes. A high sensitivity Strep-HRP conjugate from Roche (Ref: 11 089 153 001, 500 U mL^{-1}), albumin from chicken egg white (OVA) from Sigma-Aldrich and bovine serum albumin (BSA-TYPE VH) from Gerbu were also used.

The following solutions prepared in deionized water were also employed: 0.1 M phosphate buffer, pH 8.0; 0.05 M phosphate buffer, pH 6.0; phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5; 0.025 M MES buffer, pH 5.0; 0.01 M sodium phosphate buffer solution (pH 7.5, PBST) consisting of PBS with 0.05 % Tween®20, and 0.1 M Tris-HCl buffer, pH 7.2. A commercial blocker casein solution was purchased from Thermo Scientific.

Activation of the HOOC-MBs was carried out with an EDC/sulfo-NHS mixture solution (50 mg mL^{-1} each in MES buffer, pH 5.0). The blocking step was accomplished with a 1 M ethanolamine solution prepared in a 0.1 M phosphate buffer solution of pH 8.0.

All chemicals used were of analytical-reagent grade, and deionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm).

4.1.2.3. Modification of MBs

A 3-µL aliquot of the HOOC-MBs commercial suspension was transferred into a 1.5 mL Eppendorf tube. Then, the MBs were washed twice with 50 µL MES buffer solution during 10 min under continuous stirring (950 rpm, 25 ºC). Between steps, the particles were concentrated using a magnet and, after 4 min, the supernatant was discarded. The MBs carboxylic groups were activated by incubation during 35 min in 25 µL of the EDC/sulfo-NHS mixture solution. The activated MBs were washed twice with 50 µL of MES buffer and re-suspended in 25 µL of a 25 µg mL^{-1} antiAra h 1 solution (in MES buffer). The antiAra h 1 was captured onto the activated beads during 30 min at 25 °C under continuous stirring (950 rpm). Subsequently, the antiAra h 1-modified MBs were washed twice with 50 µL of MES buffer solution. Thereafter, the unreacted activated groups on the MBs were blocked by adding 25 µL of the 1 M ethanolamine solution in 0.1 M phosphate buffer, pH 8.0, and incubating the suspension under continuous stirring (950 rpm) for 60 min at 25 °C. After one washing step with 50 µL of 0.1 M Tris-HCl buffer solution (pH 7.2) and two more with 50 µL of the commercial blocker casein solution, the antiAra h 1-coated MBs were re-suspended in 25 µL of a variable concentration of the Ara h 1 standard or sample under study (prepared in commercial blocker casein solution) and incubated during 45 min (950 rpm, 25 °C). Then the modified MBs were washed twice with 50 µL of the commercial blocker casein solution and immersed in a biotin-antiAra h 1 solution (1:10000 in commercial blocker casein solution) during 45 min (950 rpm, 25 °C). After two washing steps with 50 µL of PBST buffer solution (pH 7.5), the resulting beads were incubated during 30 min in a Strep-HRP (1:1000) solution in PBST, pH 7.5. Finally, the modified-MBs were washed twice with 50 µL of PBST buffer solution (pH 7.5) and re-suspended in 45 µL of 0.05 M sodium phosphate buffer solution (pH 6.0)

4.1.2.4. Amperometric measurements

The modified MBs were captured on the SPCE surface according to the procedure described earlier. The magnet holding block with the SPCE where the modified MBs were captured, was immersed into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer pH 6.0 and 1.0 mM HQ (prepared just before the electrochemical measurement). Amperometric measurements in stirred solutions were performed by applying a detection potential of −0.20 V vs the Ag pseudo-reference electrode upon addition of 50 µL of a 0.1 M H_2O_2 solution until the steady-state current was reached (approx. 1 min). Unless otherwise indicated, each data point given for the amperometric signals represents three independent measurements and the error bars show the standard deviations of measurements.

4.1.2.5. Analysis of real samples

The suitability of the magnetoimmunosensor for the determination of Ara h 1 in real samples was evaluated by analyzing food extracts, samples containing no target protein spiked with increasing concentrations of an Ara h 1 standard or quantities of fried peanuts, different food samples with a variable concentration of endogenous Ara h 1 and saliva samples.

To perform these studies different types of food samples, bought in local supermarkets, were used: halzenuts; peanuts (raw, fried and chocolate-covered); chocolate bars with roasted peanuts, nougat, caramel and milk chocolate; chocolate chip cookies with caramelized almonds; peanut creams; and peanut oil. To extract the endogenous Ara h 1 from these samples, 0.5 g of accurately weight of samples (previously blended) were introduced in plastic tubes and incubated overnight at 60 °C under continuously stirring (950 rpm) with 5.0 mL of Tris-HCl pH 8.2. In the particular case of chocolate samples, they were frozen at −20 °C before blending, and 0.5 g of skimmed milk powder (Central Lechera Asturiana®) was added before extraction to bind the high amount of phenolic compounds (e.g. tannins) present in this matrix which have demonstrated to interfere in the Ara h 1 determination. Subsequently, samples were subjected to a first centrifugation step (3,600 rpm) during 10 min. An

aliquot (1 mL) of the supernatant was further centrifuged at 10,000 rpm for 3 min (4 °C) and the resulting supernatant appropriately diluted was used to perform the determination with the magnetoimmunosensor.

Saliva samples (~1 mL) were collected for each study participant before and after peanut butter sandwich ingestion at various time points. Participants were instructed not to eat or drink between the time courses of saliva collections.

The antiAra h 1-modified MBs, were re-suspended in a 25 μL aliquot of the diluted sample extracts or undiluted saliva samples obtained as described before and incubated at 25 ºC during 45 min with vigorous stirring (950 rpm) and similar procedures were followed.

4.1.3. Results and discussion

The fundamentals of the magnetoimmunosensor configuration as well as of the electrochemical transduction used in this work are displayed in Fig. 4.1.
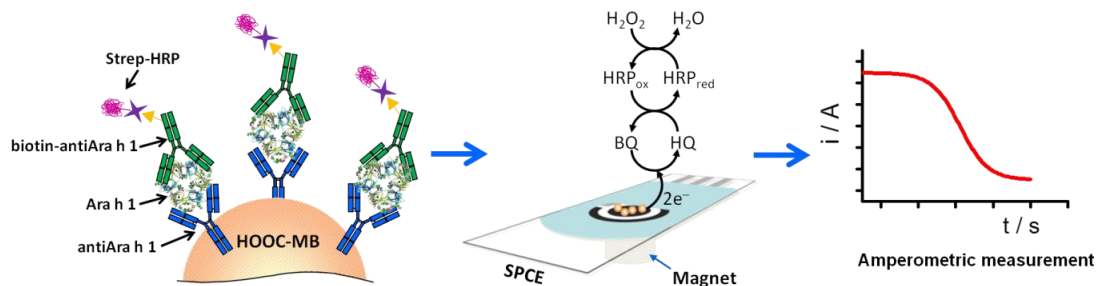

Figure 4.1. *Schematic display of the fundamentals of the developed Ara h 1 magnetoimmunosensor.*

Specific antibodies against Ara h 1 were covalently immobilized onto HOOC-MBs previously activated with an EDC/sulfo-NHS solution. After blocking the unreacted groups in the MBs with ethanolamine, the antiAra h 1-MBs were sequentially incubated in solutions containing the antigen, biotinylated detector antibody and the Step-HRP conjugate. Thus, during the incubation period, the target protein was sandwiched with the detector antibody which was labelled in a final step with an enzyme conjugate. Then, the MBs bearing the sandwich immunocomplexes were magnetically captured on the SPCE and the magnet holding block-SPCE (with the MBs captured on its surface) assembly was transferred to the electrochemical cell where the biorecognition event was monitored by the amperometric measurement of the reduction current generated in the presence of the HQ/H_2O_2 redox system. In this way, the SPCE acted only as the electrochemical transducer while all the immunoreactions occurred on the surface of the MBs.

4.1.3.1. Optimization of experimental variables

Firstly, in order to evaluate the existence of non-specific binding of the antigen, detector antibody or enzymatic tracer on the activated MBs surface, we compared the responses obtained for 0 and 250 ng mL^{-1} Ara h 1 with and without the capture antibody immobilized on the MBs.

While the non-specific adsorptions of the detector antibody and enzymatic label are negligible, the results obtained showed big non-specific adsorptions of the target antigen at antiAra h 1-free MBs. In order to minimize these nonspecific signals, we performed also the immunoreactions in different working media consisted of plain PBST, PBST supplemented with BSA 3%, 1:1 PBST buffer-diluted UHT whole milk samples and a commercial blocker casein solution. Figure 4.2 displays a comparison of the resulting amperometric measurements. As it can be seen, the antigen is immobilized to the MBs through the capture antibody only when the immunoreactions were performed in the commercial blocker casein solution or in 1:1 PBST buffer-diluted UHT whole milk samples. In the other conditions the big adsorption of the Ara h 1 protein to the unmodified MBs did not allow to conclude that a sandwich

format based-magnetoimmunosensor is being developed. Taking into account the higher signal obtained in the presence of Ara h 1 at the antiAra h 1-MBs using the commercial blocker casein solution, we selected it for further work.
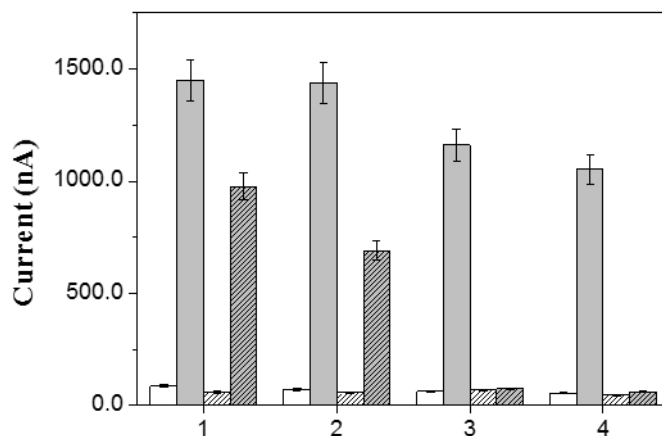

Figure 4.2. *Comparison between the amperometric responses obtained with the antiAra h 1-immobilized (empty bars) or unmodified-MBs (dense bars) in the absence (white bars) or in the presence (grey bars) of 250 ng mL^{-1} Ara h 1 by performing the antigen recognition and labeling with the biotinylated detector antibody in pure PBST buffer solution (1), PBST solution supplemented with BSA (3% w/v) (2), commercial blocker casein solution (3) and 1:1 PBST buffer-diluted UHT whole milk samples (4). Error bars estimated as the triple of the standard deviation (n = 3).*

Once the non-specific adsorptions were minimized and demonstrated the feasibility of the magnetoimmunosensor for Ara h 1 determination using a sandwich format, optimization of all other experimental variables involved in the magnetoimmunosensor preparation and functioning was accomplished by taking as selection criteria the ratio between the amperometric responses measured at −0.20 V in the presence (signal) of 250 ng mL^{-1} Ara h 1 and in the absence (blank) (S/B ratio). All the measurements were carried out. The detection potential value was that optimized previously for the same electrochemical detection system and the experimental conditions used in the HOOC-MBs activation procedure, the successive washing steps and the unreacted carboxylic groups blocking step were chosen according to the protocol given by the MBs supplier. Table 1 collects the ranges tested for all the checked variables as well as the selected corresponding values**.**

Table 1 *Optimization of the different experimental variables affecting the performance of the amperometric magnetoimmunosensor for Ara h 1. See text for other used variables.*

Experimental variable	Tested range	Selected value
V_{MBs}, μL[*]	1−6	3
[antiAra h 1], μg mL^{-1}	0−100	25
$t_{antiAra\ h\ 1}$, min	15−60	30
$t_{Ara\ h1}$, min	15−60	45
[biotin-antiAra h 1], μg mL^{-1}	0−2.5	0.5
$t_{biotin-antiAra\ h\ 1}$, min	15−60	45
[Strep-HRP], dilution factor[*]	1:500−1:5,000	1:1,000
$t_{Strep-HRP}$, min[*]	15−60	30
Number of incubation steps	1−3	3

[*]Experimental variable optimized in previous works [Esteban-Fernández de Ávila et al., 2013].

As examples of these variables optimization as it is shown in Fig. 3a), the S/B current ratio increased with the incubation time in the capture antibody solution up to 30 min and showed a decrease for higher incubation times, which is most likely due to the sterically hindered binding of the antigen when high amounts of <u>capture</u> antibody are immobilized.

Apart from the optimization of all the variables presented in Table 1 and with the aim of simplifying as much as possible the assay protocol, the effect of the number of steps used in the sandwich immunoassay was investigated. The procedures tested were: 1) a single step involving antigen capture and labeling by means of 30 min incubation of the antiAra h 1-MBs in a mixture solution containing Ara h 1, biotin-antiAra h 1 and Strep-HRP; 2A) two steps involving 30 min incubation of the immunosensor with the Ara h 1 solution, followed by a 30 min incubation step in the mixture solution containing biotin-antiAra h 1 and Strep-HRP; 2B) two steps involving 30 min incubation of the immunosensor in a mixture solution containing Ara h 1 and biotin-antiAra h 1, followed by a 30 min incubation step in the Strep-HRP solution, and 3) three steps with 30 min sequential incubations in Ara h 1, biotin-antiAra h 1 and Strep-HRP solutions. Fig.4.3b) shows clearly as the methodology involving three separate incubation steps provided the highest S/B current ratio which was probably due to a lower efficiency of the immune- and labeling reactions due to the high steric hindrance existing when the target antigen, detector antibody and enzymatic label were mixed in homogenous solution before being incubated with the MBs. Accordingly, the protocol in which the immunoreagents were added separately was employed for the implementation of the magnetoimmunosensor
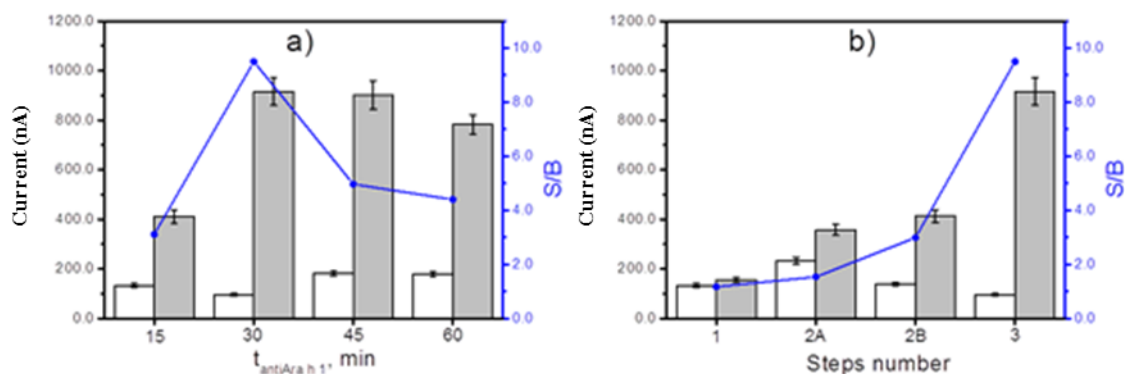
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Figure 4.3. *Effect of the capture antibody solution incubation time (a) and the number of incubation steps used to perform the sandwich immunoassay for Ara h 1 determination (b) on the S/B current ratio. Current ratios measured for 0 (white bars) and 250 ng mL^{-1} (grey bars) Ara h 1 and error bars estimated as triple of the standard deviation (n=3).*

4.1.3.2. Analytical characteristics

A calibration graph was constructed for Ara h 1 in commercial blocker casein solution under the optimized experimental conditions. A linear relationship between the measured current and the Ara h 1 concentration was found over the $20.8 - 1,000.0$ ng mL^{-1} range, with a slope value of (3.03 ± 0.03) nA mL ng^{-1}, and an intercept of (130 ± 13) nA $(r = 0.999)$. The LOD and the determination limit (LQ), 6.3 and 20.8 ng mL^{-1} respectively, were calculated according to the 3s and 10s criteria, where s was estimated as the standard deviation of ten amperometric signals measured without target Ara h 1.

Amperometric measurements for 500 ng mL^{-1} Ara h 1 made with 6 different magnetoimmunosensors prepared on the same day yielded a relative standard deviation (RSD) value of 6.3 %, showing a good reproducibility of the magnetoimmunosensor fabrication procedure and the signal transduction methodology used.

The storage stability of the magnetoimmunosensors was evaluated by preparing on the same day different antiAra h 1-MBs which were stored at 4 ºC in Eppendorfs containing 50 μL of filtered PBST. Each working day two replicates of the prepared conjugates were incubated in solutions containing each 0 and 250 ng mL^{-1} Ara h 1. A control chart was constructed by setting as the central value the average current value calculated from 10 measurements made the first day of the study at both Ara h 1 concentrations, while the upper and lower limits of control were set at $\pm3\times$SD of these initial values. The obtained results (not shown) demonstrated that the magnetoimmunosensors response remained within the control limits for 25 days indicating good storage stability of the capture antibody-modified MBs.

4.1.3.3. Selectivity of the immunosensor

The selectivity of the magnetoimmunosensor was evaluated towards Ara h 2 (the other major allergen in peanuts) and other non-target proteins such as BSA and OVA. The tests were performed by comparing the current values measured with the magnetoimmunosensor for 0 and 250 ng mL^{-1} Ara h 1 in the absence and in the presence of the potential interfering compounds. Figure 4.4.clearly shows that none of the tested proteins significantly interfere in the Ara h 1 determination.
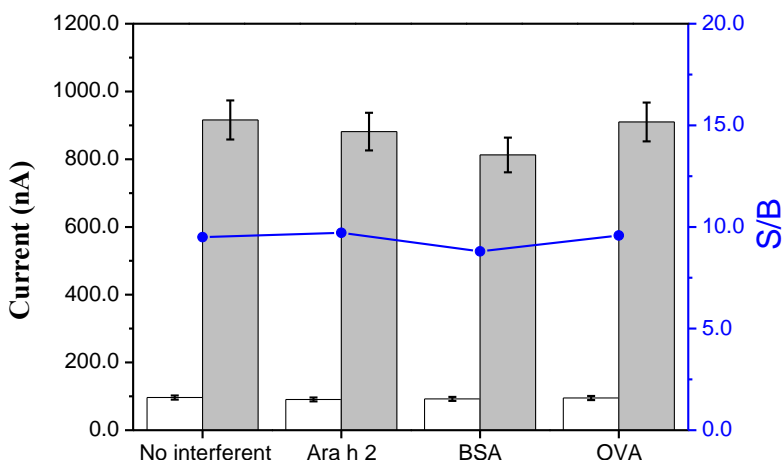
Figure 4.4. *Magnetoimmunosensor selectivity towards Ara h 1. Current values were measured for 0 (white bars) and 250 (grey bars) ng mL^{-1} Ara h 1 in the absence or in the presence of 250 ng mL^{-1} Ara h 2, 50 mg mL^{-1} BSA and 130 mg mL^{-1} OVA. Supporting electrolyte, 0.05 M sodium phosphate solution, pH 6.0; E$_{app}$ = –0.20 V vs Ag pseudo-reference electrode. Other conditions as described in Table 1 (selected values column). Error bars estimated as triple of the standard deviation (n=3).*

4.1.3.4. Determination of Ara h 1 in food extracts

Because of the absence of a reference material, the usefulness of the developed methodology for the analysis of real samples was evaluated by determining Ara h 1 in spiked target protein-free samples (prepared extracts and raw samples) and in different food extracts with variable content of endogenous Ara h 1.

For recovery experiments, a cookie extract (with no detectable content of Ara h 1) was spiked with different increasing amounts of the purified standard Ara h 1(final concentrations: 50, 100 and 250 ng mL^{-1}). Mean recoveries of (100±8), (99±8) and (103±8) %, respectively, were obtained, which demonstrated fairly well the usefulness of the magnetoimmunosensor for the accurate quantification of Ara h 1 in food extracts. Furthermore, cookie samples were spiked with increasing amounts of peanut (final concentrations: 0.05, 0.1, 0.5. 1.0 and 2.5% (w/w)) and extracts prepared and analysed. As it is shown clearly in figure 4.5., even the lowest amount of peanut (0.05%) could easily be detected with the developed magnetoimmunosensor.
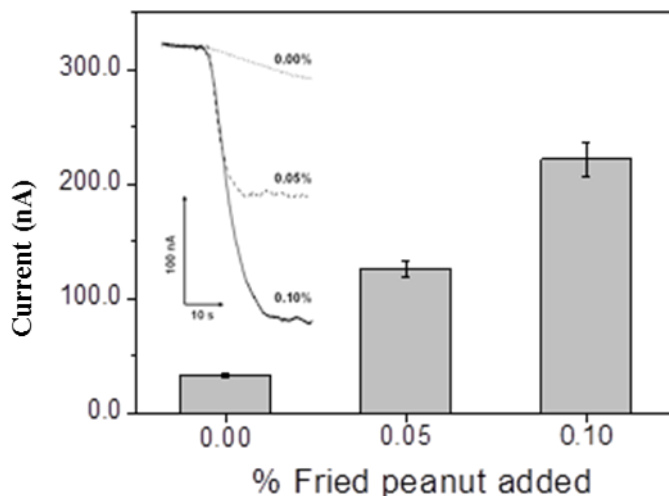
Figure 4.5. *Amperometric response measured with the Ara h 1 magnetoimmunosensor for extracts corresponding to cookie samples unspiked and spiked with 0.05 and 0.10% fried peanuts. Left inset: corresponding amperometric traces. Error bars were estimated as triple the standard deviation (n=3).*

Regarding the determination of endogenous Ara h 1 in the food extracts, the possible existence of a matrix effect was tested by constructing a calibration plot from the extracts prepared (see schematic diagram in figure 4.6.) adequately diluted with blocker casein solution and spiked with growing amounts of a standard Ara h 1 solution up to 750 ng mL^{-1}. All the slope values obtained for the respective calibration plots in the different extracts assayed were statistically similar to that obtained for buffered standard Ara h 1 solutions and, therefore, it could be concluded that no significant matrix effect was apparent once the sample dilution factors required to perform the analysis, were made. Accordingly, Ara h 1 determination could be accomplished by simple interpolation of the measured current obtained for the samples diluted into the calibration plot constructed with buffered Ara h 1 standards. The respective dilution factors for each analyzed extract, needed to fit the target analyte into the linear range of the calibration graph, are specified in Table 2.
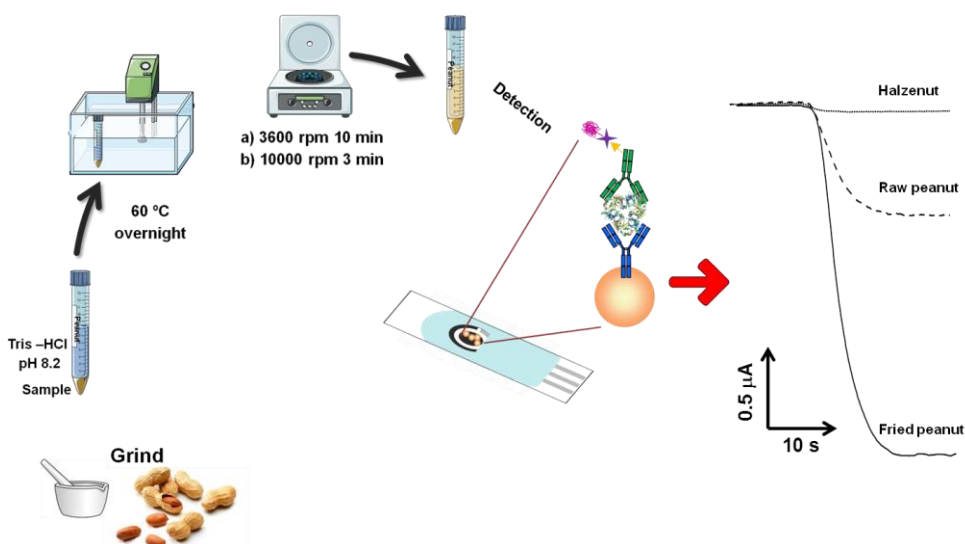
Figure 4.6. *Schematic display of the protocol used to prepare the food extracts exemplified for peanuts case and amperometric traces recorded with the magnetoimmunosensor for diluted fried peanuts, raw peanuts and raw halzenuts extract prepared and diluted as specified in Table 2.*

The results obtained in the analysis of all these samples with the magnetoimmunosensor were compared with those provided by a commercial ELISA kit using the same immunoreagents (see Table 2). It is important to remark that the same sample dilution step was needed when the protocol recommended for the ELISA was applied. A hypothesis testing carried out demonstrated the absence of statistical significant differences in the mean values estimated for all the samples analyzed with both methods at the significance level 0.05.

It is worth to mention also that as it has been described before the detected Ara h 1 levels were up to 2.5-fold higher in roasted than in raw peanuts which can be attributed to the increased efficiency of Ara h 1 extraction with roasting or to the fact that the recognized binding epitopes by the selected antibodies were more accessible in roasted peanut. Moreover, the data obtained are in good agreement also with the range of Ara h 1 content described in the literature for peanut extracts from 0.5 to 15 mg g^{-1} depending on the extraction conditions and fit perfectly with the content found by Peng et al. in roasted peanuts from two different commercial brands (mean contents of 7,004 and 7,281 ng g^{-1}, respectively) using a sandwich ELISA.

Table 2. *Determination of the Ara h 1concentration (in mg g^{-1}) in different food extracts using the developed amperometric magnetoimmunosensor and comparison with the results obtained by a commercial ELISA spectrophotometric kit.*

Extract	Dilution factor	ELISA	Magnetoimmunosensor
Fried peanuts	1/1000	7.5 ± 1.3	7.4 ± 1.0
Raw peanuts	1/1000	3.0 ± 0.6	2.9 ± 0.2
Chocolate-covered peanuts	1/250	0.028 ± 0.004	0.026 ± 0.003
Chocolate bars with roasted peanuts	1/250	0.16 ± 0.03	0.155 ± 0.001
Peanut cream 1	1/500	1.8 ± 0.4	1.8 ± 0.3
Peanut cream 2	1/1000	4.7 ± 0.5	4.3 ± 0.6
Peanut oil	1/100	*nd	*nd
Raw halzenuts	1/100	*nd	*nd

*nd = not detectable

Taking into account that exposure to this allergen through saliva (kissing, utensils) can cause local and systemic allergic reactions, the feasibility of the developed methodology to determine the Ara h 1 in saliva samples was also evaluated. To perform these studies 2 different participants consumed a sandwich prepared with 2 tablespoons of a commercial peanut buffer. One milliliter of saliva was collected before the sandwich ingestion to confirm that at baseline allergen could not be found. Further saliva samples were collected at various time points after the ingestion: 5, 15, 30, 45 and 60 min. After evaluating that although with a 64 % lower sensitivity it was possible to perform the determination of Ara h 1 in raw undiluted saliva samples, the content of the allergen in the saliva samples collected was determine by a calibration plot constructed previously in saliva samples with no detectable content of allergen spiked with increasing concentrations of Ara h 1. It is important to mention that similar calibration curves were obtained for spiked saliva samples corresponding to different individuals, thus demonstrating that the magnetoimmunosensor response did not show significant differences depending on the type of saliva used. These results pointed to the possibility of using a representative saliva sample to build a standard calibration curve for subsequent saliva measurements. The results from the 2 participants evaluated are shown in figure 4.7. As it can be seen both had a similar content of Ara h 1 at 5 min (mean values of 713 and 740 ng mL^{-1}, respectively) and undetectable levels at 60 minutes after ingestion, results in agreement with those reported by Maloney et al. using a spectrophotometric ELISA

methodology. These results demonstrate the ability of the developed magnetoimmunosensor to perform the determination directly in raw undiluted saliva samples which made it an ideal tool for practical patient care advice which allowed knowing in an easy way how much peanut protein is residual in saliva after ingestion of peanut and the time course over which it diminishes.
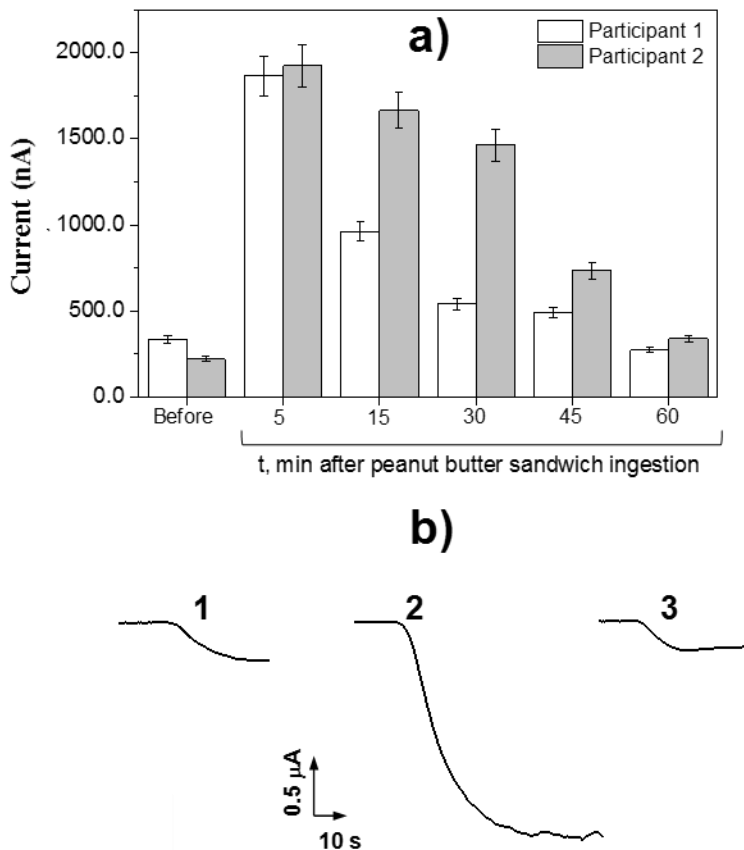
Figure 4.7. *Amperometric measurements obtained with the magnetoimmunosensor in saliva samples from two different participants before and at various time points after ingestion of a sandwich of peanut butter a). Amperometric traces recorded with the Ara h 1 magnetoimmunosensor for saliva samples from the participant 1 before (1) and after 5 (2) and 60 (3) min of the peanut butter sandwich ingestion b). Error bars estimated as the triple of the standard deviation (n = 3).*

All these applicability results demonstrated the high selectivity, accuracy, reliability and reproducibility of the developed magnetoimmunosensor to determine Ara h 1 in complex matrices (food extracts and saliva samples) of great relevance nowadays to estimate potential exposure to peanut allergens.

Compared with the two electrochemical sensors reported so far for Ara h 1 determination the major advantage of the presented approach is the whole assay time, both are integrated immunosensors which involve long protocols to modify the electrode surface, 19 and 16 h, respectively, in comparison with the 4 h which required the proposed magnetoimmunosensor. It is worth to mention also that the LOD achieved is only slightly higher than that reported by Alves et al. (6.3 vs 3.8 ng mL^{-1}) but lower than the one described by Huang et al. (20 ng mL^{-1}). Furthermore, the storage stability of these integrated immunosensors has not been evaluated and Huang et al. did not prove even the utility of the developed immunosensor. The shorter assay time of the developed magnetosensors is very attractive in order to implement a quick tool for screening purposes which reliable positive results for samples containing this major peanut allergen in a few hours.

Although with a similar sensitivity, in comparison with available commercial ELISA spectrophotometric kits the electrochemical immunosensor methodology is remarkably faster, the whole detection process lasting about 2 h (once the antiAra h 1-MBs are prepared), than the commercial kit (4 h). Moreover, the spectrophotometric methods are difficult to be automated and hence hardly used *in situ* and not recommended to monitor routinely the food quality. Conversely, the use of the disposable magnetoimmunosensor simplified largely the whole analytical procedure, requiring smaller sample volumes, and can be easily automated and performed with portable and cost-effective instrumentation which makes it a very attractive and user-friendly tool to determine routinely the food quality and to perform decentralized analysis.

4.1.4. References

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4.2. FIRST AMPEROMETRIC ARA H 2 MAGNETOIMMUNOSENSING PLATFORM FOR DETECTION OF ALLERGENIC TRACE AMOUNTS OF PEANUT IN FOODS

4.2.1. Introduction

Peanuts are the seeds of the peanut plant (*Arachis hypogaea*). The widespread use of peanut products, the severity of the symptoms and its persistence in afflicted people has made peanut allergy a major health concern. Unlike other food-induced allergies, e.g. allergies triggered by milk or egg proteins which mainly affect children and disappear when growing up, this allergy is often a lifelong condition and is the most common food-related cause of fatal allergic reactions in Western countries. There is evidence than the prevalence of peanut allergy is increasing worldwide.

To date, there is no cure for peanut allergy and no therapeutic treatment is available to reduce the severity of this allergy. Strick avoidance of peanut and peanut-ingredient remains the mainstay of management. However, avoidance of peanuts is very difficult because peanut seeds are currently widely used as source of human food ingredients due to their high quality protein (22−30%) and oil content (44−56%). Moreover, the ingestion of peanuts often happens accidentally because of mislabeling of products, rework processes which include peanut containing foods or cross-contamination during processing, which represent a potential risk for peanut allergic individuals. Thus, reliable methods for detection and quantification of peanut allergens are necessary for ensuring the compliance of food labeling and for improving consumer protection.

Over 13 allergenic components identified in peanuts Ara h 1, 2, 3, 6, 8 and 9 are considered the most important markers of peanut sensitization and are predictive of an allergic response. Among these, Ara h 2, a 17.5 kDa 2S albumin protein, which was determined to contribute 5.9–9.3 % to the total protein content of a peanut, has been identified both, as the most offending peanut allergen and as an important predictor of clinical reactivity to peanut.

The detection of peanut allergens in food products is sometimes a difficult task, as they are often only present unintentionally and in trace amounts, or can be masked by compounds of the constituting food matrix. Moreover, insufficient knowledge is available on threshold levels (established by human oral challenge studies), and therefore there is general agreement that the detection limits for their detection need to be as low as possible. Indeed, the analytical community and especially standardization bodies are looking for validated methods that can detect food allergens at the low mg kg^{-1} range (<10 mg kg^{-1}). At present, the main analytical techniques used to detect peanuts allergens can be classified into protein-based or DNA-based assays. Protein-based assays detect either a specific peanut allergen (Ara h 1 or Ara h 2), using enzyme-linked immunosorbent assays (ELISAs), or total soluble peanut proteins. DNA-based techniques detect the presence of allergens by amplifying a specific DNA fragment of a peanut allergen gene through polymerase chain reaction (PCR). However, false positive results (due to cross-reactivity with other nuts) as well as large difference in quantitative results between available ELISA kits and the high numbers of replicates for samples or an external standard required by all the PCR methods described have hindered their application to processed foods or complex food matrices.

Electrochemical immunosensors have been emerging as powerful alternatives to these methods for the detection and quantification of allergenic proteins, but their application in this field is still scarce [Alves et al., 2015]. To the best of our knowledge none electrochemical immunosensor has been described so far for the determination of Ara h 2. Nowadays, the use of magnetic beads (MBs) are known to be a powerful tool in the development of electrochemical immunosensors avoiding the need for applying laborious protocols for electrochemical substrate modification and improving their performance in terms of sensitivity, reduced assay time and minimization of matrix effects which is essential for the analysis in complex matrices such as food extracts. The main objective of this work was the development of the first electrochemical magnetoimmunosensing platform for the selective and sensitive determination of Ara h 2, the most potent peanut allergen, and the evaluation of its analytical performance. The

magnetoimmunosensor is based on a sandwich configuration, using two monoclonal mouse IgG antibodies against Ara h 2 onto carboxylic acid-modified magnetic beads (HOOC-MBs). The electrochemical detection of the enzyme reaction product was carried out at disposable screen-printed carbon electrodes (SPCEs) using hydroquinone (HQ) as electron transfer mediator and H_2O_2 as the enzyme substrate. The applicability of the developed disposable magnetoimmunosensor was evaluated by determination of the endogenous Ara h 2 content in food extracts and spiked samples at trace level (5.0 mg kg^{-1}) thus demonstrating its potential applicability in the implementation into portable and multiplexed electrochemical platforms for food safety and consumer protection.

4.2.2. Materials and methods

4.2.2.1. Apparatus and electrodes

Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments) controlled by software CHI812B; screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens), consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode, were employed as transducers and specific cable connector (ref. DRP-CAC also from DropSens, S.L.) acted as interface between the SPCEs and the potentiostat. All measurements were carried out at room temperature.

A Bunsen AGT-9 Vortex was used for the homogenization of the solutions. Was employed a Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik) and magnetic separator Dyal MPC-S (product no. 120.20, Dynal Biotech ASA). The attraction of the modified-MBs to the SPCE surface was controlled for a neodymium magnet (AIMAN GZ) embedded in a casing of teflon (homemade). Furthermore Centrifuges Cencom (J.P. Selecta S.A.) and MPW-65R were employed in the extraction steps.

4.2.2.2. Reagents and solutions

All the reagents used were of the highest available grade. Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, Tris-HCl, NaCl and KCl were purchased from Scharlab. Tween®20, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), ethanolamine, hydroquinone (HQ), hydrogen peroxide (30%, w/v), albumin from chicken egg white (OVA) were purchased from Sigma-Aldrich. 2-(N-morpholino)ethanesulfonic acid (MES) and bovine serum albumin (BSA Type VH) were purchased from Gerbu and commercial blocker casein solution (a ready-to-use, PBS solution of 1% w/v purified casein) was purchased from Thermo Scientific (Catalog number: 37528).

Carboxylic acid-modified MBs (HOOC-MBs, 2.7 μm Ø, 10 mg mL^{-1}, Dynabeads® M-270 Carboxylic Acid) were purchased from Dynal Biotech ASA. The capture antibody (Mouse monoclonal IgG1, 1C4, clone 1C4 G4 A9, AbC), the detector antibody (Polyclonal rabbit antiserum raised against natural purified Ara h 2, AbD) and the Ara h 2 standard components of the Ara h 2 ELISA Kit (1C4/AH2), used for comparison purposes, and the Ara h 1 standard, were purchased from Indoor Biotecnologies, Inc. Peroxidase-conjugated AffiniPure F(ab´)$_2$ Fragment Goat anti-Rabbit IgG (F(ab´)$_2$-HRP), Fc Fragment Specific was purchased from Jackson Laboratories.

All buffer solutions were prepared with Millipore Milli-Q purification system (18.2 MΩ cm). Phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, 0.01 M sodium phosphate buffer solution consisting of PBS with 0.05 % Tween®20 (pH 7.5, PBST); 0.05 M phosphate buffer, pH 6.0; 0.1 M phosphate buffer, pH 8.0; 0.025 M MES buffer, and 0.1 M Tris-HCl buffer, pH 7.2. Activation of the HOOC-MBs was carried out with an EDC/sulfo-NHS mixture solution (50 mg mL^{-1} each in MES buffer, pH 5.0). The blocking step was accomplished with a 1 M ethanolamine solution prepared in a 0.1 M phosphate buffer solution of pH 8.0.

4.2.2.3. Modification of MBs

A 3 µL aliquot of the HOOC-MBs commercial suspension was transferred into a 1.5 mL Eppendorf® tube. Then, the MBs were washed twice with 50 µL MES buffer solution during 10 min under continuous stirring (950 rpm, 25 ℃). Between each step the particles were concentrated using a magnet and, after 4 min, the supernatant was discarded. The carboxylic groups of MBs were activated by incubation during 35 min in 25 µL of the EDC/sulfo-NHS mixture solution. The activated MBs were washed twice with 50 µL of MES buffer and re-suspended in 25 µL of a 50 µg mL^{-1} antiAra h 2 solution (in MES buffer). The AbC was captured onto the activated beads during 15 min at 25 °C under continuous stirring (950 rpm). Subsequently, the AbC-modified MBs were washed twice with 50 µL of MES buffer solution. Thereafter, the unreacted activated groups on the MBs were blocked by adding 25 µL of the 1 M ethanolamine solution (in 0.1 M phosphate buffer, pH 8.0) and incubating the suspension under continuous stirring (950 rpm) for 60 min at 25 °C. After one washing step with 50 µL of 0.1 M Tris–buffer (pH 7.4) and two with 50 µL of the commercial blocker casein solution the AbC-MBs were used the same day of preparation to perform the determination or resuspended in 50 µL of filtered PBS buffer (pH 7.5) and kept at 4 °C until the day of carrying out the sandwich immunoassay.

Order to develop the immunosensor assay, the AbC-coated MBs were re-suspended in 25 µL of a mixture solution containing a variable concentration of the target antigen and the AbD in solution (1/1,000 in commercial blocker casein solution) during 45 min (950 rpm, 25 °C). After two washing steps with 50 µL of PBST buffer solution (pH 7.5), the resulting beads were incubated with F(ab´)$_2$-HRP (1/10,000) solution in PBST (pH 7.5) during 30 min (950 rpm, 25 °C). After was performed two washing with 50 µL of PBST (pH 7.5).

Finally, the modified-MBs were re-suspended in 45 µL of 0.05 M sodium phosphate buffer solution (pH 6.0) and immobilized on the SPCE surface by placing the neodymium magnet under the working electrode surface.

4.2.2.4. Amperometric measurements

To perform the amperometric measurements the SPCE was positioned on a homemade casing of teflon with neodymium magnet encapsulated, the final resuspended modified MBs were captured on the SPCE surface according to the procedure described earlier. The magnet holding block with the SPCE where the modified MBs were captured, was immersed into an electrochemical cell containing 10 mL 0.05 M phosphate buffer pH 6.0 and 1.0 mM HQ (prepared just before the electrochemical measurement). Amperometric measurements in stirred solutions were performed by applying a detection potential of −0.20 V vs. Ag pseudo-reference electrode upon addition of 50 µL of a 0.1 M H$_2$O$_2$ solution until the steady-state current was reached (approx. 100 s). The amperometric signals given through the manuscript corresponded to the difference between the steady-state and the background currents.

4.2.2.5. Analysis of real samples

The developed Ara h 2 amperometric magnetoimmunosensor was applied to the analysis of different food samples containing an unknown amount of endogenous Ara h 2 and of samples free of peanuts (wheat flour) spiked at trace levels.

Different types of foodstuffs, purchased in local supermarkets, were used: wheat flour, hazelnuts; peanuts (peanut flour, raw, fried and chocolate-coated); chocolate bars with roasted peanuts, nougat, caramel and milk chocolate; multicereals bars with up to 59 % (w/w) of whole roasted peanuts; chocolate chip cookies with caramelized almonds; peanut creams and peanut oil.

To perform the studies with spiked samples, peanut-free wheat flour (verified using a commercial Ara h 2 ELISA spectrophotometric kit) was fortified with different amounts of peanut flour that consisted of

100% raw peanut (unknown variety) from a commercial retailer (Frinuts), obtaining a series of mixtures with 0.05%, 0.025%, 0.01%, 0.0075%, 0.005%, 0.001%, 0.0005% and 0.0001% w/w of peanut.

In all the food matrices assayed the following protocol was used for the extraction of proteins present in peanuts: 0.5 g of accurately weighted ground sample (previously blended) were introduced in plastic tubes and incubated in 5.0 mL of Tris-HCl (pH 8.2) overnight at 60 ºC under continuous stirring (950 rpm). For chocolate samples, they were frozen at -20 °C before blending, and 0.5 g of skimmed milk powder (Central Lechera Asturiana®) were added during the extraction in order to avoid the masking of the target protein by tannins. Subsequently the aqueous phase was isolated by centrifugation, a first centrifugation step (3,600 rpm) during 10 min and a second step (10,000 rpm) during 3 min (4 ºC) of a 1 mL aliquot of the first supernatant. The resulting supernatant appropriately diluted was used to perform the determination with the magnetoimmunosensor.

The same food extracts were also analyzed by an ELISA methodology involving the use of the same immunoreagents for comparison purposes.

4.2.3. Results and discussion

The fundamentals of the magnetoimmunosensor configuration as well as of the electrochemical transduction used in this work are displayed in figure 4.8.
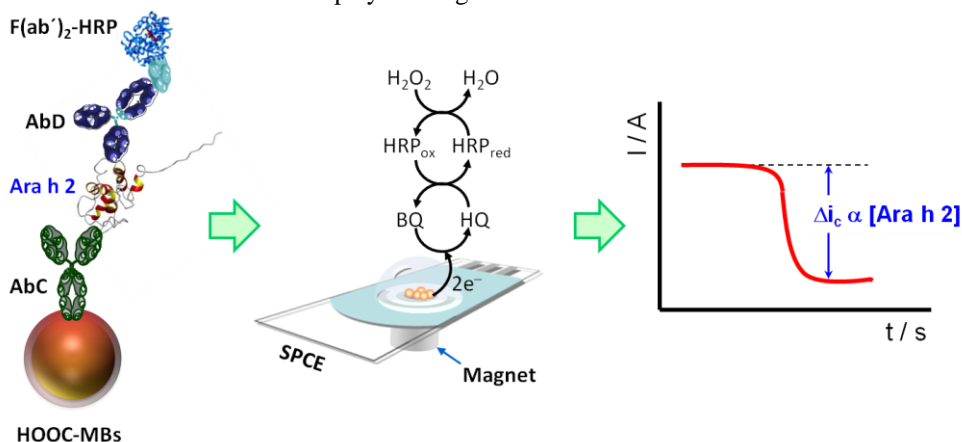

Figure 4.8. *Schematic display of the fundamentals of the developed Ara h 2 magnetoimmunosensor.*

Specific monoclonal mouse antibodies against Ara h 2 (AbC) were covalently immobilized onto HOOC-MBs previously activated with an EDC/sulfo-NHS solution. After blocking the unreacted groups in the MBs with ethanolamine, the AbC-MBs were sequentially incubated in solutions containing the target analyte, the polyclonal rabbit detector antibody (AbD) and a secondary HRP-labelled anti-rabbit IgG (F(ab´)$_2$-HRP). Thus, during the incubation period, the target protein was sandwiched with the detector antibody which was labelled in a final step with other HRP-conjugated secondary antibody. Then, the MBs bearing the sandwich immunocomplexes were magnetically captured on the SPCE and the magnet holding block-SPCE (with the MBs captured on its surface) assembly was transferred to the electrochemical cell where the biorecognition event was monitored by the amperometric measurement of the reduction current generated in the presence of the HQ/H$_2$O$_2$ redox system. In this way, the SPCE acted only as the electrochemical transducer while all the immunoreactions occurred on the surface of the MBs.

4.2.3.1. Optimization of experimental variables

Once evaluated the absence of non-specific binding of Ara h 2, AbD and F(ab´)$_2$-HRP in the absence of AbC immobilized on HOOC-MBs, using a commercial blocker casein solution which has proven to be

highly effective for minimization of nonspecific adsorptions and hence the feasibility of the approach for the target antigen determination using a sandwich format, the optimization of all other experimental variables involved in the magnetoimmunosensor preparation and functioning was accomplished. The adopted selection criterion was the magnitude of the ratio between the amperometric responses measured at −0.20 V in the presence (signal, S) of 5.0 ng mL^{-1} Ara h 2 and in its absence (blank, B) (S/B ratio). All the tested variables, the corresponding ranges into which they were checked and the optimized selected value are summarized in Table 3. The detection potential and the volume of MBs used per assay were optimized in previous.

Table 3. *Optimization of the different experimental variables affecting the performance of the amperometric magnetoimmunosensor for Ara h 2 (by comparison of the S/B ratio obtained in the presence of 5.0 and 0.0 ng mL^{-1} Ara h 2). See text for other used variables.*

Variable	Tested range	Selected value
[AbC], µg mL^{-1}	2.5−100	50
t_{AbC}, min	15−90	15
[AbD], dilution	1/250−1/5,000	1/1,000
[F(ab´)$_2$-HRP], dilution	1/250−1/50,000	1/10,000
$t_{Ag + AbD}$, min	15−90	45
$t_{F(ab´)2-HRP}$, min	15−90	30

Apart from the experimental variables collected in Table 3 and with the aim of simplifying as much as possible the whole protocol and reducing the assay time, the effect of the number of steps used in the involved sandwich immunoassay was investigated. The procedures tested were: 1) a single step involving antigen capture and labeling by means of 30 min incubation of the AbC-MBs in a mixture solution containing Ara h 2, AbD and F(ab´)$_2$-HRP; 2A) two steps involving 30 min incubation of the immunosensor with the Ara h 2 solution, followed by a 30 min incubation step in the mixture solution containing AbD and F(ab´)$_2$-HRP; 2B) two steps involving 30 min incubation of the immunosensor in a mixture solution containing Ara h 2 and AbD, followed by a 30 min incubation step in the F(ab´)$_2$-HRP solution, and 3) three steps with 30 min sequential incubations in Ara h 2, AbD and F(ab´)$_2$-HRP solutions. Figure 4.9. shows clearly as the methodology involving two separate incubation steps, first with a mixture solution of the target antigen and the AbD and after with the F(ab´)$_2$-HRP provided the largest S/B current ratio which was probably due to the lower efficiency of the target recognition due to the high steric hindrance existing when all the immunoreagents were mixed in homogenous solution (bars 1) or the AbD was incubated together with the F(ab´)$_2$-HRP (bars 2A). Results demonstrated also that the resulting S/B is significantly higher if the capture and labeling with the AbD is performed in the same step (bars 2B) in comparison with the results achieved with separate capture and labeling steps (bars 3), probably due to a more efficient labeling with AbD when Ara h 2 is free in solution. Accordingly, this protocol was employed for the implementation of the magnetoimmunosensor.
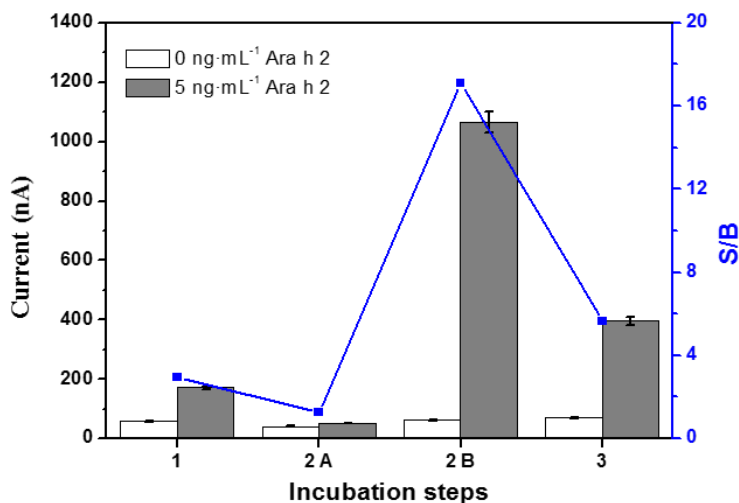
Figure 4.9. *Effect of the number of incubation steps used to perform the sandwich immunoassay for Ara h 2 determination on the amperometric responses measured for 0 (white bars) and 5 ng mL⁻¹ (grey bars) Ara h 2 standards. Error bars estimated as triple of the standard deviation (n=3).*

4.2.3.2. Analytical characteristics

Working under the optimized experimental conditions, the calibration graph shown in Figure 3 was constructed for Ara h 2 standards, giving a linear relationship (r = 0.999) between the measured current and the Ara h 2 concentration over the 87−10,000 pg mL⁻¹ range, with a slope value of (198 ± 2) nA mL ng⁻¹, and an intercept of (87 ± 8) nA. The LOD and the determination limit (LOQ), 26 and 87 pg mL⁻¹, respectively, were calculated according to the 3s m⁻¹ and 10s m⁻¹ criteria, respectively, where m is the slope of the linear calibration plot (shown in figure 4.10.), and s was estimated as the standard deviation of ten amperometric signals obtained without target Ara h 2.
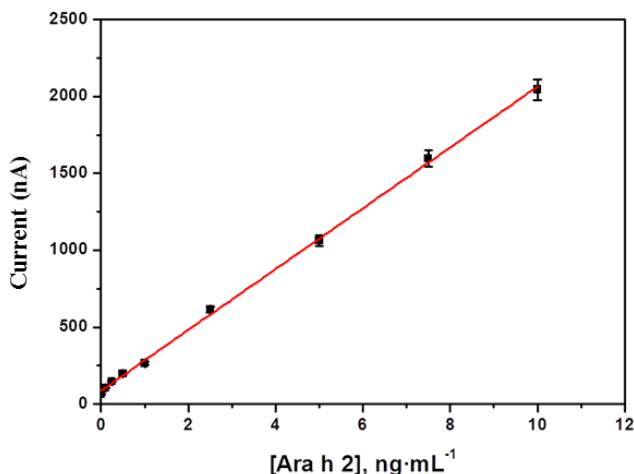

Figure 4.10. *Calibration plot constructed for Ara h 2 standards under the optimal experimental conditions. Error bars estimated as triple of the standard deviation (n=3).*

Amperometric measurements for 2.5 ng mL^{-1} Ara h 2 made with 10 different magnetoimmunosensors prepared on the same way a relative standard deviation (RSD) value of 3.3 %, showing a great reproducibility of of the magnetoimmunosensor fabrication and the signal transduction protocols used. The storage stability of the AbC-MBs kept at 4 ºC in microcentrifuge tubes containing 50 μL of filtered PBST was also evaluated. Each working day two replicates of the prepared conjugates were incubated in solutions containing each 0 and 2.5 ng mL^{-1} Ara h 2. A control chart was constructed by setting as the central value the average current value calculated from 10 measurements made the first day of the study at both Ara h 2 concentrations, while the upper and lower limits of control were set at ±3×SD of these initial values. The obtained results (not shown) demonstrated that the magnetoimmunosensors response remained within the control limits for 50 days. This remarkable storage stability suggests the possibility of preparing sets of B AbC-MBs conjugates and storing them under the above mentioned conditions until their use for the magnetoimmunosensor preparation is required.

4.2.3.3. Selectivity of the immunosensor

The selectivity of the magnetoimmunosensor was evaluated towards Ara h 1 (the other major allergen in peanuts) and other non-target proteins such as BSA and OVA which can coexist with it in food extracts. These tests were performed by comparing the current values measured with the magnetoimmunosensor for 0 and 2.5 ng mL^{-1} Ara h 2 in the absence and in the presence of these potential interfering compounds. The results, displayed in figure 4.11., clearly suggest that no significant interference was apparent in the Ara h 2 determination due to presence of the three tested non-target proteins even at the large concentrations assayed for BSA and OVA. It is worth to mention also the absence of cross-reactivity with the cupin Ara h 1 although it has been tested at a concentration 100 times higher and the presence of short similar structural motifs with Ara h.
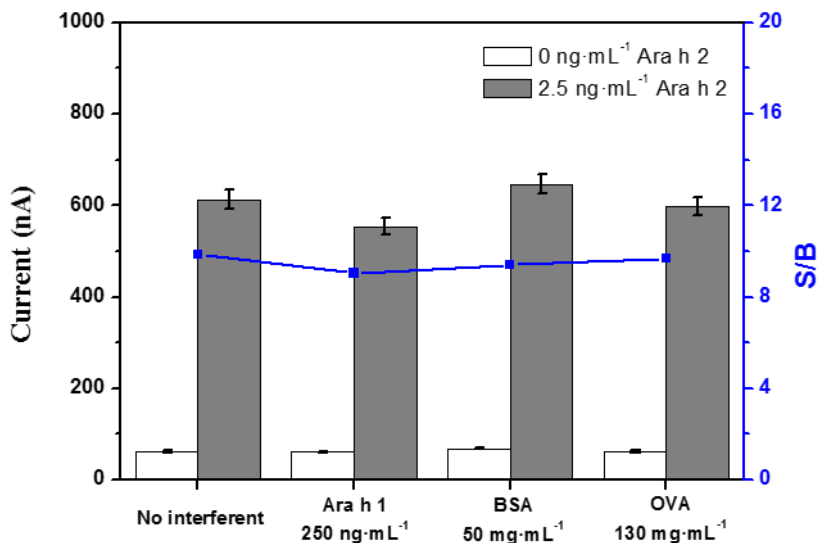

Figure 4.11. *Magnetoimmunosensor selectivity towards Ara h 2. Current values were measured for 0 (white bars) and 2.5 (grey bars) ng mL^{-1} Ara h 2 in the absence or in the presence of 250 ng mL^{-1} Ara h 1, 50 mg mL^{-1} BSA and 130 mg mL^{-1} OVA. Supporting electrolyte, 0.05 M sodium phosphate solution, pH 6.0; E_{app} = −0.20 V vs Ag*

pseudo-reference electrode. Other conditions as described in Table 1 (selected values column). Error bars estimated as triple of the standard deviation (n = 3).

4.2.3.4. Determination of Ara h 2 in real samples

The usefulness of the developed methodology for the analysis of real samples was evaluated by determining the target allergen in different food extracts with variable unknown content of endogenous Ara h 2 and in spiked target protein-free samples following the protocol displayed in figure 4.12.
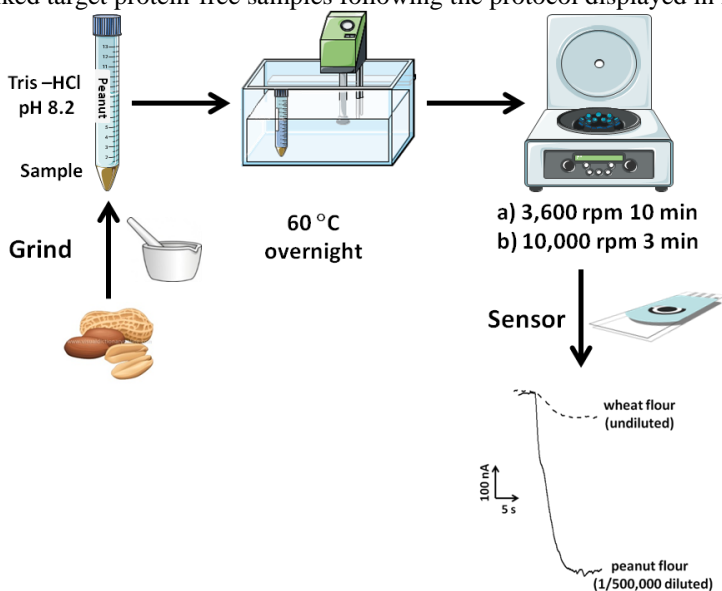

Figure 4.12. *Schematic display of the protocol used to prepare the food extracts (exemplified for peanuts case) and amperometric traces recorded with the magnetoimmunosensor for undiluted wheat flour and 1/500,000 diluted-peanut flour extracts prepared.*

Regarding the determination of endogenous Ara h 2 in the food extracts, no statistically significant differences were observed between the slope values of the calibration plots constructed with Ara h 2 standards and the calibration graphs obtained for all the prepared extracts adequately diluted with blocker casein solution and spiked with growing amounts of a standard Ara h 2 solution up to 2.5 ng mL^{-1}. Accordingly, we could conclude that no significant matrix effects were apparent once the sample dilution factors required to perform the analysis were made, confirming also the effectiveness in the use of MBs and blocker casein solution to minimize the nonspecific adsorption of components from complex samples. Therefore, Ara h 2 quantification could be accomplished by simple interpolation of the measured current from the diluted samples into the calibration plot constructed with Ara h 2 standard solutions.

The results obtained in the analysis of all these samples with the magnetoimmunosensor, together with the dilution factors applied to each analyzed extract (in order to fit the target analyte concentration into the linear range of the figure 4.10. calibration graph) were compared with those provided by a commercial ELISA kit using the same immunoreagents (see Table 4). Although an excellent correlation was found between the Ara h 2 concentrations obtained with the ELISA and those measured with the amperometric magnetoimmunosensor, the presented methodology could measure the samples twice as fast as compared to the ELISA protocol (2 vs 4 h once the AbC-MBs and AbC-plate were prepared). Moreover, the spectrophotometric method is rather expensive and some steps are difficult to automate. Conversely, the use of the disposable magnetoimmunosensor simplified largely the whole analytical

procedure and can be easily automated and performed with portable and cost-effective instrumentation simplifying its use *in situ* which makes it a very attractive and user-friendly tool to monitor routinely the food quality and to perform decentralized analysis in comparison with the ELISA method which is the most commonly used by the food industry and official food control agencies to assess the presence of allergens like peanut in food products.

Table 4. *Determination of the Ara h 2 concentration (in mg g^{-1}) in different food extracts using the developed amperometric magnetoimmunosensor and comparison with the results obtained by a commercial ELISA spectrophotometric kit.*

Extract	Dilution factor	ELISA	Magnetoinmunosensor
Fried peanuts	1/100,000	(4.4 ± 0.3) $RSD_{n=3} = 6.4\%$	(4.3 ± 0.3) $RSD_{n=3} = 2.7\%$
Raw peanuts	1/100,000	(4.1 ± 0.4) $RSD_{n=3} = 4.3\%$	(4.3 ± 0.4) $RSD_{n=3} = 3.6\%$
Chocolate-covered peanuts	1/1,000	(0.034 ± 0.006) $RSD_{n=3} = 6.5\%$	(0.033 ± 0.005) $RSD_{n=3} = 6.5\%$
Chocolate bars with roasted peanuts	1/10,000	(0.38 ± 0.02) $RSD_{n=3} = 2.4\%$	(0.31 ± 0.04) $RSD_{n=3} = 5.5\%$
Multicereals bars with roasted peanuts	1/100,000	(3.4 ± 0.5) $RSD_{n=3} = 6.1\%$	(3.5 ± 0.6) $RSD_{n=3} = 6.5\%$
Peanut cream 1	1/100,000	(3.6 ± 0.8) $RSD_{n=3} = 8.8\%$	(3.6 ± 0.3) $RSD_{n=3} = 3.4\%$
Peanut cream 2	1/100,000	(1.4 ± 0.2) $RSD_{n=3} = 6.3\%$	(1.3 ± 0.3) $RSD_{n=3} = 8.6\%$
Peanut oil	1/1,000	0.0023 ± 0.0004 $RSD_{n=3} = 7.0\%$	0.00224 ± 0.0004 $RSD_{n=3} = 6.5\%$
Peanut flour	1/500,000	11 ± 1 $RSD_{n=3} = 4.0\%$	10.9 ± 0.7 $RSD_{n=3} = 2.5\%$
Wheat flour	--	ND	ND
Raw halzenuts	--	ND	ND

ND: non detectable

For recovery experiments, a wheat flour (with no detectable content of the target allergen, see table 4) was spiked with different increasing amounts of a peanut flour (final concentrations: 0.0001, 0.0005, 0.001, 0.005, 0.0075, 0.01, 0.025 and 0.05% (w/w)) and the corresponding extracts were prepared and analyzed. As it is shown clearly in figure 4.13., in which results obtained with a previously developed Ara h 1 magnetoimmunosensor was also presented, the presented Ara h 2 allowed clearly the detection of 0.0005 % (5.0 mg kg^{-1}) peanut, a concentration 100–200 times lower to that detected with previously reported magnetoimmunosensor (0.05%) and integrated immunosensor (0.1 %) for Ara h 1 determination. This greater sensitivity for peanut detection, even using a similar methodology, could be attributed to the use of different immunoreagents and labeling protocol (use of F(ab´)$_2$-HRP instead of Strep-HRP) and to a more efficient extraction of the smaller target protein (16.5 vs 65 kDa) from the food samples.

This important improvement in sensitivity represent a major advantage with the previously reported Ara h 1 magnetoimmunosensor taking into account the serious public health problem that the degree of contamination of commercial food samples with peanuts, whether fraudulent or accidental, can cause in sensitive individuals. Moreover it is important to emphasize since a level of 10 mg kg^{-1} is within the range of detection limits that are relevant for the detection of potentially hazardous residues of undeclared allergens in foods, lower detection limits, like the one demonstrated in this work may result very convenient, since minimal amounts of the target allergen can be critical.
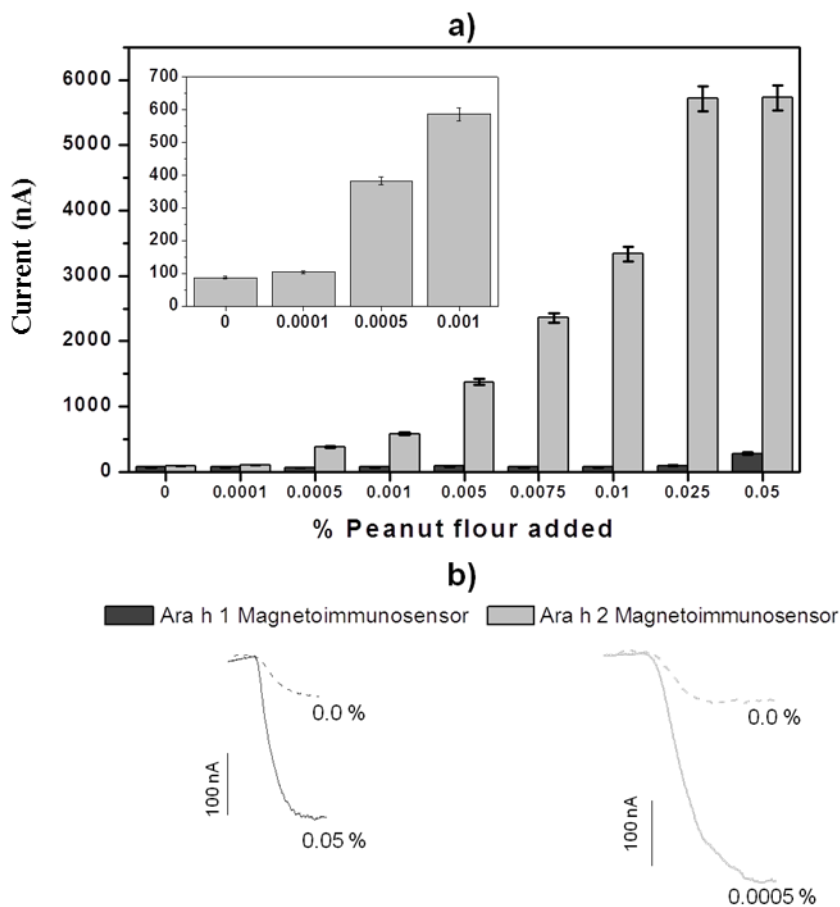
Figure 4.13. *Amperometric responses measured with Ara h 1 and Ara h 2 magnetoimmunosensors for extracts from unspiked wheat flour and spiked with (0.0001–0.05)% (w/w) peanut flour (a). Amperometric traces recorded with the Ara h 1 magnetoimmunosensor for extracts from an unspiked wheat flour and spiked with 0.05% (w/w) peanut flour (left) and with the Ara h 2 magnetoimmunosensor for extracts from an unspiked wheat flour and spiked with 0.0005% (w/w) peanut flour (right) (b). Error bars were estimated as triple the standard deviation (n=3).*

Moreover, it is important to mention that the detection level achieved in this work of about 5.0 mg kg^{-1} peanut incurred in wheat flour, in the range of some of the most promising PCR-based approaches reported for sensitive peanut determination, in the range between 2 and 10 mg kg^{-1}, is achieved without performing any amplification step.

All these applicability results demonstrated the high selectivity, accuracy, reliability and reproducibility of the developed magnetoimmunosensor to accurately determine Ara h 2 in complex matrices, even in real food samples containing peanut material in trace amounts (low mg kg^{-1} range), of great relevance nowadays to estimate potential exposure to peanut allergens.

4.2.4. References

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5. CONCLUSIONS

Electroanalysis is a powerful analytical tool for food quality and safety, the development of nano and biosensors are an important research area and will remain so in the future. Nanostructures have successfully shown a great potential for being used in nanosensors and the versatility and high applicability makes them clear candidates to be further used in these devices. Compared with other methods (HPLC/UV, GC-MS, Spectrophotometry), electrochemical methods are characterized by simplicity, high sensitivity and good selectivity, low-cost instrumentation, speed up the detection, enable high-throughput analysis, and to be used for critical control points monitoring in food production.

In this thesis, we demonstrated the potentialities of nanosensors in two spheres of food-related applications: (i) nanosensors aimed at food quality control, (ii) nanosensors used for safety control in food.

In detail, with regard to the first sphere namely quality assessment of food systems:

-The applicability of a novel sensing unit based on a disposable screen printed carbon electrode (SP) coated by an electrospun nylon-6 nanofibrous membrane, for the *in-situ* determination of AA in both standard solution and real fruit samples, was demonstrated. The sensor displays high sensitivity, reproducibility and selectivity towards AA with a good stability and a fast response. In short, the membrane in this sensing unit acts as a partial barrier-to-diffusion from the matrix to the electrode surface, especially towards phenolic compounds, protecting the electrochemical signal from the distortion caused by the oxidation of these chemical species. In addition, the membrane shields the electrode against the mechanical damage to which it is exposed when introduced in a complex solid matrix as a fruit pulp. The methodology here developed is straightforward, rapid and inexpensive for the assessment, especially when compared to traditional analytical determinations that require a sample pre-treatment. Moreover, due to the simplicity of the apparatus, it could be used like a portable device to be applied in the field.

-The immobilization of xanthine oxidase onto nanofibrous membranes through a very simple methodology and its use as coating of rhodium-carbon home-made electrodes operating at low potentials, allow for a very specific amperometric detection of purines (i.e., xanthine), an important chemical marker of the freshness of meat products, especially fish. The increase in current was attributed to the reduction of hydrogen peroxide on the electrode surface, constantly regenerated by the enzyme through the oxidation of xanthine to uric acid. The rate of increase of redox amperometric signals (-μA s_{-1}) was considered as a measurement of the reaction rate, and therefore of the analyte concentration, the results indicated that this device can be a valid alternative for the selective detection of xanthine or other purines.

-A novel method to measure purine derivatives was developed. Owing to this scheme measuring the regenerated current of the copper electrode after dissolving the surface passive layer by these purines, several problems including crucial operating potential was solved, and gave us the possibility to improve the selectivity and sensitivity of the method for the detection of purine derivatives present in fish as a function of time, for the determination of the fish freshness. The methodology here developed is straightforward, rapid and inexpensive for the assessment, especially when compared to traditional analytical determinations that require a sample pre-treatment. Moreover, due to the simplicity of the apparatus, it could be used like a portable device to be applied in the field.

With regard to the second sphere namely safety assessment of food systems:

-A novel amperometric sensor for the determination of bisphenol A (BPA) was fabricated using a glassy carbon electrode modified with multi-walled carbon nanotubes functionalized with hydroxyl groups. The catalytic activity of the modifier towards the oxidation of BPA was demonstrated by cyclic voltammetry, giving a well-defined peak at 0.55 V in sodium glycine buffer solution (pH 8.0).

The flow injection analysis (FIA) system of BPA exhibited a linear response in the $0.1 - 2.4$ μg mL^{-1} concentration range, with a detection limit of 0.11 μg mL^{-1}. The current reached the steady-state value with a very fast response time (less than 5 s). The proposed method was successfully applied for the determination of BPA in real samples (water contained in plastic and baby bottles) with satisfactory

Conclusions

results, in optimum agreement with those obtained by an independent HPLC method.

-A disposable amperometric magnetoimmunosensor for the sensitive determination of Ara h 1 was described for the first time. This magnetoimmunosensor allows a LOD of 6.3 ng mL^{-1} and has demonstrated successful applicability to Ara h 1 determination in complex matrices such as diluted food extracts and undiluted saliva samples. This great analytical performance, together with the flexibility and simplicity of the MBs and disposable mass-produced electrochemical transducers-based technology mean important advantages for an easy integration of the presented methodology into portable and multiplexed electrochemical platforms for sensitive and selective determination of the most important food allergens, which will help to reduce the risk of inadvertent allergens presence of food.

-A disposable amperometric magnetoimmunosensor for the high sensitive determination of Ara h 2 was described for the first time in this work. This magnetoimmunosensor allows a LOD of 26 pg mL^{-1} for the target allergenic protein and has demonstrated successful applicability for the accurate determination of the endogenous content of Ara h 2 in different food extracts (results benchmarked against a commercially available ELISA kit) and for the detection of peanut traces (5 mg kg^{-1}) in spiked wheat flour samples. This great analytical performance, together with the flexibility and simplicity of the MBs and disposable mass-produced electrochemical transducers-based technology, mean important advantages for an easy implementation of the presented methodology in any analytical food quality and safety laboratory performing routine determinations. Moreover, this high sensitive and quite fast magnetoimmunosensor could also find application in inspection programs to enforce accurate labeling of commercial food products, or to monitor the effectiveness of cleaning processes from production units of the food industry, thereby protecting both producers and consumers against peanut adulteration, misrepresentation and unintentional ingestion of hidden allergens.

APPENDIX 1: LIST OF PAPER, ORAL COMMUNICATION AND POSTERS

1. C. A. Fuenmayor, S. Benedetti, A. Pellicanò, M. S. Cosio, S. Mannino, 2014. Direct In Situ Determination of Ascorbic Acid in Fruits by Screen-Printed Carbon Electrodes Modified with Nylon-6 Nanofibers. Electroanalysis 4: 704-710.

2. M. S. Cosio, A. Pellicanò, B. Brunetti, C. A. Fuenmayor, 2015. Amperometric Detection of Bisphenol A by Multi-Walled Carbon Nanotubes and Hydroxyl Groups modified Glassy Carbon Electrode. Submitted to Electroanalysis

3. Pellicanò A, 2013. Micro- and Nanosensors for the rapid evaluation of contaminants in food. In Proc.s of the 18th Workshop on the Developments in the Italian Phd Research on Food Science, Technology and Biotechnology, Conegliano (Italy), 25-27 September, 2013.

4. Pellicanò A, 2014. Electrochemical sensors for assessment of food quality and safety. In Proc.s of the 19th Workshop on the Developments in the Italian Phd Research on Food Science, Technology and Biotechnology, Bari (Italy), 24-26 September, 2014.

5. Pellicanò A, 2015. Electrochemical sensors for assessment of food quality and safety. In Proc.s of the 20th Workshop on the Developments in the Italian Phd Research on Food Science, Technology and Biotechnology, Perugia (Italy), 23-25 September, 2015.

6. S. Benedetti, M. S. Cosio, C. A. Fuenmayor, A. Pellicanò, 2013. Nanoemulsion for Olive Oils Quality Control by Electrochemical Methods. Publication at AIDIC meeting, Milan, (Italy) 2013.

7. V. Ruiz-Valdepeñas Montiel, S. Campuzano, A. Pellicanò, R.M. Torrente-Rodriguez, Á.J. Reviejo, J.M. Pingarrón, 2015. Novel Amperometric magnetoimmunosensing platforms for fast, sensitive and selective determination of food allergens, Oral communication, 20th Reunión de la Sociedad Española de Quimica Analitica, Santiago de Compostela (Spain), 1-3 july, 2015.

8. V. Ruiz-Valdepeñas Montiel, S. Campuzano, A. Pellicanò, R. M. Torrente-Rodríguez, A. J. Reviejo, M. S. Cosio, J. M. Pingarrón, 2015. Sensitive and selective magnetoimmunosensing platform for determination of the food allergen Ara h 1. Analytica Chimica Acta 880: 52-59.

9. V. Ruiz-Valdepeñas Montiel, A. Pellicanò, S. Campuzano, R. M. Torrente-Rodríguez, A. J. Reviejo, M. S. Cosio, J. M. Pingarrón, 2015. Electrochemical detection of peanuts at trace levels in foods using a magnetoimmunosensor for the allergenic protein Ara h 2. Submitted to Analyst