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Scientific Research

Development of a QCM (Quartz Crystal Microbalance) Biosensor to the Detection of Aflatoxin B1

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ABSTRACT

In this study, we have used a direct immunoassay where the simple binding between antigen and an antibody is detected. Immunoassays were performed in a drop system, monitoring the frequency decrease of the quartz-crystal microbalance device because of mass increasing during immunoreaction. The QCM sensor was coated on both sides by gold electrodes, only one side of the crystal (liquid side) was in contact with the solution; the other side (contact side) was always dry. We tested a piezoelectric immunosensor for aflatoxin B1 (AFLA-B1) mycotoxin detection through the immobilization of DSP-anti-AFLAB1 antibody (AFLA-B1-Ab anti AFLAB1) on gold-coated quartz crystals (AT-cut/5 MHz). The DSP (3,3'-Dithiodipropionic-acid-di-N-hydroxysuccinimide ester) was used for the covalent attachment of the proteins. The piezoelectric crystal electrodes were pretreated by DSP for 15 min, rinsed with water and dried in a gentle flow of nitrogen gas. Then the DSP-coated crystals were installed in a sample holder and exposed to the anti-AFLAB1 antibody and to the AFLA-BI. Frequency and resistance shifts (Δf and ΔR) were measured simultaneously. Δf versus AFLA-BI concentrations in the range of 0.5 - 10 ppb exhibited a perfect linear correlation with a coefficient of above 0.998.

Keywords: Immunosensor; Quartz Crystal Microbalance; Aflatoxin B1; Antibody; Gold Electrodes

1. Introduction

The term mycotoxins includes numerous secondary metabolites with high toxicity, products in suitable microclimatic conditions by microscopic fungi and filamentous, better known by the term "mold", that colonize the plants and/or the foodstuffs during their growth [1]. Aflatoxin B1 (AFLA-B1) is an example of a group of highly toxic difurancoumarin derivatives that are produced by many strains of *Aspergillusflavus* and *A. parasiticus* which often contaminate a variety of food and animal feed stored. The four major aflatoxins have been designated as B1, B2, G1 and G2 based on their fluorescence under UV light and their relative chromatographic mobility during thin layer chromatography. AFLA-B1 has been classified as a group 1 human carcinogen and aflatoxins G1, G2 and B2 belong to a group human carcinogens [2]. These toxins exhibit carcinogenic, teratogenic and mutagenic properties and have now been isolated from a wide variety of agricultural products [3,4]. AFLA-B1 can enter the food chain mainly through the ingestion of contaminated human or animal food. The intake of AFLA-B1 over a long period of time, even in low concentrations, may be very deleterious to health [5]. The Food and Agricultural Organization 2004 report on mycotoxins [6] revealed that as of December 2003, at least 99 countries worldwide had regulations in place for permitted mycotoxin levels in food/or feed, and have set limits for AFLA-B1 alone or for the sum of aflatoxins B1, B2, G1 and G2. The maximum permissible level for AFLA-B1 in food was set at 2 μg/kg (2 ppb). Analytical methods for the determination of mycotoxins in food products generally provide a first stage of extraction of the toxin from the matrix (with a solution of methanol-water or ethanol-water, depending on the type of analysis later), a second purification step, and finally the revelation by an appropriate instrumental technique. Typically, the methods used are of type chromatographic or immunochemical but in recent years are being developed analytical techniques that use instead of antibodies, such as biological elements, aptamers. In particular for the determi-

nation of OchratoxinA (OTA) have been developed on solid phase extraction columns containing aptamers specific for the OTA [6]. Generally tests are employed qualitative or semi-quantitative rapid screening for as many samples (tendentially with immunochemical methods) and subsequent phases of confirmation with a precise quantification for positive samples (tendentially with chromatographic methods), these include thin-layer chromatography (TLC) [7] and high-performance liquid chromatography (HPLC) [8]. Though these techniques have excellent sensitivities they typically require skilled operators, extensive sample pre-treatment and expensive equipment [9]. The goal of more recent studies has been to simplify and expedite the method of detection while attempting to maintain or improve the sensitivity. Responding to the need to achieve high sensitivity and move to the use of disposable probes, several electrochemical immunosensors have been reported in literature for the detection of AFB1 in corn and barley [10-12] and AFM1 in milk [13]. Among the immunochemical approaches, the enzyme-linked immunosorbent assay (ELISA) method is the most widely applied. Spectrophotomeric ELISAs specific for AFLAB1 [14,15], total aflatoxins [16,17] and AFLAM1 [18,19] have been developed and their simplicity, adaptability and sensitivity have been demonstrated. In order to achieve higher sensitivity and move to the use of disposable probes, electrochemical immunosensors for aflatoxins based on indirect competitive ELISA format have been proposed [20]. These immunosensors require the use of labeled secondary antibodies for detection. To achieve label-free immunosensors, direct electrochemical immunosensors for AFB1 based on electrochemical impedance spectroscopy [21], optical waveguide lightmode spectroscopy [22] and room temperature ionic liquids [23] have been reported. However, results on ELISAs need several incubation, washing, and separation steps. Because of the labor of ELISAs, a great interest in developing label-free and less time-consuming on-line detection methods, such as the use of biosensors, is undiminished. With this purpose, piezoelectric quartz crystals are convenient for affinity-based sensors, particularly for immunosensors. The combination of low cost with increased sensitivity, selectivity, simplicity, and possible reusability make piezoelectric immunosensors a valuable alternative to other existing like optical (e.g., plasmon resonance or molecular fluorescence) and electrochemical immunosensors. The search for a simple and label-free piezoelectric immunosensor is of considerable interest. The mass sensing of the quartz-crystal microbalance (QCM) removes the need of any labelling step for the signal transduction and display detection sensitivities up to a 1- to 100-fold linear ΔF vs. mass range with limit detections as few as nanograms per milliliter levels. The QCM immunosensor is usually comprised of a quartz crystal with an antigen or antibody immobilised on its surface, which allows the label-free detection with a direct quantification of the immunocomplex (Ab-Ag). A QCM that responds to changes in mass on the electrode surface and has a sensitivity in the ngand is used for a wide range of applications in the medical field [24,25]. Currently, the piezoelectric immunosensors are used to determine tumor markers in clinical diagnostics, since they allow performing the analysis in real time. Chou *et al.* [26] and Zhang *et al.* [27] have proposed respectively a piezoelectric immunosensor for the human ferritin and one for hCG (human chorionic gonadotropin). In this investigation, a piezoelectric immunosensor for the detection of AFLA-B1 was developed through the drop-coating of DSP-anti-AFLAB1 antibody (AFLA-B1-Ab anti AFLAB1) on gold-coated quartz crystals (AT-cut/5 MHz). Details of the preparation, characterization and application of the immunosensor are described.

2. Experimental Section

2.1. Instrumentation

Research Quartz Crystal Microbalance (RQCM) and the piezoelectric quartz crystals (AT-cut, 5 MHz, 1-inch diameter) were from Inficon (USA).The quartz crystals were obtained by cutting the quartz with an angle of 35˚ 25′ relative to the axis crystallographic z. The main advantage of this cut is the obtainment of quartzes little sensitive to temperature, at least in a range that goes from 10˚C to 50˚C. The crystals are coated on both sides by gold electrodes, the crystals were installed in a sample holder and exposed to the anti-mycotoxin antibody and to the analyte (mycotoxin). Frequency and resistance shifts (Δf and ΔR) were measured simultaneously. Only one side of the crystal was in contact with the solution; the other side was always dry. All date is recorded and displayed graphically using an integrated software based on Windows system. The heart of this system is an oscillator, a phase locked loop (PLO, Phase Lock Oscillator) which allows creating a signal whose phase has a fixed relationship with that of a reference signal. The circuit also includes the ability to adjust the electrical capacity of the crystal, and this is essential for accurate measurements of thin films and for applications that provide a measure of the analyte in the flow. The PLO uses an internal oscillator indicated as a voltage controlled oscillator (VCO, Voltage Controlled Oscillator) for driving the crystal.

2.2. Immunochemicals and Chemicals

Polyclonal rabbit antibody for Aflatoxin B1 (Rabbit anti-Aflatoxin B1) was from Sigma Aldrich (St. Louis, USA), monoclonal rabbit antibody immobilized on beads which

were extracted from immunoaffinity columns were from R-Biopharms.r.l, (Italy). Aflatoxin B1 from *A. flavus* (*Aspergillusflavus*), 3,3'-Dithiodipropionic-acid-di-N-hydroxysuccinimide ester (DSP),acetone (C_3H_6O) and sepharoase beads, similar to those usually used for the coating of Protein A in the production of affinity columns for the Fc region (Fragment crystallizable) immunoglobulin (IgG), were purchased from Sigma-Aldrich. Working buffer was the phosphate buffer saline (PBS) of pH = 7.4 (1.4 mM KH_2PO_4; 8.0 mM Na_2PO_4; 136 mM NaCl; 2.7 mM KCl). Caution AFLA-B1 is a potent carcinogen molecule, and extreme caution is therefore necessary to avoid contact with this. Contaminated materials must be appropriately discarded.

2.3. Materials and Methods Used for the Coating of Quartz Crystals

The piezoelectric crystal electrodes were pretreated with 20 ul DSP (3,3'-Dithiodipropionic-acid-di-N-hydroxysuccinimide ester) for 15 min rinsing with water, dried in a gentle flow of nitrogen gas and then were installed in a sample holder. The DSP was used for the covalent attachment of the proteins. The DSP binds covalently to proteins, including antibodies, which generally have different primary amines in the amino acid side chains (ad. Example amines present in the chain of Lysine R), these residues and the N-terminal of each polypeptide are available as targets for the DSP. This phase functionalization of quartz crystals is extremely critical, you must obtain a homogeneous coating of the surface to be treated. Clean gold surfaces were immersed in a 3 mL solution of hydrogen peroxide (30%), ammonia (25%) and deionized water heated to a temperature of about 75˚C for 5 minutes. Immediately rince liberally with deionized water and dry in a gentle flow of nitrogen gas. Then the DSP-coated crystals were installed in a sample holder and exposed to the anti-mycotoxin antibody and to the analyte (mycotoxin). Frequency and resistance shifts (Δf and ΔR) were measured simultaneously. The frequency shifts were recorded as a function of time at an interval of 300 s for a longtime monitoring (about 5 h).

2.4. Immobilization of AFLA-B1 Antibody

The coating of quartz was carried out under a hood to avoid contamination. We have developed two procedures for immobilization of Ab-specific antigen (Ab anti-aflatoxin) on the surface of the quartz crystals: absorption of specific antibodies directed and use of antibodies immobilized on the beads (**Figure 1**). As regards the direct absorption, antigen specific antibodies were directly added in drops on the surface of the quartz crystals previously coated with DSP.

The antibodies immobilized on the solid support were taken from immunoaffinity columns and added in a drop on quartz pre-treated with DSP.

2.5. Method of Extraction of Aflatoxin from Contaminated Matrices

For the extraction of aflatoxin B1 from real samples (peanuts) considered in this work applies a standard protocol: the homogenized sample with a water-alcohol solution, then you make a dilution of the extract by the addition of a phosphate buffer solution (PBS). The purification is carried out by passing it through the immunoaffinity column (IAC) containing antibodies specific for AFB1; the mycotoxin is then eluted with methanol and quantified via RQCM. Initially were weighed directly homogenizer 5 g of sample (accuracy ± 0.1 g), and added 0.5 g of sodium chloride (NaCl) and 2 mL of extraction solvent (methanol: water in the proportions 80:20 v/v). The sample was agitated at high speed for 3 minutes. The extract was filtered on a paper filter and 2 mL of filtrate is taken and diluted with 2 mL of PBS (Phosphate Buffered Saline). The diluted sample was applied to the immunoaffinity column as described in the next section. The aflatoxin B1 was eluted directly into 5 mL volumetric flask, following a two-step procedure:

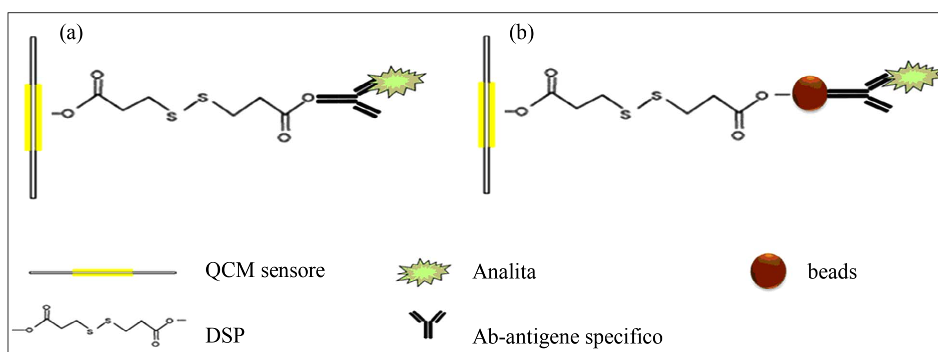


Figure 1. Diagram of immobilization of Ab on the surface of the electrode of gold of the quartz crystal. (a) Coating of the gold surface with DSP and Ab-specific antigen; (b) Coating of the gold surface with DSP and beads with monoclonal Ab specific mycotoxin.

- By applying 1 mL of methanol and leaving the IAC flowing by gravity.
- Waiting for 1 minute, and applying a second portion of 1 mL of methanol.

Subsequently we collected the eluting solvent residue passing, with a 10 mL syringe, through the IAC, a volume of air equal to two /three times the volume of the same. It was brought to volume with water, the sample was then stored at 4˚C until analysis with RQCM.

3. Results and Discussion

3.1. Characterization of the Quartz Crystal Surface

For accurate results of the QCM the characterization of the gold surface of the quartz crystals are highly recommended. The surface of the quartz crystal functionalized with the DSP was characterized AFM (Atomic Force Microscope) is shown in **Figure 2**. The phase imaging is able to bring out details hidden in the normal image reconstruction, highlighting edges and grooves which can be correlated with the different physic-chemical characteristics of the sample.

From the phase imaging shows that the treated surface is homogeneous, the gradation of gray indicates the thickness of the coating, the lightest areas are those that present a greater layer DSP, 90 nm thick coating.

3.2. Standard Curve for Aflatoxin B1

For the determination of mycotoxins through an immunosensor-QCM was initially developed a direct immunoassay which used a polyclonal antibody, by which it was possible to identify the mycotoxin AflatoxinB1 in solutions at different concentrations (standard solutions) prepared in the laboratory. In these experiments, conducted essentially in order to define and characterize our system, we used the standard concentrations of AflatoxinB1 of 0.5, 1, 5, 10 ppb (ng/mL). Applying the methodology described were conducted immunoassays and direct from the data obtained we were able to construct the standard curve shown in **Figure 3**. We report the follow-

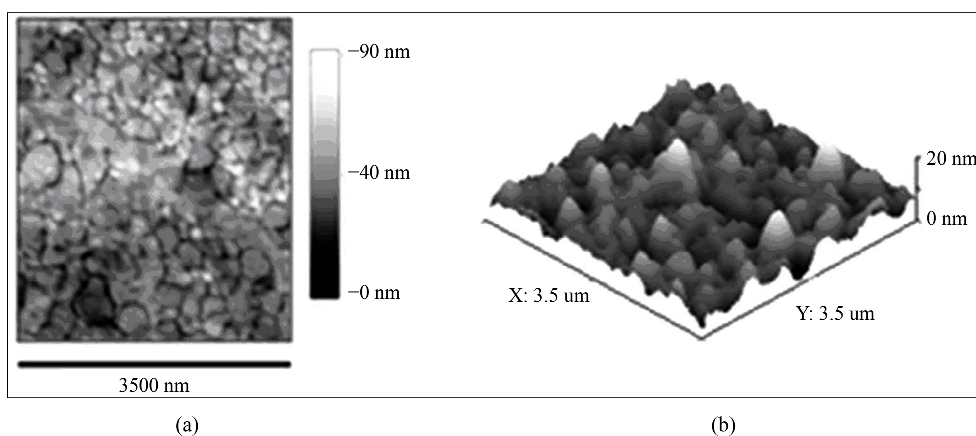


Figure 2. AFM image of surface of the quartz crystal functionalized with the DSP: (a) Phase-imaging; (b) Topography 3D image.

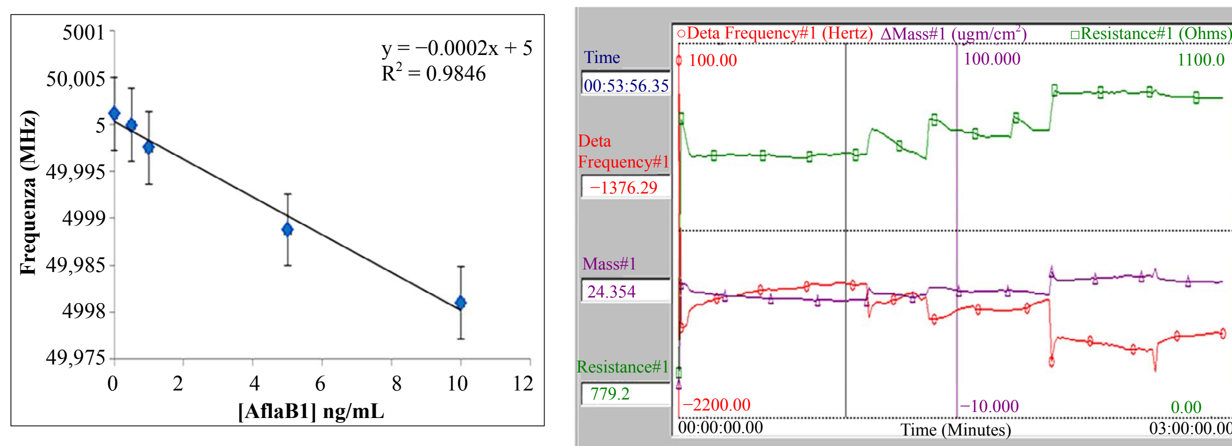


Figure 3. Standard curve by immunoassay using polyclonal antibodies directed to different concentrations of AflaB1 (on left) and relationship between Δf (red line) a function of mass (violet line) and resistance (green line).

ing values:

- LOD = 0.3 ng/ml
- Working range: 0.5 - 10 ng/ml

The detection limit (LOD) is estimated from the mean of the blank, the standard deviation of the blank and some confidence factor. The value of is given by the equation:

$$x_L = x_{b1} + k s_{b1} \quad (1)$$

where x_{b1} is the mean of the blank measures, s_{b1} is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired.

The RQCM allows to measure simultaneously the Δf and ΔR, the frequency has a decreasing trend, as described by Sauerbrey equation [28] with increasing mass deposited on the surface of the quartz crystal is a reduction of the resonance frequency of the crystal.

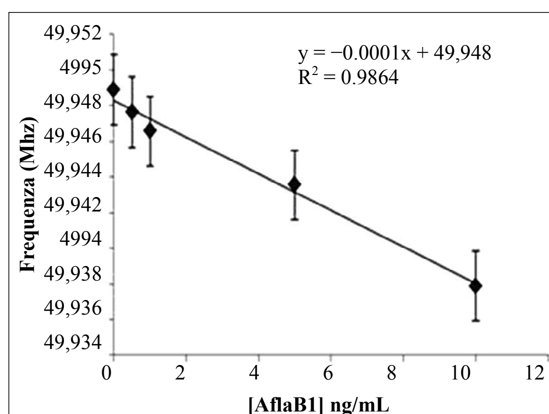
3.3. Standard Curve for Aflatoxin B1 (Immunoassay Performed with a Monoclonal Antibody mAb Anti-Aflatoxin B1 Immobilised on Beads)

Using the protocol described before and through the use of another type of antibody specific for the Aflatoxin B1 (a monoclonal antibody), we conducted a series of experiments, especially in order to improve the sensitivity of our system and could construct a second standard curve. In this case, we used monoclonal antibodies to the Aflatoxin B1 immobilized on beads. The results obtained are shown in **Figure 4**. We have built a standard curve for the Aflatoxin B1, obtaining very encouraging results about the possibility to propose our system as a screening test for this mycotoxin. As shown by the graphs, the two different systems have similar results especially in terms of measurement sensitivity. The system using polyclonal antibody would seem to be more sensitive and certainly better meets the analytical needs required for the determination of Aflatoxin B1.

Monoclonal antibodies, in fact, generally show a greater selectivity and affinity for the antigens against which they are produced, compared to polyclonal immunoglobulins. For the extraction of aflatoxin B1 from real samples (peanuts) considered in this work, we used a standard protocol described previously. In **Figure 5** we have a graph that relates the frequency as a function of concentration, each point on the curve corresponds to the addition of Ab specific monoclonal AflaB1 and to the sample of Aflatoxin B1 diluted 1:100, 1:50, 1:10 and 1:5, the curve has a decreasing trend which corresponds to the decrease of the resonance frequency of the quartz crystal. For the development immunoassay direct we used the monoclonal antibodies immobilized on beads as generally show greater selectivity for the antigens against which they are produced, compared to the polyclonal immunoglobulins.

4. Conclusion

These results, since they are related to a direct immunoassay using monoclonal antibodies specific for Aflatoxin B1 (mAb anti-AflaB1) immobilized on beads, bound on the surface of quartz pre-treated, by means of which increases the mass on the surface of the quartz crystals is a reduction of the resonance frequency of the crystal, are of particular importance, since they show the applicability and feasibility of direct immunoassay format with immobilized antibodies. Unfortunately, we have found that the step of functionalization of the quartz crystals affect the success of the experiment, however, since the greater advantage of the QCM results in a label-free system that allows the identification of different analytes, in our system that characteristic not only is preserved, but, being



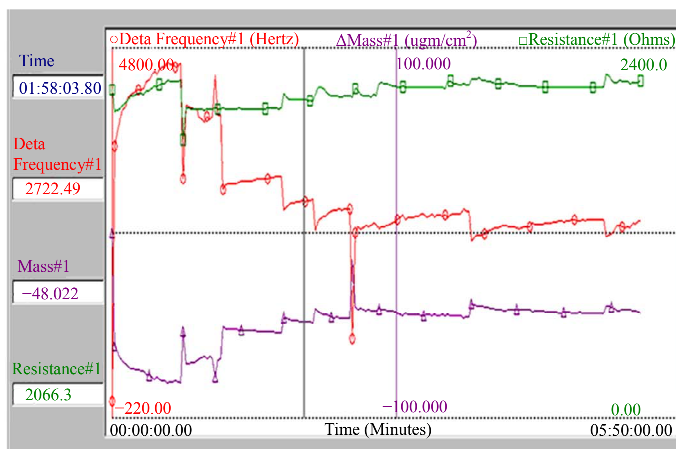


Figure 4. Standard curve by direct immunoassay using monoclonal antibodies immobilized on beads (on left) and relationship between Δf (red line) a function of mass (violet line) and resistance (green line).

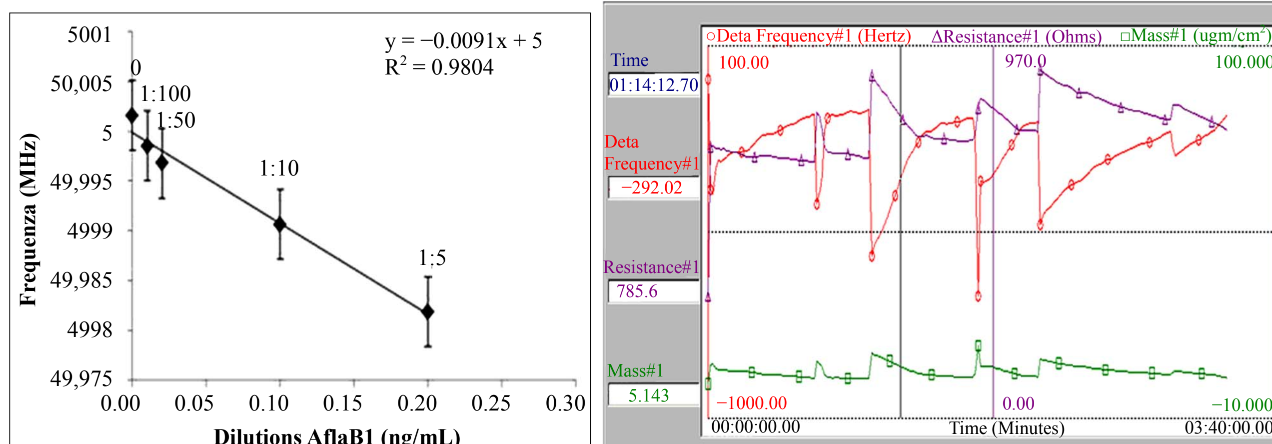


Figure 5. Standard curve by direct immunoassay using monoclonal antibodies for AFLAB1 applied in the sample (on left) and relationship between Δf (red line) a function of mass (green line) and resistance (violet line).

applied to the identification of mycotoxins, can assume new application scenarios of the QCM, based on the use of antibodies immobilized on a solid support in the field of food security and, therefore, human health. Frequency and resistance shifts (Δf and ΔR) were measured simultaneously. Δf versus mycotoxins concentrations in the range of 0.5 - 10 ppb exhibited a perfect linear correlation with a coefficient of above 0.998. Monoclonal antibodies, in fact, generally show a higher and better specificity for antigens against which they are produced, as compared to polyclonal immunoglobulin. The QCM based sensing label free procedure for mycotoxin detection, developed in our laboratory, can be considered a simple, cost effective, real time and no time and labor consuming technique in comparison with conventional assay procedures, as GC chromatography. It is important to remember the QCM measures frequency changes and not mass changes, then a strictly keeping constant of the experimental conditions while a typical QCM immunosensor experiment is necessary.

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Biographies

Katia Spinella received her MSc degree in industrial biotechnology from University of Rome, TorVergata, in 2012. She is pursuing her PhD in materials for health, environment and energy in the University of RomeTor-Vergata. She is currently working onthe development aptamers based biosensors for food contaminants.

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Giuseppe Palleschi is the head of Chemistry Department of the University of Rome "Tor Vergata". Since 1989 member of the Scientific Committee for the "Emergency Plan for National Defense Pollution of hydrocarbons or other harmful substances caused by marine accidents", Department of Civil Protection of the Presidency of the Council of Ministers. The activity of prof. Palleschi, Professor of Analytical Chemistry, is mainly focused on the development and application of chemical sensors, biosensors and immunosensors for food, environmental and clinical analysis.

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