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Abstract

Mycotoxins, such as aflatoxins and ochratoxin A, are presently considered as the most important chronic dietary risk factor, higher than food additives or pesticide residues. Therefore, the serious health and economic consequences of mycotoxin contamination have created the need for rapid, sensitive, and reliable techniques to detect such dangerous molecules within foodstuffs.

We here report on the development of an innovative immunosensing method for mycotoxin detection, based on antibody-immobilized microcantilever resonators, a promising label free biosensing technique. A considerable part of the work is devoted to show the effect on microcantilever resonance frequency of the composition of the incubation buffer, as well as of the washing and drying procedure. We show the feasibility of using microcantilever resonator arrays to effectively identify total aflatoxins and ochratoxin A, at low concentrations (3 ng/mL and less than 6 ng/mL, respectively), with relatively low uncertainty (about 10%) and good reproducibility for the same target concentration. Furthermore, the developed immunosensing method shows limited cross-reactivity to different mycotoxins, paving the way to a highly specific technique, able to identify different mycotoxins in the same complex matrix. To our knowledge, this work represents the first example in literature of successfully immunodetection of low concentrations of multiple mycotoxins by microcantilever resonator arrays.

1. Introduction

Mycotoxins are secondary metabolites produced by molds with severe toxic, carcinogenic, mutagenic, and immunosuppressive effects. Around 25-50% of the crops harvested worldwide are contaminated with mycotoxins. Mycotoxins are presently considered as the most important chronic dietary risk factor, higher than food additives or pesticide residues (Van Egmond et al., 2007). Among the known mycotoxins, aflatoxins (AFs) and ochratoxin A (OTA) are of greatest concern due to their frequent occurrence in foods and their severe effects on animal and human health (Eaton and Gallagher, 1994).

AFs are a group of highly toxic difuranocoumarin derivatives produced by *Aspergillus* species. AFs can be found in cereals, oilseeds, crude vegetable oils, tree nuts, figs and other dried fruits, spices, coffee, cocoa beans and milk. Chronic aflatoxicosis causes cancer, with the liver as the primary target organ, immune suppression, teratogenicity and other symptoms (Bennett and Klich, 2003). AFB_1, the major AF produced by toxigenic strains, is the most potent hepatocarcinogen known in mammals and is classified by the International Agency of Research on Cancer (IARC) as human carcinogen (group 1) with a role in aetiology of liver cancer.

Another mycotoxin, OTA is widely distributed and mainly produced by *Aspergillus ochraceus*, *A. carbonarius* and *Penicillium verrucosum* (Spadaro et al., 2011). OTA occurs in various products such as cereals, beans, groundnuts, spices, dried fruits, coffee, milk, grape juice, beer and wine, as well as in pork meat. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic to several animal species and to cause kidney and liver tumors in mice and rats. OTA is suspected to be involved in the Balkan Endemic Nephropathy (BEN), a fatal kidney disease occurring in some areas of south-eastern Europe and to be associated with urinary tract tumors.

National and international institutions and organizations, such as the World Health Organization

(WHO), the Food and Agriculture Organization (FAO), the US Food and Drug Administration (FDA) and the European Commission (EC), have recognized the potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication and the economic consequences of mycotoxin contamination were well demonstrated. Regulations have been established in many countries to protect consumers from the harmful effects of these natural toxins, as well as to ensure fair practices in food trade. The European Union (EU) has established with the Commission Regulations 1881/2006 and 165/2010 severe limits for major mycotoxins in many products at high risk of contamination. As regards the AFs (which include AFB_1, AFB_2, AFG_1 and AFG_2), the Maximum Levels (MLs) set by the EC in food for direct human consumption are from 4 to 15 $\mu g\ kg^{-1}$ for the sum of AFs, whereas for OTA the ML adopted is ranging from 2 to 10 $\mu g\ kg^{-1}$. This regulatory limits force all Member States to monitor and control mycotoxin levels in foodstuffs in order to reduce the intake of this toxic metabolites. The serious health and economic consequences of AF and OTA contamination have created the need for rapid, sensitive, and reliable techniques to detect mycotoxins within foodstuffs. Several analytical methods have been used for AFs and OTA monitoring, such as thin-layer chromatography (TLC) (Stroka et al., 2000), high-performance liquid chromatography (HPLC) coupled to fluorescence or mass spectrometry detectors, and immunological methods (Maragos and Busman, 2010). Although generally regarded as robust and sensitive, the need for well-equipped laboratory facilities and the requirements for highly-trained personnel strongly limit the routine implementation of chromatography-based approaches (Krska et al., 2008). Over the past years most laboratories have tended to move towards using antibody-based affinity column clean-up which is relatively simple to carry-out and provides sample extracts generally free of interferences (Spadaro et al., 2010).

Cantilever-based microbalances, with molecular recognition coatings for target immobilization, are one of the most promising classes of label free biosensor platforms (Waggoner and Craighead, 2007). The mass adsorbed to the cantilever surface is monitored by the change in resonance frequency of the

sensor: the obtained mass resolution is in the nano- to zeptogram range, when vibrational curves are monitored directly in liquid environment (for real-time measurements) or in vacuum conditions (to minimize dumping effects and therefore enhancing mass sensitivity), respectively (Arlett et al., 2011). Cantilever biosensors can incorporate different molecular recognition strategies, representing a technological platform adaptable to different targets, such as microorganisms, biomolecules and nucleic acids (Johnson and Mutharasan, 2012). Furthermore, such technique was recently demonstrated to be successfully integrable as a diagnostic tool on a Lab-On-Chip (LOC) platform, to reduce assay times and limits sampling and/or sample preparation, providing compact and portable objects for biomolecule (Ricciardi et al., 2010a; Waggoner at al. 2010) or microorganism (Ricciardi et al., 2010b) identification. In spite of a considerable literature on microcantilever (MC) biosensing, few works reported on the successful detection of residues of immunogenic small molecules such as pesticides (Alvarez et al., 2003; Raiteri et al., 1999; Suri et al., 2008), toxins (Tark et al., 2010), hormones and antibiotics (Tan et al., 2010). Probably in consideration of the high mass sensitivity and precision needed to detect small targets with low molecular weight, the aforesaid papers reported the use of the static approach, where the deflection of the cantilever due to surface stress variation generated by antigen/antibody formation is monitored. However, static mode is often subjected to important restrictions, such as stabilization problems due to thermal drift (Lochon et al., 2006) and difficulty to link the deflection change due to specific molecular adsorption to the amount of the adsorbed molecules (Eom et al., 2011). This implies that immunodetection by MC resonators is to be preferable when a quantitative study on molecular interactions is aimed (Eom et al., 2011).

The present work deals with the development of an immunosensing method based on antibody-immobilized MC resonator arrays, to detect low concentrations of total AFs and OTA in buffer solutions, with the aim of developing a new diagnostic tool to identify different mycotoxins within

foodstuffs. FRASE SU SVILUPPI/MOTIVAZIONI? PRIMA VOLTA IN LETTERATURA? The results are presented into two subsections: first, the optimization of the method (buffer, washings, reproducibility) for AFs (AFB_1, AFB_2, AFG_1 and AFG_2) detection is reported; second, the optimized method is applied to OTA detection and the cross-reactivity of the immunosensing platform to the different mycotoxins is tested.

2. Materials and Methods

2.1 Reagents

3-aminopropyltriethoxysilane (APTES, anhydrous, 99% Aldrich), glutaraldehyde (GA, 25% v/v water solution), toluene (anhydrous, 99.8% Aldrich), sulphuric acid (95-97% w/w) and hydrogen peroxide (30% w/w) were purchased from Sigma-Aldrich. ACS reagents (essay ≥99.5%) were chosen to prepare borate buffer, in particular ortoboric acid and sodium chloride were used and purchased from Sigma-Aldrich too. Recombinant Protein G, purified from Streptococcus, was from PIERCE. Phosphate-Buffered Saline (PBS) was from GIBCO®.

Two mL of each analytical standards of AFB_1, AFB_2, AFG_1, AFG_2 and OTA were purchased from Sigma-Aldrich (St Louis, MO, USA) at the concentration of 2 µg/mL in acetonitrile (CH_3CN). The standards were dried by heating and re-dissolved in 3 mL HPLC grade methanol (MeOH, Sigma-Aldrich) and phosphate buffer (1:2 v/v) solution (PBS “Dulbecco”, Sigma-Aldrich). Working standard solutions at different concentrations were prepared by serial diluting the standard solutions, to test the buffer effect on the developed immunosensing MC-based platform. The solutions were stored at 4°C in the dark. Total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A ELISA kits were purchased from Euroclone (Pero, Italy). The same anti-aflatoxins antibody of the ELISA kit was used

for MC sensors, while the anti-ochratoxin A antibody (IgG1-lambda) was purchased from Antibodies-online (Aachen, Germany).

2.2 Microcantilever fabrication and functionalization

MC resonators were fabricated in clean room (class 1000) using a combination of bulk and surface micromachining processes on Silicon-On-Insulator (SOI) wafers: starting from the backside, a membrane is obtained by a KOH etching, and then MCs are released by a Reactive Ion Etching (RIE) process on the frontside. Each array consists of eleven MCs, with length, width and thickness respectively in the range of 460-600 μm, 50-70 μm, 5-10 μm. For details concerning the fabrication processes, please refer to (Canavese et al., 2007).

A silicon oxide film was grown on silicon MC arrays by thermal oxidation at 1100 °C in O2 atmosphere for 3 h. Before exposure to organosilanes, the SiO_2/Si MC arrays were cleaned by piranha solution (75% H_2SO_4: 25% H_2O_2) for 15 min to remove organic contaminants, followed by a deep rinsing in deionized water and dried with N_2. The substrate has to be freshly cleaned before the deposition of any APTES, in order to obtain a good reaction yield. After that, MC arrays were incubated (1% (v/v) APTES solution in toluene reflux at 70 °C for 10 min) following an anhydrous protocol (Ar flux and glassware dried in oven at 140 °C for at least 4 h), in order to reduce the presence of water that, acting such a catalyst, causes APTES hydrolysis in ethanol and trisilanols. Silane-coated MC arrays were rinsed deeply with toluene and well dried under N_2 flux. Just after the reaction, MC arrays were incubated in a 0.5 % (v/v) GA solution in borate buffer 0.1M (pH 8.5) for 1 h using an orbital shaker at 40 rpm. Borate buffer pH 8.5 ensures that amino groups exposed at MC surface are not protonated ($–NH_2$), so available to react with GA aldehyde groups (–CHO). 300 μL of sodiumcyanoborohydride solution (5M) in NaOH were added after 15 minutes, in order to create a

more stable bond, reducing the imine formed by the reaction between $–NH_2$ and –CHO groups. After incubation, MCs were rinsed several times with deionized water, dried under nitrogen stream and stored into a desiccator.

Then, functionalized MC arrays were incubated overnight in a protein G solution (50 µg/mL in PBS) at 4 °C. After the incubation, MCs were deeply washed in PBS-Tween 20 (0,05%), rinsed with deionized water for three times and dried in a N_2 stream. Protein G coated MCs were incubated in anti-aflatoxins or anti-ochratoxin A antibody at RT for 90 minutes using an orbital shaker at 40 rpm. After the antibody binding, MC arrays were washed as described before (PBS/water) and dried with nitrogen. The target molecule binding was performed incubating at RT for 90 minutes on orbital shaker, followed by a final washing step (PBS-Tween and deionized water) and dried with nitrogen.

2.3 Microcantilever experimental set-up and data analysis

Resonance curves were monitored thanks to the same apparatus described elsewhere (Ricciardi et al., 2010c): MCs were characterized in a vacuum environment (using a series of a membrane and turbomolecular pump – by Varian Inc. Vacuum Technologies) where a piezoelectric actuator (by PI Ceramics) was employed for the excitation and a position sensitive detector (PSD - by Hamamatsu) as detector. Output signal was amplified and filtered by means of a phase-loop lock in (by EG&G) and stored to a personal computer, together with the frequency of the oscillation piloted by the function generator (by HP) that gives the stimulus signal to the piezo disk. Cantilever arrays were attached to the actuator with double sided tape and held to a fix temperature thanks to a thermoelectric cooler-based temperature controller (by ILX Lightwave).

The measurement procedure as well as the fitting of data with a Lorentzian curve were performed by a software in LABVIEW® environment, able to extract the resonant frequency f and the quality factor Q

from fitting the measured amplitude of the cantilever vibration as function of the excitation frequency.

The experiments mainly consisted in characterizing the first flexural resonant mode (typically in the range of 50-80 kHz) of the MCs comprising each array in vacuum before and after target molecule immobilization. Using the following well-known equation, it is possible to link the consequent mass increment Δm to the shift of the resonant frequency Δf, simply as:

$$\Delta m = -\frac{1}{2}\frac{\Delta f}{f_0} m \qquad (1)$$

where f_0 and m are the resonant frequency and the effective mass before the binding event, respectively. Although this simple relation has been recently applied to correctly gain quantitative information about biomolecular interactions (Oliviero et al., 2008, Ricciardi et al., 2010c), a deep knowledge of antibody structure and surface density, of mass contribution from non-specific bindings (Waggoner and Craighead 2007), of stiffness and thickness of the added layer (Gupta et al., 2006, Tamayo et al., 2006), of the uniform localization of binding areas (Johnson and Mutharasan 2012), is needed to correctly use it. Due to the lacking in information and literature about mycotoxins immunodetection with Ab-immobilized MC resonators, we here consider eq. 1 just as a rough approximation and thus preferred to directly link the measured frequency shift to sample concentration.

Since each MC has slightly different geometrical dimensions (due to inherent tolerances in the fabrication process), and thus slightly different frequency, it is recommended to take into account of the relative frequency deviation $\Delta f / f$ rather than the absolute frequency shift of each MC. To compare different bioexperiments, we calculate the mean relative frequency shift $\overline{\Delta f / f}$ over the eleven cantilevers comprising the array, and use the standard deviation of the mean as uncertainty.

2.4 Reference analysis with HPLC

Liquid chromatography was used to quantify the standards for AFs and OTA for microcantilever immunosensor analysis. In particular, the analysis of AFs was performed with a Varian Model 212-LC micro pump (Hansen Way, CA, USA), coupled with a Varian autosampler Model 410 Prostar equipped with a 100 µL loop. The analytical column used for LC separation was a Pursuit XRs Ultra C18 (100 mm x 2.0 mm, 2.8 µm particle size, Varian). The chromatographic conditions were: column temperature at 30°C, and mobile phase consisting of eluent A (water with 0.1% acetic acid) and eluent B (methanol with 0.1% acetic acid), using a flow rate of 0.2 mL/min. A gradient elution was applied as follows: 0-25 min: from 90% A to 15% A; 25-28 min: from 15% A to 90% A; 28-30 min: 90% A. The injection volume was 10 µL. The LC system was coupled to a triple quadrupole mass spectrometer, Varian 310-MS, and was operated in the positive electrospray ionization mode (ESI^+). The ionization source conditions were: needle voltage of 6 kV, capillary voltage of 55-77 V, source temperature of 50 °C, desolvation temperature of 300°C, cone gas flow rate of 50 psi, desolvation gas flow rate of 20 psi with nitrogen. Multiple reaction monitoring (MRM) mode of operation was used. The $[M+H]^+$ ions of aflatoxins were used as parent ions. The most intense daughter ions, resulting from collision-induced dissociation with argon, were used to detect and quantify aflatoxin content. The argon pressure was set at 1.8 psi. The most intense daughter ions detected were: m/z 284.9 at 14 eV of collision energy (CE) for AFB_1, m/z 286.9 at 18 eV CE for AFB_2, m/z 242.9 at 18 eV CE for AFG_1, m/z 245 at 24 eV CE for AFG_2.

OTA analysis was performed in a HPLC apparatus Agilent 1100 series (Agilent, Waldbronn, Germany) equipped with G1379 degasser, G1313A autosampler, G1316A column thermostat set at 27°C, G1321A Fluorescence Detector set at excitation and emission wavelengths of 333 and 460 nm, respectively, G1311 quaternary pump and Agilent Chemstation G2170AA Windows XP operating

system. An analytical column RP-18 (XTerra Waters, Milford, MA, USA; 150 mm x 4.6 mm i.d., 5 μm) with a pre-column was used. The mobile phase, eluting at 1 mL/min, consisted of an isocratic mixture of acetonitrile:water:acetic acid (99:99:2) for 18 min. 100 μL of standard were injected into the HPLC column and the retention time of OTA was ca. 6.23 min.

3. Results and discussion

3.1 Optimization of a microcantilever-based immunosensing method for aflatoxins (AFs) detection

Mycotoxins are commonly stocked in acetonitrile buffer solutions, because when such molecules are highly concentrated they can be well solved in highly polar organic solvents only. On the other hand, it is well known that proteins are highly sensitive to such reagents: for immunosensors, this fact represent a serious risk because even a partial unfolding or denaturation of the antibody structure can drastically modify the mechanism of antigen recognition (both in terms of sensitivity and selectivity). Therefore, we checked the behavior of Ab-immobilized MC resonators, when incubated with PBS buffer solutions containing CH_3CN, without any target molecule. Solutions of acetonitrile/PBS in the range 0,05-12,5% v/v were prepared to simulate the dilution of mycotoxin stock solutions (2 μg/mL) to a typical operative range of 1-250 ng/mL (part-per-billion, ppb). Cantilever sensors were set in the prepared buffer solutions for two hours to simulate a typical experiment of antigen/antibody binding. Incubations were repeated twice, to evidence and confirm the monitored effect. Anti-AFs antibodies were used for this preparatory experiment.

As reported in Section 2.2, our immunoassay procedure comprises four steps, before the final incubation with the antigen-containing solution: the oxidation to active the Si surface, the chemical

derivatization with APTES and GA to expose aldehyde groups, the binding of protein G to induce a correct orientation of the antibody, and the immobilization of the antibody as the biorecognition element. Figure 1 reports the frequency of three MC sensors after the aforementioned steps (labeled on the *x* axis as “oxidized”, “APTES+GA”, “PtG”, and “Ab”, respectively) and after two incubations of two hours each (both labeled as “Buffer”) with: pure PBS, CH_3CN/PBS at 0.05% v/v and CH_3CN/PBS at 12,5% v/v. Please, note that in Fig. 1 MC resonant frequency is normalized to the value after oxidation, to show a comparison in terms of relative shifts. As expected, all the MCs experienced negative frequency shifts after the chemical functionalization with APTES and GA, and after the binding of protein G and antibody. As pointed out in Section 2.2, eq. 1 can be used to link this shift to the adsorbed mass, but here the most interesting part of the graph is represented by the variation of the MC frequency after the incubation with buffers (highlighted in the box?). As it can be clearly seen in Fig. 1, while the incubations with pure PBS buffer (green triangles) properly show no noticeable effects on the sensor resonance frequency ($\overline{(\Delta f / f)}_{PBS} < 10^{-5}$), incubations with CH_3CN/PBS buffers (red circles and black squares) always exhibit remarkable positive shifts ($\overline{(\Delta f / f)}_{CH_3CN/PBS} \geq 10^{-4}$), indicating a decrease in the oscillator mass. This behavior probably means that when our Ab-immobilized MCs are exposed to CH_3CN/PBS solutions, even at concentration as low as 0.05% v/v, antibody and protein G may unfold and unbind from the surface. This interpretation can be strengthen by the fact that the MC final frequencies after incubations with CH_3CN/PBS solutions are close to the ones registered after the chemical functionalization with APTES and GA: as a matter of fact, a pretty much higher activation energy would be need to break such bonds, due to their covalent nature.

Since the presence of acetonitrile, even in traces (0.05% v/v), was demonstrated to drastically affect the development of the immunoassay, the mycotoxin standards were dried by heating and re-dissolved in a solution containing methanol, a lower polar organic solvent than acetonitrile, and PBS (1:2 v/v). Five

solutions containing 0/100%, 20/80%, 50/50%, 80/20% and 100/0% of MeOH/PBS v/v were prepared and eight concentrations of AFs or OTA (0.0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 ng/mL) were added to each solution. The evaluation of the activity of the antibodies to different concentrations of methanol was preliminary tested by ELISA, for each mycotoxin and MeOH/PBS concentration. The experiments, not reported here, showed an effective bioactivity of anti-OTA antibodies up to 50% methanol, while the anti-AFs antibodies were effective up to 80% methanol. Experiments were always performed twice. We finally decided to use a 20/80% v/v of MeOH/PBS, to exclude any adverse effect of the solvent on the immunorecognition mechanism: all the measurements hereafter reported were done with such buffer solution. This preliminary activity was optimized, bearing in mind the analyses on real samples, where AFs and OTA extractions guarantee higher recovery in presence of higher methanol concentration (Imperato et al., 2011).

The histogram of the mean relative frequency shift of four MC resonator arrays, respectively incubated with 0, 0.3, 3 and 30 ng/mL of total AFs solutions, is reported in Fig. 2. The value correspondent to 0 ng/mL (ppb) resulted extremely small, $\overline{(\Delta f / f)}_{0\,ppb} = (-0,4 \pm 1,6) \times 10^{-5}$, while the others varied between $\overline{(\Delta f / f)}_{0.3\,ppb} = (-2,0 \pm 1,5) \times 10^{-5}$ and $\overline{(\Delta f / f)}_{30\,ppb} = (-10,2 \pm 9,0) \times 10^{-5}$, following almost linearly the correspondent variation of target concentration (please, note that since columns of the histogram are equally space, *x* axis in Fig. 2 follows a pseudo-logarithmic scale). By the way, the quantification of total AFs at different concentrations using the histogram in Fig. 2 would be invalidated by the remarkably large uncertainty (up to 90% of relative error) calculated over each MC array. Such a large deviation between the measured resonance frequency shift of each MC comprising the array can be ascribed to an inefficient washing and/or drying procedure in removing water content and possible salt residuals, as well as excess of unbound mycotoxins. As a matter of fact, it is overall well established that non-specific bindings often represents the primary constraint of cantilever-based biosensing

(Waggoner and Craighead, 2007), where the absence of a labeled secondary molecular recognition can limit the assay specificity. Furthermore, variation of biomolecule hydration and conformation (Sharma and Kalonia, 2004), as well as salt precipitation (Liu et al., 2009) possibly occurring with the dip-and-dry method, can drastically alter the quantitative response and the repeatability of the assay. Therefore, in addition to that of the incubation buffer, a very careful evaluation of the influence of washing and drying procedures on cantilever biosensing experiments is needed.

The protocol was then improved as follows: after standard washings (PBS-Tween and doubly distilled water) MCs were put in vacuum for 5 minutes, then rinsed again with doubly distilled water and finally dried again in a stream of nitrogen. The histogram reported in Fig. 3 shows the measurements of four MC resonator arrays incubated with 0 and 3 ng/mL of total AFs solutions, respectively before (RUN I) and after (RUN II) the implementation of the improved procedure. The mean relative frequency shift of each array resulted respectively: $\overline{(\Delta f / f)}^{I}_{0ppb} = (-0,4 \pm 1,6) \times 10^{-5}$ and $\overline{(\Delta f / f)}^{I}_{3ppb} = (-2,8 \pm 1,2) \times 10^{-5}$ for RUN I, $\overline{(\Delta f / f)}^{II}_{0ppb} = (-0,5 \pm 0,1) \times 10^{-5}$ and $\overline{(\Delta f / f)}^{II}_{3ppb} = (-2,7 \pm 0,3) \times 10^{-5}$ for RUN II. Thanks to the optimization of washing and drying procedure, the uncertainty over the MC arrays was drastically decreased, reaching a remarkably low relative error close to 10% for the value related to 3 ng/mL. In such conditions, the quantification of low concentrations of total AFs is successfully achievable, as shown in Fig. 3 RUN II. In addition, the mean values remains definitely unchanged, indicating a good reproducibility of the MC resonator arrays when incubated with the same target concentration.

In conclusion, thanks to the optimization of the incubation buffer and the washing/drying procedure, the proposed method based on MC resonator arrays show the possibility to successfully detect low concentrations of total AFs such as 3 ng/mL (a value lower than current UE legal limit), with relatively low uncertainty and good reproducibility.

3.2 Implementation of the method to ochratoxin A (OTA) detection

This second part deals with the implementation of the MC-based immunosensing method developed for total AFs to the detection of another dangerous mycotoxin: ochratoxin A (OTA).

Fig. 4 reports the histogram of the mean relative frequency shift of four MC resonator arrays, respectively incubated with 0, 0.6, 6 and 60 ng/mL (ppb) of OTA solutions. As for total AFs detection, an almost linear correlation with target concentration in this range is shown (please, note that *x* axis of Fig. 4 follows a pseudo-logarithmic scale, as it was for Fig.2). Incubations with solutions containing OTA concentrations of 6 and 60 ng/mL caused relative shifts of $\overline{(\Delta f / f)}_{6\,ppb} = (-5{,}8 \pm 0{,}7) \times 10^{-5}$ and $\overline{(\Delta f / f)}_{60\,ppb} = (-23{,}8 \pm 4{,}7) \times 10^{-5}$ respectively, thus resulting fairly distinguishable from the background signal correspondent to 0 ng/mL: $\overline{(\Delta f / f)}_{0\,ppb} = (-1{,}0 \pm 1{,}7) \times 10^{-5}$. The use of the previously optimized buffer and washing/drying procedure guaranteed an acceptably low relative uncertainty: 12% and 20%, respectively. On the other hand, the mean relative frequency shift of the MC array incubated with 0.3 ng/mL OTA resulted $\overline{(\Delta f / f)}_{0.3\,ppb} = (-2{,}2 \pm 0{,}9) \times 10^{-5}$, a value that can hardly be distinguishable from the background level (0 ng/mL). Therefore, we can estimate that the Limit Of Detection (LOD) of the method would be between 0.6 and 6 ng/mL, a lower range than current EU maximum limits of 2-10 ng/mL. A correct calculation of our method LOD would need a previous identification of the concentration dynamic range, as well as the use of more data for a correct fitting, and is beyond the scope of the present work.

To exploit the possible use of MC resonator arrays as mycotoxin sensors, a first evaluation of the selectivity of the technique was made, by checking the cross-reactivity of the developed immunosensing method to different target molecules. To this aim, three MC arrays were functionalized with the same antibody (anti-AFs) and incubated with MeOH/PBS buffer solutions containing different

concentrations of AF and OTA: 3/0, 0/0, and 0/6 ng/mL. The correspondent mean relative frequency shifts are reported in the histogram of Fig. 5. The incubation of the MC array coated with anti-AF antibody with a solution containing the four aflatoxins as *target* molecules caused a well measurable shift of $\overline{(\Delta f / f)}_{OTA=0}^{AF=3} = (-2,2 \pm 0,5) \times 10^{-5}$, absolutely equivalent to the other values previously reported for similar experiments with the same concentration of AFs: 3 ng/mL. Such result confirms again the good reproducibility of the method to the same target concentration. On the other hand, the incubation with a solution containing a *competitor* molecule, OTA at 6 ng/mL, induced a very small frequency change, $\overline{(\Delta f / f)}_{OTA=6}^{AF=0} = (0,4 \pm 0,9) \times 10^{-5}$, definitely comparable in its absolute value with the one due to the solution without antigens, $\overline{(\Delta f / f)}_{OTA=0}^{AF=0} = (-0,5 \pm 0,1) \times 10^{-5}$, as well as to other values previously reported for similar incubations in buffer solution. Therefore, we can assume that the cross-reactivity of the developed method based on MC resonator immunosensors to different mycotoxins would be comparable to the experimental frequency fluctuations induced by a blank sample. The determination of such limited cross-reactivity of the here proposed immunosensing method represents the first step to develop a highly specific technique, able to identify different mycotoxins in the same complex matrix.

4. Conclusions

The development of a microcantilever-based immunosensing method for mycotoxin detection was here reported. A considerable part of the work was devoted to show the effect on microcantilever resonance frequency of the composition of the incubation buffer, as well as of the washing and drying procedure. We showed the feasibility of using microcantilever resonator arrays to effectively identify total aflatoxins and ochratoxin A, at low concentrations (3 ng/mL and less than 6 ng/mL, respectively), with

relatively low uncertainty (about 10%) and good reproducibility for the same target concentration. Furthermore, the developed immunosensing method showed a limited cross-reactivity to different mycotoxins, paving the way to a highly specific technique, able to identify different mycotoxins in the same complex matrix. To our knowledge, this work represents the first example in literature of successfully immunodetection of low concentrations of multiple mycotoxins by microcantilever resonator arrays.

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Figures

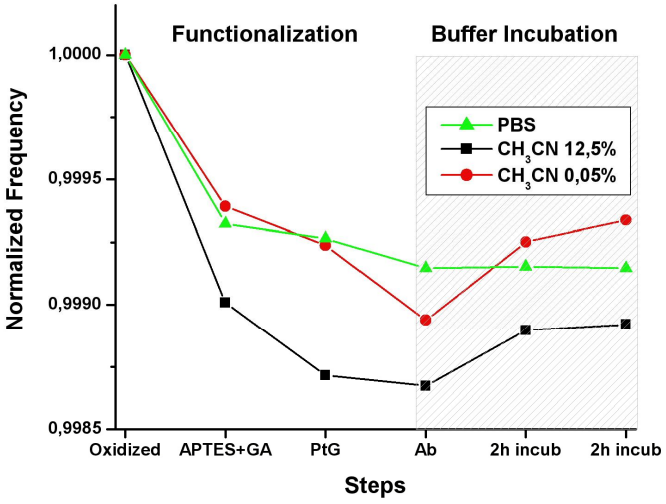
Fig. 1. Effect of buffer (MeOH/PBS) composition on MC resonant frequency. *x* axis refers to the different steps of the immunoassay procedure: Si surface activation ("oxidized"), chemical derivatization ("APTES+GA"), protein G binding ("PtG"), antibody immobilization ("Ab"), incubation with MeOH/PBS solution ("buffer") . Please see text for further details.

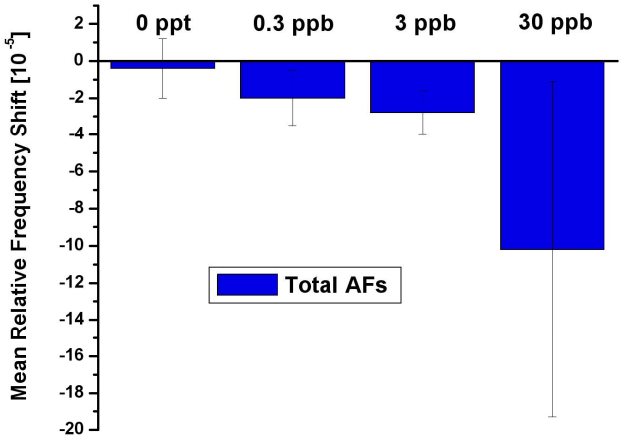
Fig. 2. Immunosensing of total aflatoxins (AFs) in the range 0-30 ng/mL (ppb).

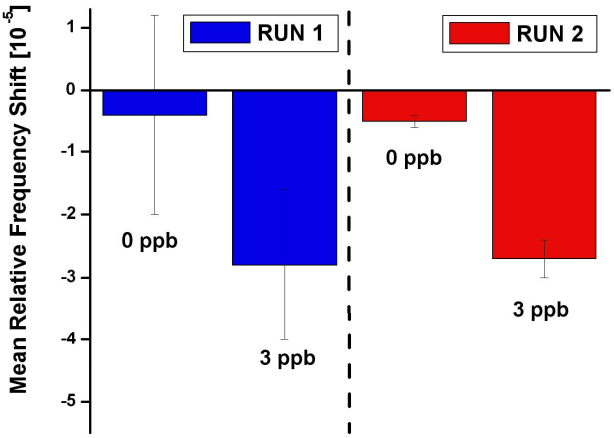
Fig. 3. Influence of washing/drying procedure on uncertainty and reproducibility of total AFs detection by MC resonator arrays. Details of differences between RUN I and RUN II are given in the text.

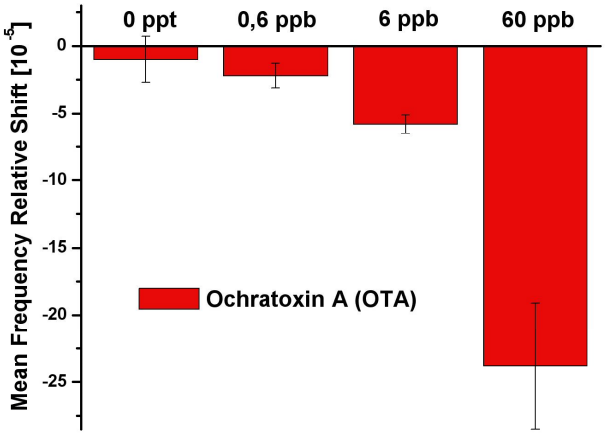
Fig. 4. Immunosensing of ochratoxin A (OTA) in the range 0-60 ng/mL (ppb).

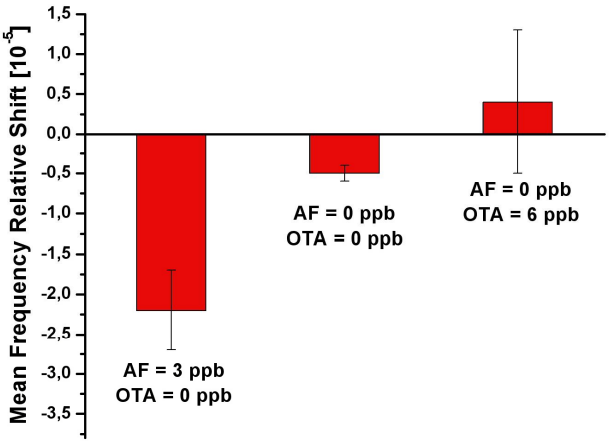
Fig. 5. Histogram of mean relative frequency deviation of MC resonator arrays incubated with MeOH/PBS buffer solution containing different concentration of AF/OTA: 3/0, 0/0, and 0/6 ng/mL.


Functionalization
Buffer Incubation
Normalized Frequency
1,0000
0,9995
0,9990
0,9985
PBS
CH3CN 12,5%
CH3CN 0,05%
Oxidized
APTES+GA
PtG
Ab
2h incub
2h incub
Steps


0 ppt
0.3 ppb
3 ppb
30 ppb
Mean Relative Frequency Shift [10-5]
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
Total AFs


Mean Relative Frequency Shift [10 -5]
RUN 1
RUN 2
1
0
-1
-2
-3
-4
-5
0 ppb
3 ppb
0 ppb
3 ppb


Mean Frequency Relative Shift [10 -5]
0 ppt
0,6 ppb
6 ppb
60 ppb
0
-5
-10
-15
-20
-25
Ochratoxin A (OTA)


Mean Frequency Relative Shift [10-5]
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
-3,5
AF = 3 ppb
OTA = 0 ppb
AF = 0 ppb
OTA = 0 ppb
AF = 0 ppb
OTA = 6 ppb