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Microcantilever Resonators for Ochratoxin A Detection in Food and Drinks

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Abstract: Mycotoxins food contamination represents a serious risk for consumers health. They are secondary metabolites of fungi that can be present in a wide range of foodstuffs. Ochratoxin A (OTA) is one of the most toxic compound and it is classified as a possible carcinogenic molecule. The harmful effects of OTA on human and animal health lead to a big boost to develop and optimize highly sensitive and accurate methods for OTA detection. An innovative and rapid detection method based on microcantilever resonators for ochratoxin A identification in food matrix has been developed. This work demonstrates the possibility to apply microcantilever technology in food safety field, showing for the first time in literature the successful detection of one of the most dangerous mycotoxin in different food matrixes both solids and liquids, such as green coffee, grape juice and wine. Sensing performances are discussed in terms of calibration plot and limit of detection.

Keywords: coffee; grape juice; immunosensing; mycotoxins; nanobiosensors; wine; microcantilever

1. Introduction

Ochratoxin A (OTA; $C_{20}H_{18}ClNO_6$) is a fungal metabolite, which can contaminate a wide number of different foodstuffs representing a serious threat to human health. In 1993, the International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogen for humans (group 2B) [1]. OTA is one of the most important mycotoxins, produced mainly by fungi of the genera *Aspergillus* (e.g., *Aspergillus ochraceus* and *Aspergillus carbonarius*) and *Penicillium* (e.g., *Penicillium verrucosum*) [2]. These species are ubiquitous and capable of growing on different plant products and under different climatic conditions, therefore OTA contamination is worldwide spread [3]. The chemical structure of OTA is very stable, as it is a 3,4-dihydromethylisocoumarin derivative linked with an amide bond to the amine group of an L-beta-phenylalanine. The mycotoxin can be found as a natural contaminant of a wide range of foods, such as cereals, beer, wine, cocoa, coffee, dried fruit and spices, as well as food of animal origin, such as milk and meat, as a result of contamination of animal feed [4,5]. Human exposure to OTA is mainly due to consumption of plant origin foodstuffs, and the three major sources of OTA are cereals, wine and coffee [6]. The presence of OTA in wine is well documented [7] and represents a worldwide problem [8]. About 10% of the total dietary intake originates from wine consumption [9]. OTA contamination in coffee was reported for the first time in 1974[10]. Since coffee is actually the second most consumed hot beverage in the world [11], the presence of OTA in this foodstuff represents a serious threat to human health.

OTA shows immunotoxic, nephrotoxic, hepatotoxic, teratogen and carcinogenic effects in animals and it is suspected to be the etiological agent of the Balkan Endemic Nephropathy (BEN), a fatal kidney disease occurring in some areas of south-eastern Europe, and to be associated with urinary tract tumours [12,13]. The European Union (EU) established, with the Commission Regulation 1881/2006, severe limits for the presence of ochratoxin A in products that are associated with a high risk of contamination. The Maximum Level of OTA in food for direct human consumption set by the EC is ranging from 2 to 10 µg/kg. In particular, the threshold of contamination is set at 5.0 µg/kg for roasted coffee and at 2.0 µg/kg for grape juice and wine [14].

Traditional analytical methods for mycotoxin quantification are highly sensitive and robust but very expensive because they require well-trained personnel and high-priced instrumentations. They are based on chromatographic techniques, such as Thin-Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) associated with fluorescent or mass spectrometry detectors [15,16], GC-MS [17]. In order to reduce the assay cost and allow a major food contamination control, unconventional methods are explored [18]. Biosensors could represent a valid alternative because of their rapidity, specificity, sensitivity and ease of use. In the last years, efforts were devoted to the development of novel analytical methods to detect OTA, but research activity was mainly focused on optical and electrochemical biosensors [19–24].

Cantilever-based biosensors are considered one of the most promising technology and attracted large interest for applications such as point of care diagnostics, food and environmental monitoring and homeland security. In fact, they combine cheapness, speed, sensitivity, specificity, portability, and label-free advantages [25]. Cantilever biosensors consist in singly-side clamped micro-beams opportunely functionalized with specific receptors in order to bind target species to its surface. They can work in static or dynamic mode: in static mode, only one side of the cantilever is activated and the target molecule can bind only on one side of the sensor, generating a surface stress and a deflection of the beam [26]. Dynamic mode cantilevers quantify the mass of the target bound to the surface through a shift in resonance frequency [27]. In fact, microcantilever (MC) oscillates with a specific resonance frequency, which changes when molecules land on the device proportionally to the mass added on the sensing area [28]. Examples of detection of ultrasmall masses are reported in literature, reaching a sensitivity ranging from femtogram down to zeptogram range [29]. When a specific antibody is attached on their surface, MC resonators can be used as biosensors, as successfully shown in literature to detect microorganisms [30], proteins [31–34] and small molecules [35–37].

The capability of MCs to detect OTA with high specificity and sensitivity was demonstrated by Ricciardi et al. [38], but the measurements were limited to buffer solutions.

Few biosensor performances are reported in literature for OTA identification in food samples, and they are mainly based on colorimetric and fluorescence detection. A QCM-D based biosensor was implemented for OTA detection in red wine with a LOD and a LOQ of 0.16 and 0.55 µg/kg, and a linear range between 0.2-40 µg/kg. The indirect competitive assay required an amplification of the QCM-D signal which was obtained by applying secondary antibodies conjugated with gold nanoparticles [39]. OTA in wine sample was successfully detected by Arduini et al. (2016) [40] using a portable fibre optic spectrometer reaching a LOD of 0.3 µg/kg. No cantilever detection in wine was reported in literature. An optoelectronic biosensor was used to quantify OTA in beer, another complex and slightly alcoholic matrix, reaching a detection limit of 2.0 ng/mL and a dynamic range 4.0-100 ng/mL [41].

In the present article, the possibility to detect OTA at low concentrations in food samples using MC resonators was investigated. In particular, the potential of nanobiosensors to detect OTA was evaluated in three food matrices, often contaminated by OTA: green coffee, grape juice and wine. In parallel to biosensing assays, OTA concentrations were quantified through a validated HPLC method [42] in order to directly correlate the signals of MCs and the amount of OTA extracted from the samples.

2. Results

Ochratoxin A was quantified with both HPLC apparatus and MC-based biosensing platform. The resonance frequency shifts, due to mass addiction to sensor surface, were monitored after each surface activation step, in order to check the effectiveness of chemical and biological functionalization. The quantification of OTA with MCs was performed by monitoring the resonance frequency before and after antigen binding. MCs-based platform for OTA detection was previously optimised [38], demonstrating high sensitivity, good reproducibility, and specificity of the method in buffer solutions. Therefore, a first set of experiments was carried out to evaluate how the MC signal is influenced by the different food composition and extraction protocol, i.e. the so-called *matrix effect*. Figure 1 reports the mean relative frequency shifts of four cantilever arrays, incubated respectively in deionized (DI) water, green coffee, grape juice and red wine, without any spiking of OTA. Each value is the mean of twenty-two microcantilever resonance shifts. MCs mean relative frequency shift for green coffee was found to be $\overline{(\Delta f/f)}_{coffee}$= (-1.39±1.80) x 10^{-5}, thus very similar in average to DI water $\overline{(\Delta f/f)}_{water}$= (-1.39±0.02) x 10^{-5}, but with a remarkably higher uncertainty. On the other hand, the resonance changes of MCs incubated in OTA-free grape juice and wine were found to be rather similar in terms of mean value and relative uncertainty, even if slightly larger respect to DI water and coffee: $\overline{(\Delta f/f)}_{juice}$ = (-2.29±0.89) x 10^{-5} and $\overline{(\Delta f/f)}_{wine}$ = (-2.18±1.1) x 10^{-5}. All arrays showed a limited negative frequency shift respect to the blue continuous line representing the instrumental uncertainty (0.7 x 10^{-5} as previously evaluated by Ferrante et al. (2017) [37]). Such behaviour is consistent with an increase in oscillating mass, due to small unspecific adsorption on sensing surfaces of salts and biomolecules from the extracted samples.

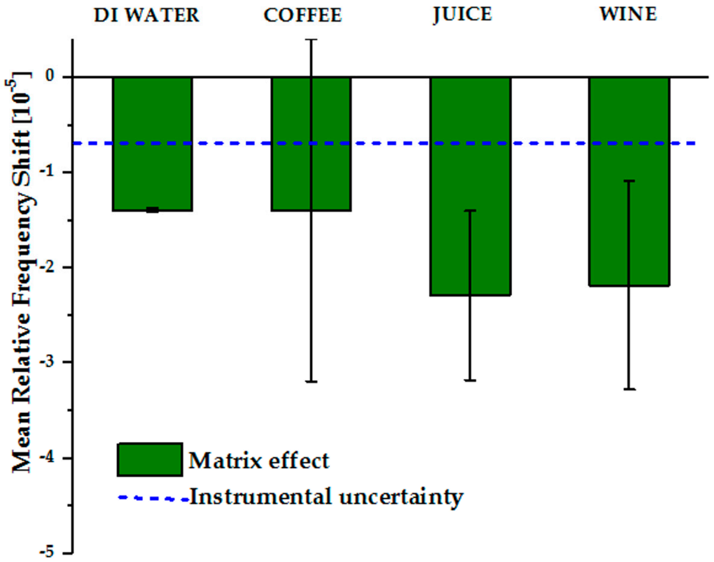

Figure 1. Matrix effect on microcantilever signals

2.1 Immunodetection of OTA in green coffee

Four different concentrations precisely quantified after OTA extraction with HPLC-FD (0 μg/kg, 3.9 μg/kg, 6.3 μg/kg, and 12.6 μg/kg) were used to investigate the biosensing performance. MCs mean relative frequency shifts were $\overline{(\Delta f/f)}_{0\mu g/kg}$= (-1.39±1.80) x 10^{-5}; $\overline{(\Delta f/f)}_{3.9\mu g/kg}$= (-2.01±1.03) x 10^{-5}; $\overline{(\Delta f/f)}_{6.3\mu g/kg}$= (-5.44±2.00) x 10^{-5}; $\overline{(\Delta f/f)}_{12.6\mu g/kg}$= (-16.4±3.94) x 10^{-5}. As often found in immunosensing experiments, the calibration plot shown in Figure 2 is well fitted by a sigmoid function, even if some more data would be needed for a quantitative fit.

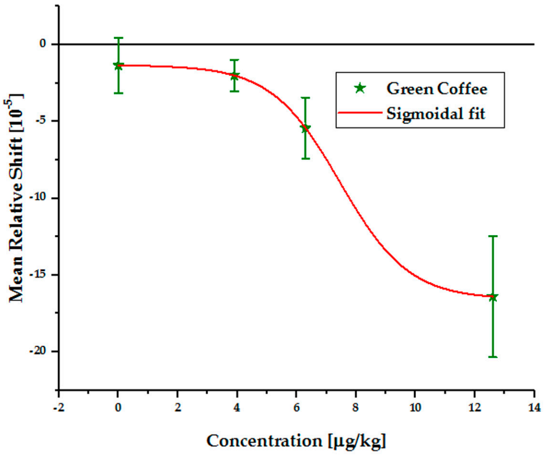

Figure 2 Immunosensing of OTA in green coffee, range 0-12.6 µg/kg.

In such a sigmoid curve, three regimes of interest can be described: a first zone for concentration lower than 4 µg/kg, where the matrix effect alone is comparable with the signal coming from the contaminated samples; a linear range, approximately between 4 and 11 µg/kg, where doubling the concentration of the target molecule, a nearly three-fold increase in frequency shift is obtained; a saturation, for concentration higher than 11 µg/kg, where all binding sites of MC sensors are occupied, thus leading to a constant signal regardless to the OTA contamination. Even if the sample contaminated with 3.9 µg/kg produces a mean relative frequency shift higher (as absolute value) than the uncontaminated one, the quantification of such concentration would be statistically unreliable, due to the relative high uncertainties of data, in particular of the blank.

2.2 Immunodetection of OTA in grape juice and wine

Before applying MCs to an expensive and complex matrix as wine to discriminate between contaminated and non-contaminated samples, four cantilever arrays were incubated in grape juice extracts containing different amounts of OTA; their mean relative frequency shifts are reported in **Error! Reference source not found.**

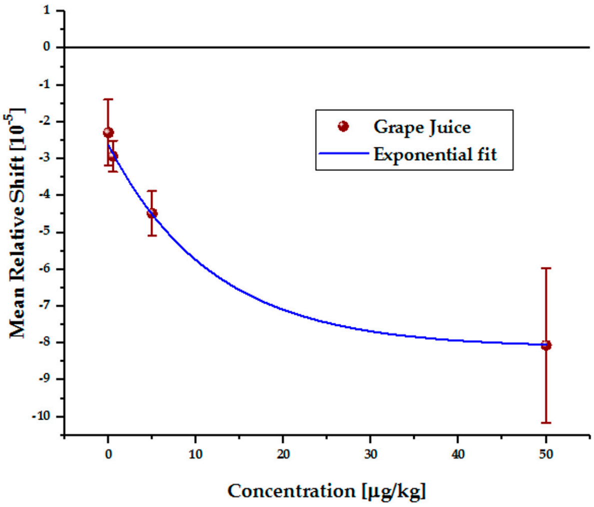

Figure 3 Immunodetection of OTA in grape juice using MCs

The OTA concentration varied from 0 to 50 µg/kg to investigate the capability of MCs to detect OTA in food matrix over a larger range, and to better assess the linear range for the target molecule. As for the case of green coffee, all OTA positive samples exhibited higher signal respect to blank solution: $\overline{(\Delta f/f)}_{0\mu g/kg}$= (-2.29±0.89) x 10^{-5}, $\overline{(\Delta f/f)}_{0.5\mu g/kg}$= (-2.93±0.42) x 10^{-5}, $\overline{(\Delta f/f)}_{5\mu g/kg}$= (-4.48±0.60) x 10^{-5}, $\overline{(\Delta f/f)}_{50\mu g/kg}$= (-8.06±2.1) x 10^{-5}. The calibration plot is well fitted by an exponential decay function (R^2=0.98 in Figure 3), instead of a sigmoid one, showing that not just the blank signal and the LOD are primarily influenced by the matrix effect, but the whole MCs biosensing performance is affected by it. A linear range is found for concentrations lower than 6 μg/kg, while, similarly to the previous case, the LOD is estimated at 5 μg/kg.

In addition, the analysis focused on the sensor capability to discriminate between contaminated and non-contaminated wines. Control wine with no OTA was compared to two samples, independently spiked with 2 μg/kg of OTA (the maximum level established by European legislation) and quantified with LC-MS, in order to evaluate the repeatability of the method. **Error! Reference source not found.**4 reports the histograms of the mean relative frequency shifts of MCs arrays: $\overline{(\Delta f/f)}_{0\mu g/kg}$= (-2.18±1.1) x 10^{-5}, $\overline{(\Delta f/f)}_{2\mu g/kg(a)}$= (-7.07±0.03) x 10^{-5} and $\overline{(\Delta f/f)}_{2\mu g/kg(b)}$= (-7.29±0.06) x 10^{-5}. Signals from both contaminated samples are nearly identical, while being clearly discernible from the control wine. Thus, MC arrays show the needed sensitivity and precision to detect OTA in wine.

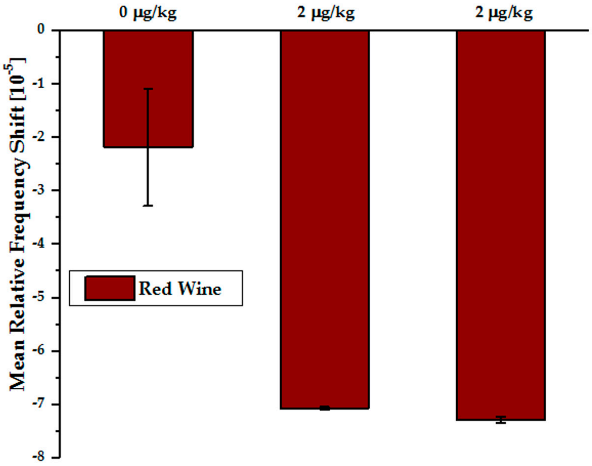

Figure 4. Immunodetection of OTA in wine with MCs

Error! Reference source not found.5 resumes the different performances of OTA immunodetection in real food samples by MC arrays, focussing on the lowest concentration ranges tested. Even if more data would be useful, it is clear that the matrix effect has a strong influence on the measurements.

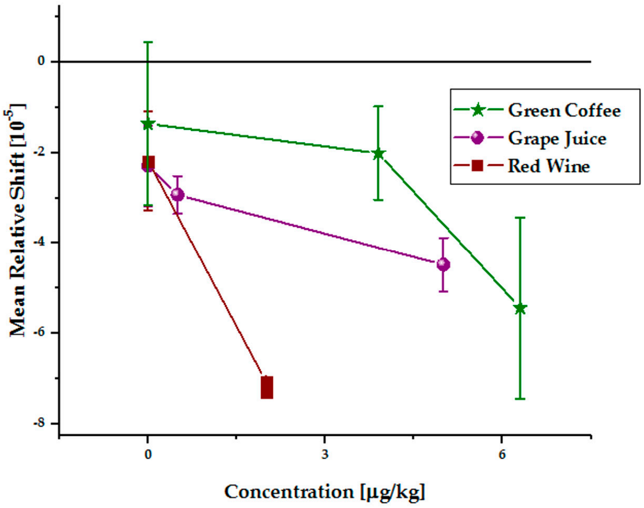

Figure 5. MCs detection of OTA in different food matrices

3. Discussion

To evaluate the microcantilever biosensor performances, the matrix effect was investigated on resonance frequency shifts, by using sample extracts previously confirmed to be OTA free. Green coffee matrix led to negligible resonance frequency shifts, similar to those produced incubating MCs with deionized water. Wine and grape juice instead gave a higher signal indicating a non-specific absorption of molecules on the biosensing platform. Such unspecific phenomena are typically large for very complex matrixes like grape juice and wine, for which the immunoaffinity column is not always able to remove all the matrix complex components. The same problem was encountered also by Todescato et al. [43] with a plasmonic based optical biosensor for OTA detection in grape juice. In this matrix, the quantification was not achieved because of the interference of some components present in the extract, which resulted in high background signal in the control and a consequent non-specific signal in spiked samples.

The detection of OTA in a raw matrix like green coffee could be useful to detect contaminated batches prior to industrial processing. Usually coffee roasting takes place far away from production sites. Therefore, a rapid and cost-effective method for the screening of raw coffee is highly desirable.

Four different concentrations were used to obtain the calibration curve, and the results fitted with a sigmoid curve. The signal produced by the lowest contamination (3.9 μg/kg) was higher respect to the blank one, but the high uncertainty of the measurements did not allow a statistical reliable discrimination of samples contaminated with such amount of OTA.

As commonly accepted in the literature, the limit of detection (LOD) of a linear bioassay can be evaluated as the concentration corresponding to three times the standard deviation of the blank signal. Considering the signal at 3.9 μg/kg as the lower limit of our linear range, we could estimate the LOD of MCs for green coffee at 5 μg/kg. Commission Regulation (EC) No 1881/2006 regulates the maximum level of OTA contamination in roasted and soluble coffee to 5 μg/kg and 10 μg/kg, respectively. Being such values close to the previously estimated LOD, it is clear that MCs could be hardly used to identify OTA in coffee as a confirmatory tool, instead of HPLC. The probable small recovery fraction of OTA in the extracts, coupled with the high uncertainty induced by the green coffee matrix alone (as already seen in Figure 1), could be the reasons for the shifting of the linear range to relatively high concentrations. Nevertheless, coffee-roasting industries could benefit from a screening platform that could analyse batches rapidly in order to reject highly contaminated raw material.

OTA intake for wine consumption poses great concern to legislator and a rapid screening method is needed to enhance consumer health protection. The possibility to detect OTA in a fast and easy way without the need of costly and complicated instrumentation could help wine producers to gain efficient mycotoxin control. Moreover, consumer attention to safe food is steadily increasing, and a fast and portable sensor would enable people to analyse the products right before the consumption. While at low concentrations the resonance changes of MCs incubated in OTA-contaminated grape juice samples are overall similar respect to green coffee ones, the saturation values are very different, probably because of the binding of OTA with other components of coffee which behaved as signal enhancers at high concentrations, as previously reported for roasted coffee in Suárez-Quiroz et al. [44]. In grape juice, the linear range is found for concentrations lower than 6 μg/kg, while the LOD is estimated at 5 μg/kg. Such high LOD did not allow the use of cantilever biosensors for discrimination of safe or contaminated samples considering the legal limit of 2 μg/kg in grape juice.

Cantilever biosensors instead showed a good capacity to discriminate wines with 2 μg/kg of OTA with good sensitivity and reproducibility.

By considering all the results, MC arrays show the sensitivity and precision needed to satisfy EU requirements for OTA detection in red wine, while for green coffee and grape juice they could be used as screening platform to quickly reject highly contaminated samples.

4. Conclusions

The development of microcantilever biosensing platform for detection of OTA in food samples is here reported. The capability of MCs to detect OTA within the European legal limit range, as well as the analytical protocol optimization using buffer solutions were previously tested and results are reported elsewhere [38]. Here we demonstrated for the first time the possibility to successfully detect the mycotoxin in three different food matrices using MCs resonators. The matrix heavily influenced the measurements, so that a direct quantification was hardly achievable. For green coffee and grape juice, the limit of detection (LOD) was found to be close to legal limit (5 µg/kg), while for wine MC resonators were able to precisely discriminate between contaminated (2 µg/kg) and non-contaminated samples. Therefore, thanks to their capability to be used as rapid and cheap tests, MC arrays demonstrated their feasibility as a screening platform to quickly reject highly contaminated samples, thus drastically limiting routine analysis with HPLC.

5. Materials and Methods

5.1 Reagents

Sulphuric acid (95–98% w/w), hydrogen peroxide (30% w/w), 3-aminopropyltriethoxysilane (APTES, anhydrous, 99%), glutaraldehyde (GA, 25% v/v water solution), sodium hydroxide, Dulbecco’s phosphate buffered saline (PBS),Tween 20, HPLC grade acetonitrile and LC/MS grade methanol, OTA analytical standards ortoboric acid and sodium chloride used to prepare borate buffer were purchased from Sigma-Aldrich (St Louis, MO, USA). Pierce™ Recombinant Protein G, purified from *E. coli* was from Thermo Scientific (Waltham, MA, USA). Toluene anhydrous, 99.8%, was from Carlo Erba Reagents (Milan, Italy). The monoclonal antibody anti-Ochratoxin A was from Covalab (Lyon, France).

Polyethylene glycol (PEG), $NaHCO_3$ were purchased from Merck (Merck, Darmstadt, Germany). OtaClean select immunoaffinity columns were obtained from LCTech (Dorfen, Germany). HPLC eluents were degassed for 5 min and filtered through mixed cellulose ester 0.22 µm-filters (Advantec MFS, Pleasanton, CA, USA) before use.

5.2 Cantilever fabrication and functionalization

MC arrays are composed by 11 resonators each 5-7 µm thick, 460-600 µm long, and 50-70 µm wide. They are produced starting from Silicon-On-Insulator (SOI) wafers using a combination of surface and bulk micromachining techniques as described by [45]. The backside of the wafer was etched by KOH creating a membrane successively removed by a Reactive Ion Etching (RIE) from the front side, which allows the releasing of free-standing cantilevers. Finally, MCs were subjected to thermal oxidation for 3 h in O_2 atmosphere at 1100 °C. Before biochemical functionalization, MC arrays were cleaned in piranha solution (3:1 sulphuric acid: hydrogen peroxide) for 15 minutes, rinsed thrice in deionized water and dried in a stream of nitrogen. Surface silanization was performed in anhydrous conditions dipping MCs in 1% APTES solution in anhydrous toluene for 10 minutes at 70 °C. After MCs rinsing in toluene three times, the arrays were dried in air and immersed in GA solution 0,5% v/v in sodium borate buffer 0.1M pH 8.5 for 60 min at 40 rpm. After 15 minutes, sodium cyanoborohydride solution 5M in NaOH 1M was added to the buffer at a concentration of 0.1% v/v in order to reduce imines, stabilizing the bonding, as described by Ricciardi et al., 2010a.

Activated MCs were incubated overnight at 4 °C in 100 µl of protein G solution (50 µg/mL in DPBS), washed three times with PBS-Tween 20 0,05%, rinsed three times in deionized water and dried in a stream of nitrogen. In order to remove buffer salts, MCs were put in vacuum and rinsed three times in deionized water before each frequency characterization. Cantilever arrays are ready to functionalization with the monoclonal anti-OTA antibody. The covalent binding surface-antibody was performed at room temperature on orbital shaker for 90 minutes. Unbound antibodies were washed away with DPBS using the same procedure described above.

5.3 OTA extraction and clean up from coffee

10 g, 5 g and 2.5 g of naturally contaminated coffee were ground and mixed with 50 ml of 70:30 methanol: water with 3% of $NaHCO_3$ for 30 min. The samples were centrifuged 10 min at 5000 rpm/min, filtered, and diluted 1:4 in PBS. 10 ml of each solutions were purified in immunoaffinity column and dried after the elution with methanol. OTA extracts were recovered in ultrapure water.

5.4 OTA extraction and clean up from liquid samples

Extraction of OTA from wine samples was performed using the procedure reported in Prelle et al., 2013. Grape juice samples were previously analysed to verify the absence of OTA and after they were artificially contaminated with different amount of OTA. Samples were diluted 1:10 in water solution containing polyethyleneglycol (1%) and $NaHCO_3$ (5%), mixed and filtered. 10 ml of diluted extract were cleaned up through an immunoaffinity column. OTA was eluted by adding three times 0.75 ml methanol and collected in silanized clean vial. The elute was dried using a microplate evaporator with air flow at 40°C.

5.5 Cantilever resonance frequency characterization

OTA immunodetection was performed using a semiautomatic frequency read-out system, which allowed the measurement of both first and second flexural mode. After incubation in coffee and grape juice extracts, cantilevers were put in contact to a piezoelectric element (PI Ceramic, Lederhose, Germany) through a double-sided vacuum tape to correctly actuate the resonators and the measurement chamber was evacuated using a series of membrane and turbomolecular pump (Agilent Technologies, Santa Clara, CA, USA) to avoid any viscous damping effect. A function generator (Hewlett-Packard, Palo Alto, CA, USA) excites the piezodisk at different frequencies around the resonance and the vibration is correctly transferred to the sensor. The movement of cantilevers was detected exploiting the optical lever technique: a laser diode beam (Hamamatsu Photonics, Hamamatsu City, Japan) was focused on the tip of the cantilever and reflected onto a Position Sensitive Detector (Hamamatsu). Output signal was amplified and filtered by a phase-loop lock in and stored to a personal computer. A LABVIEW® program controls the measurement process and the data fitting with a Lorentzian curve, giving resonance and Q factor of vibration curves.

Resonance frequencies of sensors incubated in wine samples were monitored in ultra high vacuum conditions at 22±0.01 °C. MCs were actuated as above and the high frequency lock-in amplifier is HF2LI by Zurich Instruments (Zurich, Switzerland). The optical part is composed by a laser diode beam (Acal Technology, Wokingham, UK) and the Position Sensitive Diode (PSD by Edmund Optics Inc., Barrington, NJ, USA). This system is semiautomatic and allowed the characterization of resonance for the first and second flexural mode, giving a more accurate measurement of the mass bound to the microcantilever.

In order to compare different MCs, which present slightly different dimensions due to the fabrication process, relative frequency shifts $\delta = \Delta f/f$ were used. The mean relative frequency shift of cantilevers composing arrays was used to compare different bioassays and the standard deviation was used as uncertainty. In particular, for each cantilever the arithmetic mean of the first two flexural modes of vibration was calculated as $\boldsymbol{\delta_{12}} = \frac{\boldsymbol{\delta_1 + \delta_2}}{\boldsymbol{2}}$ and the relative uncertainty as $\boldsymbol{\sigma_{\delta_{12}}} = \frac{\boldsymbol{|\delta_1 - \delta_2|}}{\boldsymbol{2}}$.

The weighted average is given by $\boldsymbol{\overline{\delta_{12}}} = \frac{\sum \boldsymbol{\delta_{12} w_{12}}}{\sum \boldsymbol{w_{12}}}$ and the uncertainty of the array is given by $\boldsymbol{\sigma_{\overline{\delta_{12}}}} = \frac{\mathbf{1}}{\sqrt{\sum \boldsymbol{w_{12}}}}$ where $\boldsymbol{w_{12}} = \frac{\mathbf{1}}{\boldsymbol{\sigma^2{}_{\delta_{12}}}}$.

More details on experimental set-up and data analysis used can be found in Ferrante et al. 2017[37].

5.6 OTA quantification through HPLC-FLD

OTA quantification in food samples was performed using an HPLC apparatus 1100 series Agilent equipped with G1311 quaternary pump, G1379 degasser, G1313A autosampler, G1316A column thermostat and G1321A FLD-Fluorescence Detector set at excitation and emission wavelengths of 333 and 460 nm, respectively, and Chemstation G2170AA Windows XP operating system (Agilent, Waldbronn, Germany). An analytical column RP-18 (XTerra® Waters®, Milford, MA, USA; 150 mm x 4.6mm i.d., 5 µm) with a pre-column was used. The mobile phase was an isocratic mixture of acetonitrile: water: acetic acid (49:49:2) eluting at 1 mL/min, for 18 min. Samples (30 µl) were injected into the HPLC column and the retention time of OTA was ca. 6.23 min [42].

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Author Contributions: Carlo Ricciardi, Davide Spadaro and Maria Lodovica Gullino conceived and designed the experiments; Santoro Karin performed the experiments and wrote the paper, Ambra Prelle performed chemical analyses.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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