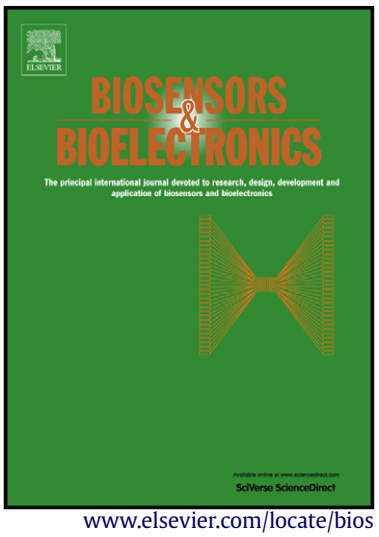

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Sensitive detection of Ochratoxin A in food and drinks using metal–enhanced fluorescence

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ABSTRACT

Easy, sensitive, rapid and low cost ochratoxin biosensors are strongly demanded in food analysis since Ochratoxin A (OTA) is a widely diffused food contaminant, highly detrimental for human health. In this work, a novel plasmonic based optical biosensor prototype for ochratoxin A is described. It exploits the metal-enhanced fluorescence phenomenon due to the silver film over nanosphere plasmonic substrate. Since ochratoxin A could be present in different food commodities, sensor performances have been tested on three different matrices (dried milk, juices, and wheat mix). Firstly, a common OTA extraction solvent and a labeling and detection protocol were defined for the analyzed matrices. Then, the efficiency of the Ag–FON surfaces in signal amplification for the detection of low ochratoxin A concentrations was defined. Using samples spiked with OTA–AF 647 or with unlabeled OTA we were able to detect the mycotoxin at concentrations lower than E.U. specifications of 0.5 μg/kg in wheat, milk and apple juice. The test performances are comparable to those of ELISA kits but the platform presented here, once optimized, present some perspective advantages, such as: low cost and time consuming, versatility of the protocol for the investigation of different matrices, employment also in non-qualified laboratories, small dimensions that allow its integration in a compact device for OTA on–site detection.

KEYWORDS

Ochratoxin A, plasmonic biosensor, Metal–enhanced Fluorescence, Ag–FON

1. INTRODUCTION

Ochratoxins are a group of mycotoxins produced as secondary metabolites by *Aspergillus ochraceus* and *Penicillium verrucosum*. The family of Ochratoxins consists of three members – A, B, and C – which differ slightly from each other in chemical structures. Ochratoxin A (OTA) is the most abundant and hence the most commonly detected member but is also the most toxic of the three (Li et al. 1997; van der Merwe et al. 1965a; van der Merwe et al. 1965b). As for other mycotoxins, OTA can contaminate a wide variety of foods as a result of fungal infection in crops, in fields during growth, at harvest, or during storage and shipment. Besides cereals and cereal products, OTA is also found in a range of other food commodities, including coffee, cocoa, wine, beer, pulses, spices, dried fruits, grape juice, pig kidney and other meat and meat products of non–ruminant animals exposed to feedstuffs contaminated with this mycotoxin.

This toxin has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic. The International Agency for Research on Cancer classified OTA in 2B group (possibly carcinogenic agent) (International Agency For Research On 1993). For this reason legal limits of OTA have been set in great number of food commodities including foods for infants and children by the European Commission under EC regulation 466/2001 (2001), 472/2002 (2002a; 2002b) and 123/2005 (2005).

OTA detection actually is performed using different strategies including high–performance liquid chromatography (HPLC) (Afsah-Hejri et al. 2012), gas chromatography (GC) (Soleas et al. 2001) thin layer chromatography, (TC) (Welke et al. 2010), enzyme linked immunosorbent assay (ELISA) (Flajs et al. 2009), and immunochromatographic assays as lateral flow strips for rapid detection (Lai et al. 2009). Analytical chromatographic detection methods, as HPLC, are rather expensive, time consuming and could be performed only in specialized laboratories by qualified staffs. Immunoenzymatic method (ELISA) belongs to the rapid detection techniques; however the disadvantage relies in the necessity of using expensive enzyme–labeling reagents and high performance laboratory instruments to detect the lower legislation limit amounts. On the other hand, approaches relying on fast detecting procedures as membrane based cards, antibody based tubes or immune cup tests, that are more suitable for an on-site screening, are usually qualitative or semi-quantitative tests, with lack of sensitivity in proximity of the lower legislation limits and may suffer matrix interface problems (Bazin et al. 2010; Kaushik 2013). Recent detection achievement have been reached based on lateral flow strategy implemented with a custom software for data analysis and quantification (EnviroLogix QuickTox test kit) but the array of analyzable matrices is restricted so far to only wheat with a limit of detection of 1 μg/kg (Davis et al. 2013).

Further efforts for the definition of direct, high sensitivity, rapid, and low cost methods for OTA detection are ongoing with the aim of defining strategies suitable for real time on site analysis and adaptable to different matrix screening. Many research groups have been developing different strategies to detect OTA in food commodities like quartz crystal microbalance (QCM), electrochemical impedance spectroscopy (EIS) (Lisdat and Schäfer 2008; Wen-Chi and Chi-Kun 2007; Zamfir et al. 2011) and label–free techniques based on the surface plasmon resonance (SPR) properties of gold surfaces (van der Gaag et al. 2003; Yu and Lai 2004).

In this work we have developed a prototypal new sensor able to detect OTA in different food commodities using a plasmonic substrate functionalized with a specific anti–OTA antibody, able to bind the complex OTA–Alexa Fluor (AF) 647. Moreover, we have developed a protocol for extraction and direct labeling of toxin in order to perform both, extraction and OTA–AF 647 conjugation, in the same solution. Plasmonic substrates, generally used for Raman spectroscopy detection, work well also as enhancers for the emission of fluorescent molecules located within about 10–20 nm from the nanostructured surface, through MEF (Metal Enhanced Fluorescence) effect (Aslan et al. 2005; Deng and Goldys 2012; Geddes and Lakowicz 2002; Lakowicz 2006; Petryayeva and Krull 2011). This phenomenon could be exploited to obtain new high sensitive fluorescent biosensors. To develop an ochratoxin A sensor we have employed the well known silver film over nanosphere (Ag–FON) plasmonic substrates (Van Duyne et al. 1993). This particular category of surfaces have been chosen because of its high enhancement factor ($\geq 10^4$), high stability over wide temperature, potential, and analyte concentration ranges, and high shelf life (higher than 40 days) (Dieringer et al. 2006). In our view, thanks to all these intriguing properties, the Ag–FON can be successfully used for MEF investigation of many relevant molecules.

We have evaluated the performance of our system on different food commodities including cereals for infants, milk, and juices; in all cases the sensor is able to detect the toxin at lowest European law limit. In this way it could be possible to detect the toxin at concentration of 5 ng/kg in labeled OTA (OTA–AF 647) spiked samples and of 50 ng/kg in OTA spiked samples, labeled after extraction.

2. EXPERIMENTAL

2.1 Chemicals and solutions

Carboxymethyl–PEG–Thiol 2000 Da (COOH–PEG_{2000}–SH) was purchased from Laysan Bio Inc.

Ochratoxin A, EDC (N–(3–Dimethylaminopropyl)–N′–ethylcarbodiimide hydrochloride) and sulpho–NHS (sulfo–N–hydroxysulfosuccinimide), CDI (1–1' carbonyldiimidazole), reagent grade methanol, H_2SO_4, NH_4OH, silver flakes, acetone, ethylendiamine, and DMSO (dimethyl sulfoxide) were purchased from Sigma Aldrich. H_2O_2 was purchased from Merck and benzenthiol (BT) from Fluka. Polystyrene nanospheres (150 nm diameter) were obtained from Fisher Scientific.

Ochratoxin A was resuspended in pure acetone at 1 mg/ml concentration and stored at 4°C. Ochratoxin requires special handling and disposal. Materials that are used for ochratoxin solution preparation were decontaminated by immersion in a 5% Acetone/5% Hypochlorite killer solution.

Specific capture and negative control antibody were: anti–ochratoxin (Abcam, clone 3C5) and anti–aflatoxin (Genetex, clone 1C6), respectively. Lyophilized Alexa Fluor (AF) 647 –NHS ester was purchased from Invitrogen. Lyophilized milk was purchased from Fluka.

Every used reagent for the preparation of the following buffer solution was from Sigma Aldrich:

MES buffer for –COOH activation: 0.1M 2-(morpholino)ethanesulfonic acid, 0.5M NaCl, pH 6.0.
PBS 1X: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
Antibody printing buffer: 0.1 M sodium phosphate, 0.3 M NaCl 0.01% Triton X100 pH 7.2.
Array blocking solution: 50 mM sodium phosphate, 2.0% w/v Bovine Serum Albumin pH 7.2.
Array washing solution: 0.5M Tris 2.5M NaCl, 0.5% w/v Tween 20, pH 9.0.

2.2 Plasmonic substrates production and functionalization

Ag–FON substrates were obtained using the following procedure: 2.5x2.5 cm^2 glass slides were cleaned first in acid piranha solution (H_2S0_4:H_2O_2 = 3:1) at ~ 80°C for 1 h and then sonicated in basic piranha solution (NH_4OH(30%):H_2O_2 = 5:1) for 1 h; the substrates were cleaned four/five times and kept in deionized water.

Glass slides were subsequently dried using an N_2 flux, and then 100 µL of 1% a polystyrene nanospheres water solution were spin–coated on top of the glass slides to form a self–assembled mask. Ag thin films were finally deposited onto the substrates in an Edwards E306A coating system with a bare pressure of $6x10^{-5}$ mbar. The metal deposition rate was ~ 1 Å/s and the final Ag thicknesses, evaluated by means of a quartz crystal microbalance, were about 50 nm, 100 nm and 150 nm.

Thanks to their metallic nature, Ag-FON substrates have been directly characterized by means of SEM microscopy (VEGA TS 5130 LM Tescan) and then the surface topography has been investigated in details using AFM microscopy (Ntegra NT-MDT).

Finally, in order to evaluate the Enhancement Factor (EF, see section 3.1) Ag–FON silver nanostructures have been functionalized with BT simply by dipping in a solution of 25 µL of BT in 25 mL of methanol for about 20 hours. Then samples were rinsed repeatedly in methanol and the EF was obtained using the equation: (Le et al. 2007)

$$EF = \frac{I^{Raman,Monolayer}}{I^{Raman,Liquid}} \frac{C_V H_{eff}}{C_S} \frac{\sigma_{Toluene}}{\sigma_{BT}}$$

where σ_{BT} is the BT differential cross section, $\sigma_{Toluene}$ is the toluene differential cross section and their ratio was directly measured, CV is the toluene volumetric density (5.69×10^{21} molecules/cm^3), CS is the BT packing density on the surface (6.8×10^{14} molecules/cm^2) (Haynes and Van Duyne 2003) and H_{eff} is the collection efficiency dependent on the collection apparatus and on the effective scattering volume of the sample.

The Ag–FON surfaces were then functionalized overnight in a close chamber at 23°C and 85% of humidity, with a 1 mM water solution of COOH–PEG$_{2000}$–SH; –COOH groups were activated with EDC and sulpho–NHS (10mM in 1X MES buffer) for 15 minutes, rinsed with ddH_2O and N_2 fluxed. Antibodies diluted in printing buffer (1 mg/ml final concentration) were spotted onto the silver activated surfaces and on commercial microarray control surfaces (e–surf glass slides, LifeLine Lab) using Microarray Spotter (Versarray Chip Writer Pro System, BioRad) with Telechem SMP3 microspotting pins (Arrayit) and left overnight into a 75% humidity incubation chamber (µBox, Quantifoil Instrument). On each slide, more replicates of the same deposition scheme were printed consisting of 4 spots of the specific anti–OTA antibody and 4 of the negative control anti–aflatoxin antibody. Slides were blocked and washed according to standard protocols indicated by the supplier. Ochratoxin detection and incubations were performed using the Grace Bio–Labs ProPlate microarray system (Sigma Aldrich) which allows to physically isolate each sub–array.

2.3 Ochratoxin A labeling and quantification

Alexa Fluor 647 (AF 647) Dye was ammino– (NH_2–) modified through incubation with ethylendiamine for 2 hours. Two different organic solvents were used to obtain the OTA–AF 647 covalent complex by adding the properly COOH–activated OTA.

In the first case, OTA coupling was performed in acetone through CDI at the following molar ratio: 25 nmoles of 1 mg/ml OTA and 450 nmoles of 50 mg/ml CDI were mixed and let react for 10 minutes at room temperature, under stirring. Subsequently, 25 nmoles of 2 µg/µl NH_2–activated AF 647 were added to OTA–CDI, mixed and let react for 2 hours.

In the second case, OTA labeling was performed in methanol with a final concentration 25% v/v, since OTA extraction from complex matrices was usually performed using this solvent (see 2.4). 500 nmoles of EDC and 500 nmoles of sulpho–NHS (both in MES buffer) were mixed with 25 nmoles of 1 mg/ml OTA, in a solution of MES buffer with 25% methanol. Sample was mixed and let react for 10 minutes at room temperature to allow –COOH groups' activation, under stirring.

Subsequently, 25 nmoles of 2 µg/µl NH_2–activated AF 647 were added to the mix and let react for 2 hours.

Serial dilutions of OTA–AF 647 were performed in PBS 1X and samples were incubated on commercial microarray slides (LifeLine Lab) and on silver slides for 1 hour at room temperature. Microarray wells were subsequently washed in Antibody washing solution and in PBS.

2.4 Ochratoxin A extraction from complex matrices and analysis

Ochratoxin A quantification was performed through spikes in a range of food matrices (i.e. dried milk, cereal–based dietary foods intended specifically for infants, and juices, all purchased from supermarkets with no preferential criteria) to verify the performance of extraction procedure and detection efficiency.

Using a single vial for each of the tested concentrations, samples from known amount of each commodity were spiked with OTA (final concentrations ranging from 0.05 µg/kg to 500 µg/kg), vortexed and let equilibrate overnight at 4° C under stirring. For each food commodity, a control sample without OTA addition was processed in parallel.

Spikes were performed either with OTA–AF 647, in order to evaluate only the extraction recovery of the labeled analyte in juices and wheat mix, or with unlabeled OTA, to define the labeling efficiency of the analyte and its subsequent detection in dried milk, comparing the performance of acetone or methanol OTA extraction and coupling procedure.
The optimized procedure was applied to all tested spiked commodities in order to obtain the maximum OTA recovery and labeling.

After the evaluation of different procedures we defined specific extraction protocols for each food matrix. Samples were processed through the addition of absolute methanol with a 1:4 ratio for milk, 5 ml of 50% methanol for 500 mg of wheat, or absolute methanol with a 1:10 ratio for juice. In all cases, samples were extracted as follow: after solvent addition at the concentrations reported above, samples were mixed for 10 min and clarified through centrifugation for 5 minutes at 16k rcf for juices and milk and 5 min at 3k rcf for wheat derived products. Supernatants were recovered and directly incubated on the antibody microarray in the case of OTA–AF 647 spikes, or further treated for OTA labeling in the case of unlabeled OTA spikes, using the linker molecules and related concentrations as described above.

3. RESULTS AND DISCUSSION

3.1 Ag–FON characterization

The morphology of Ag–FON samples has been characterized by means of SEM and AFM (Figure 1) in order to check the quality of the plasmonic substrates.

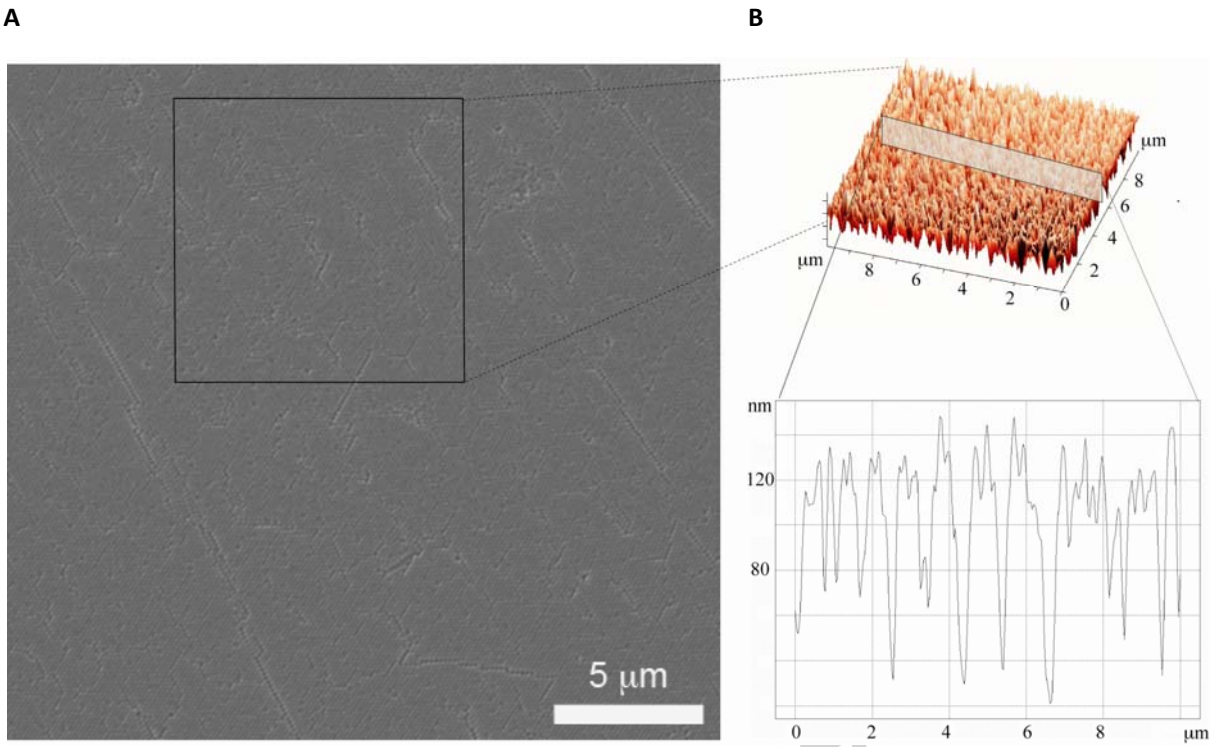

Figure 1. A. SEM topography of an Ag–FON substrate; B. 3D AFM reconstruction of 10x10 μm^2 area of the same substrate; C. Relative depth profile.

Figure 1.A shows a 25x25 μm^2 array of 150 nm polystyrene nanospheres presenting large areas with close-packing structures. The AFM image confirms the quality of the close-packed structure (Figure 1.B) and the uniformity of Ag coverage (Figure 1.C).

Ensured the quality of the surface, the most useful figure of merit to characterize a plasmonic substrate is the EF. This parameter correlates the increase in the local electromagnetic field due to the plasmonic phenomenon with the far field of the exciting light. Such local electromagnetic field is a direct consequence of the metal free electron cloud oscillations. These oscillations are responsible for the strong enhancement of the fluorescence for dyes in the proximity of plasmonic objects (Lakowicz 2006).

The EF of each Ag–FON with different silver layers (50, 100 and 150 nm) has been evaluated (at 633 nm) functionalizing many silver nanostructures for each layer thickness with BT, as described in section 2.2. Samples with 50 nm of silver present an EF of about $0.5 \pm 0.2 \times 10^5$, while Ag-FON with 100 and 150 nm of silver film present EFs of $0.9 \pm 0.1 \times 10^5$ and $1.4 \pm 0.6 \times 10^5$, respectively. Samples with 150 nm of Ag present higher EF but they also exhibit lesser reproducibility (higher standard deviation) and they suffer for a possible metal delamination during the functionalization step. Ag-FON with 100 nm of silver thickness have been finally chosen because they ensure high plasmonic enhancement factor with good reproducibility and minimum silver delamination.

The 100 nm EF value is high enough to ensure the quality of our plasmonic substrates and the enhancement of fluorescent labels near the metal structures.

3.2 Pure ochratoxin A labeling and detection

Pure ochratoxin A was labeled, using the acetone/CDI coupling procedure, as described in section 2.3 and incubated on commercial microarray or on Ag–FON slides. Binding signals were collected and analyzed. Performances of the two substrates were compared to evaluate fluorescent signal enhancement obtained thanks to MEF phenomenon. Known amounts of labeled OTA were incubated in order to test the system performance. Tested amounts ranged from 500 µg/kg to zero, including 0.5 µg/kg, which represents the European lower legislation limit of OTA in baby foods for infants and young children.

Results are summarized in figure 2: no detectable signal was observed on anti–aflatoxin antibody spots used as negative control, confirming the specificity of anti–ochratoxin antibody used in the array. Tested concentrations of pure AF 647 labeled OTA are expressed in µg/kg and fluorescent signals at 635 nm are reported, representing a mean of 3 independent experiments, each one done with a matrix of 4 spots. On plasmonic surfaces, spots appear larger and less sharp if compared to the commercial microarray slides ones, probably due to differences in wettability of the nanostructures in the substrates.

OTA labeling was performed in acetone medium, because it is one of the most efficient solvent for the formation of a peptide bound between the $–NH_2$ group of activated AF 647 and the –COOH group of OTA (Prieto-Simón et al. 2008).

In this experimental setting, the 0.5 µg/kg concentration is well detected by the Ag–FON OTA array, which furthermore allows the detection of a 10–fold lower concentration (0.05 µg/kg), not detectable if using commercial microarray slides (figure 2 and table 1) where the lower concentration is similar in signal intensity to that of the negative sample. These results confirm the signal amplification capability of the silver nanostructured surfaces if compared to conventional microarray slides. The limit of quantification for OTA detection on Ag–FON substrates is as low as 3.6 ng/kg that is 20 times lower than what observed for commercial array slides (70.7 ng/kg). Plasmonic surfaces allow detecting OTA at concentrations comparable with that of commercial ELISA kits as reported in the specific data sheets by the producers.

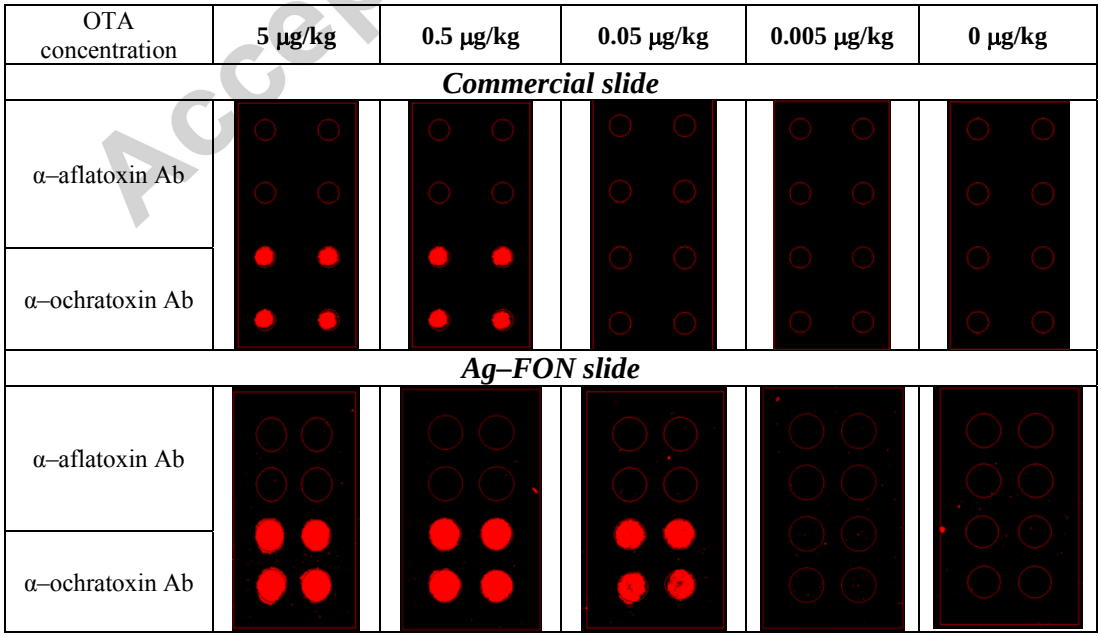

Table 1: OTA detection on Ag-FON and commercial slides

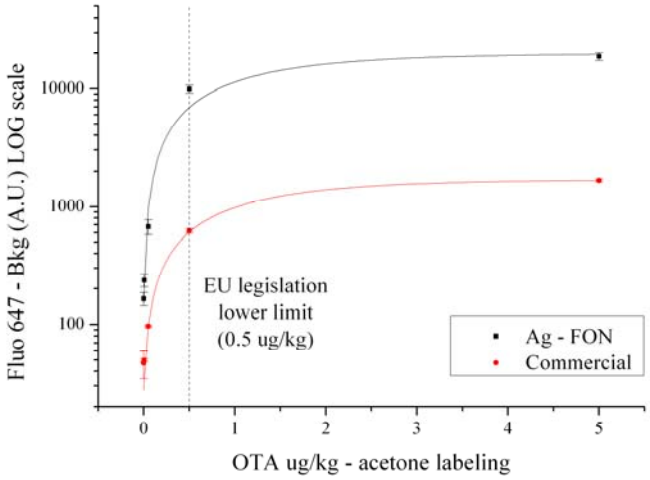

Figure 2. Tested concentration of pure OTA–AF 647 labeled in acetone, expressed in µg/kg (EU lower low legislation limit for OTA in baby foods for infants and young children: 0.5 µg/kg). Signals obtained on Ag–FON substrate (black squares) and on commercial surface (red circles) are compared and reported both as images (A), graphically (B) and numerically (**Table 1**). Mean–background fluorescence values and standard deviations (sd) are reported graphically and numerically. In ***Italic*** in the table: lower legislation limit, also plotted on the graph as dotted line.

3.3 Solvent role in ochratoxin A analysis

Since methanol is the elective solvent for the extraction of Ochratoxin A from several food matrices, pure OTA labeling in methanol was evaluated. Considering that acetone usage permits to obtain a high and efficiently amount of labeled product (figure 2), it was necessary to define the best conditions of OTA labeling for methanol (i.e. methanol concentration, linker molecule species and their concentrations in the final reaction volume) that allow reaching a comparable detection in OTA quantification.

In figure 3 and Table 2, signal intensities of methanol–labeled OTA at the concentration range used for OTA spike in food matrices were reported.

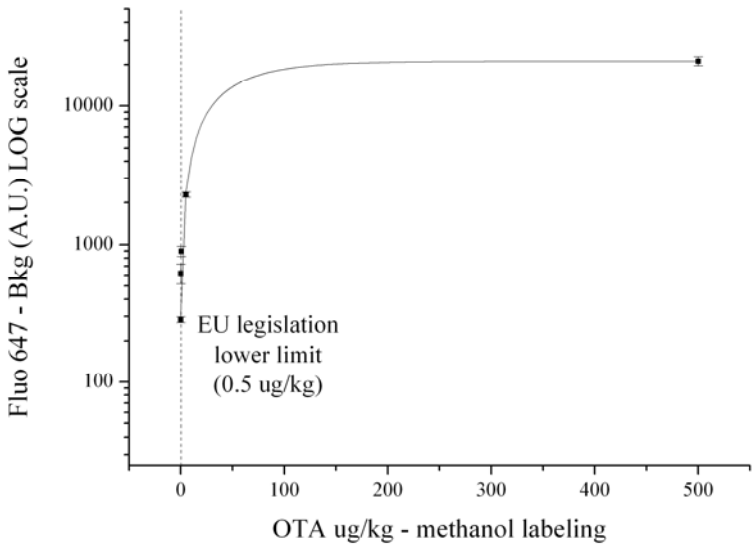

Table 2: Methanol labeled OTA detection on Ag-FON substrates

Figure 3. Tested concentration of pure OTA–AF 647 labeled in methanol, expressed in µg/kg (EU lower low legislation limit for OTA in baby foods for infants and young children: 0.5 µg/kg) on Ag–FON substrates. Mean–background fluorescence values and standard deviations (sd) are reported graphically and numerically (**Table 2**). In ***Italic*** in the table: lower legislation limit, also plotted on the graph as dotted line.

Both 0.5 µg/kg and 0.05 µg/kg of OTA are clearly detected using methanol labeling protocol, demonstrating the feasibility of this new procedure that could be used firstly to maximize OTA extraction and also to perform the coupling and detection steps after OTA extraction from complex matrices (figure 4).

Once established the feasibility of methanol usage for OTA labeling, we performed experiments to verify the best conditions of OTA recovery and labeling that could be common to all tested food commodities. Firstly, we used milk samples to evaluate OTA recovery comparing acetone and methanol, used both as extracting and labeling solvents. Several attempts of methanol percentage in extraction and labeling solutions were tested (data not shown) that allowed us to select the proper concentration of methanol in the two mixes, as reported in section 2.4.

Milk is an emulsion that contains different types of biomolecules, as lipids, proteins, minerals, vitamins and carbohydrates, thus representing a complex and varied matrix for the set–up of the experimental protocol. No regulation for OTA in milk exists, even though it has been suggested that the level of this toxin in cow's milk may exceed the tolerable daily intake of 5 ng/kg of body weight per day for small children (Skaug 1999). Also if ochratoxin A and its less toxic metabolite (OTα; (Kiessling et al. 1984)) are mainly eliminated in the urine and feces, they can also be found in milk (Boudra et al. 2007).

Dried milk samples were spiked and extracted with acetone or methanol and labeled according to the protocols reported above.

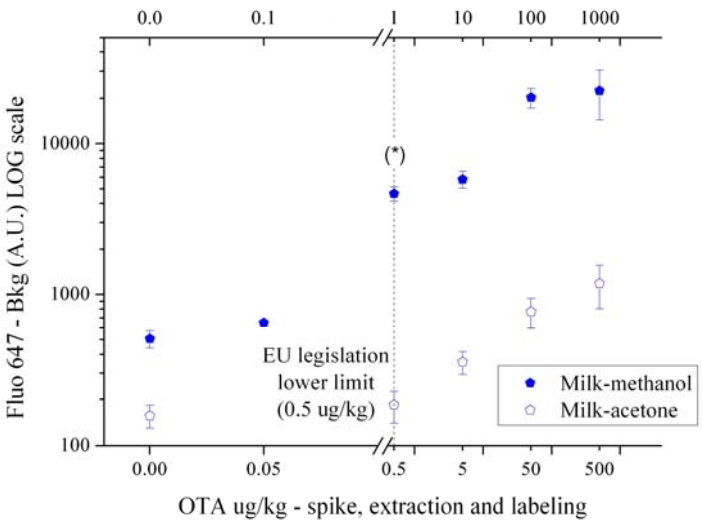

Figure 4. Dried milk, spiked with OTA at final concentrations ranging from 0.05 to 500 µg/kg were extracted in acetone (empty pentagons) or methanol (full pentagons) and labeled as in 2.4. Control samples were run in parallel for all experiments. Mean–background fluorescence values and standard deviations (sd) are reported graphically. The lower legislation limit is plotted on the graph as dotted line. *: values significantly higher than control samples, $p<0.05$, unpaired t test.

The usage of methanol both for extraction and subsequent labeling resulted in the best quantification of OTA content in milk, when compared to samples processed in acetone (figure 4). In methanol processed milk samples, OTA concentration as low as 0.05µg/kg of spiked OTA resulted to be successfully extracted, labeled and detectable in the developed MEF assay. Acetone processed samples, even at 0.5µg/kg, did not show significant fluorescence signal when compare to control samples, thus demonstrating the better efficiency of methanol usage in the experimental procedures. These results demonstrated that acetone is not able to guarantee an efficient OTA extraction even if it represents an elective organic solvent to perform COOH–activated OTA labeling. Methanol procedure, even if not associated to a fully linear relationship between fluorescent signal and OTA concentration, as can be seen from the plot in figure 4, permits to detect the toxin even at lower concentrations with respect to the ones required from the European legislation limit.

3.4 Ochratoxin A detection in food and drink commodities

Once established the best extraction and labeling solvent, Ag–FON performances in OTA quantification were evaluated analyzing different food commodities, in which OTA can be present (i.e. dried milk, juices, and wheat mix), with known labeled OTA spikes. This preliminary step aims to define the efficiency of OTA extraction and the potential presence of contaminants that would affect its quantification. Subsequently, samples from all tested matrices were spiked with unlabeled OTA and analysis was performed after extraction and labeling in real–like samples.

3.4.1 Detection of OTA–AF 647 in food and drink commodities

OTA content in grape juice and cereal–based foods for infants and young children is strictly regulated by European legislation, with limits of 2 µg/kg and 0.5 µg/kg, respectively, while is 5 ug/kg for unprocessed cereals.

To define the capability of our plasmonic surfaces in the analysis of these complex matrices, known amounts of methanol–labeled OTA–AF 647 were incubated in food commodities, treated and labeled as described in section 2.3. Figure 5 illustrates the results obtained when detecting OTA–AF 647 spiked in commercial wheat mix and in juices.

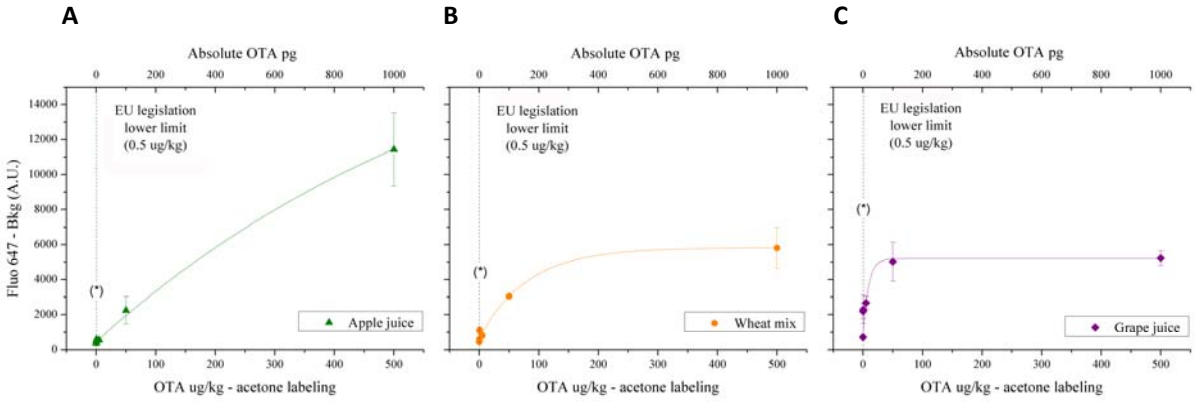

Figure 5. Samples of a commercial cereal–based food for infants, apple juice (A - green triangles), wheat mix (B - yellow circles) and grape juice (C - purple diamonds), were spiked with OTA–AF 647 at final concentrations ranging from 0.05 µg/kg to 500 µg/kg. A control sample was run in parallel for each matrix. Samples were extracted in methanol and tested on Ag–FON substrates. Mean–background fluorescence values and standard deviations (sd) are reported graphically. The lower legislation limit is plotted on the graph as dotted line. *: values significantly higher than control samples, $p<0.05$, unpaired t test.

For each of the tested food commodities, detected fluorescence of all OTA–specific signals at the E.U. limit concentration of 0.5 µg/kg were higher than those of the reference matrices (control sample with no spiked OTA, extracted and diluted as for spiked samples) (figure 5). Results suggest that, except for the differences due to matrix extraction efficiency or to extract composition that could affect OTA quantification, the experimental procedure was successful and obtained samples were suitable for direct testing on plasmonic surfaces.

3.4.2 Detection of ochratoxin A from commodities post–extraction and labeling

To evaluate the feasibility of the detection strategy simulating real sample detection, the same food commodities, spiked with unlabeled OTA, were used (milk, juices and wheat mix). Methanol extraction and labeling protocol selected in 3.3 was used to quantify OTA from spiked samples and results are illustrated in figure 6.

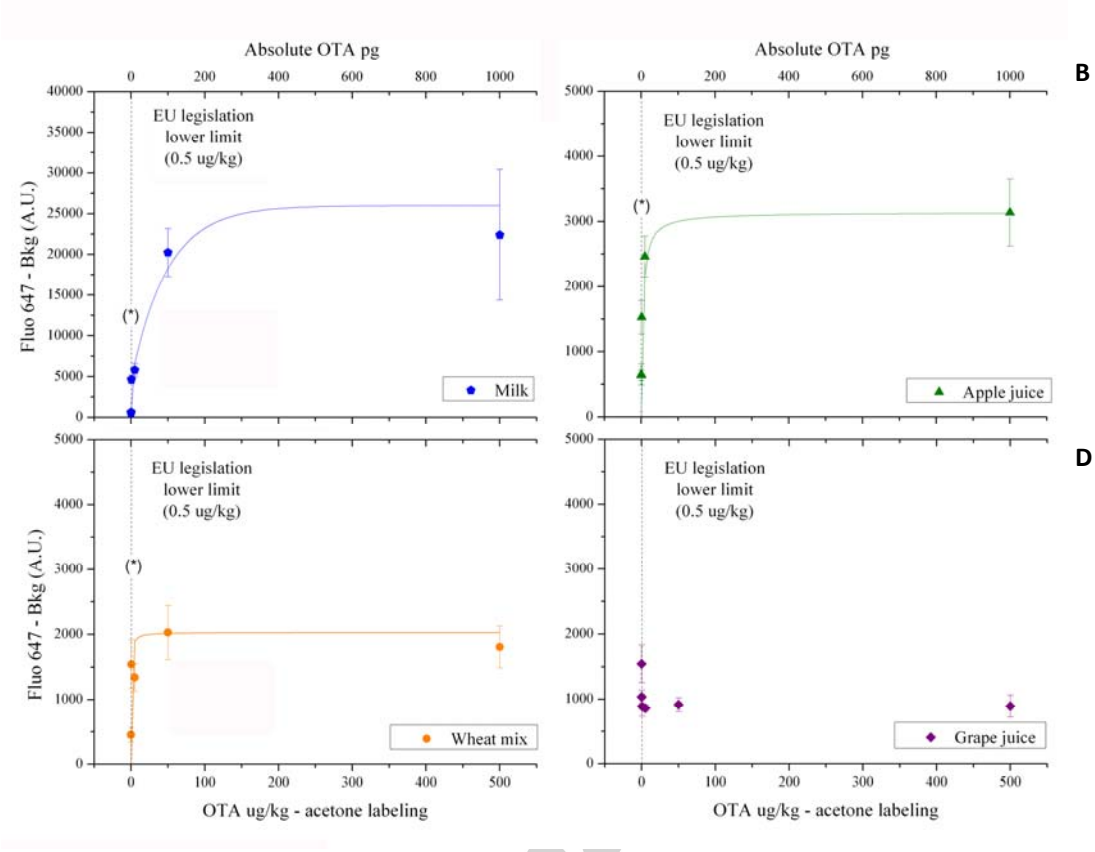

Figure 6. Dried milk (A - blue pentagons), apple juice (B - green triangles), wheat mix (C - yellow circles) and grape juice (D - purple diamonds) samples spiked with known concentrations of OTA ranging from 0.05 to 500 µg/kg, extracted in methanol and labeled as in 2.4. Control samples were run in parallel for all experiments. Mean–background fluorescence values and standard deviations (sd) are reported graphically. The lower legislation limit is plotted on the graph as dotted line. *: values significantly higher than control samples, $p<0.05$, unpaired t test. Significant differences between control and 0.5 µg/kg of OTA were obtained for milk, apple juice and wheat mix, and not for grape juice.

Results plotted show that for milk, wheat mix and apple juice matrices concentrations of 0.5 µg/kg (E.U. legislation lower tolerable limit) are statistically higher than those observed in the relative control samples. These results permit to state that OTA concentrations higher than 0.5 µg/kg are thoroughly detectable in these matrices when compared to control samples.

OTA quantification in grape juice was not successfully achieved because of the interference of some components present in the extract that resulted in a higher background in control samples and no specific signal in spiked ones. The matrix probably contains components that could interfere with the labeling reaction, impairing system performances even at higher concentrations.

For all the investigated matrices we observed that the linearity in the relationship between fluorescence signal and OTA concentration was not present, even though OTA spiked samples showed a fluorescence signal clearly above control samples.

4. CONCLUSIONS

In this work we have developed a MEF–based prototype sensor capable to detect OTA in different food complex matrices (i.e.: milk, wheat mix, juices) with high efficiency. The plasmonic surface signal enhancement allowed to detect OTA content up to concentrations of one order of magnitude lower than those specified by E.U. legislation concerning limit of exposure in food for infants and young children with a rapid and economic sensor.

Sample processing strategy developed in this work allows reducing the dilution ratios of samples before test performance. We have been able to test samples on Ag–FON substrates with dilution factors ranging from 10 to 30, depending on the tested commodity, whereas dilution factors up to 50 must be used for some commodities in commercial ELISA kits sample.

Although the system does not present a linear relationship between OTA concentration and fluorescent signal on simulating samples, in nearly every case we have been able to detect OTA also at concentrations lower than E.U. specifications. The assay performance could be affected by both the extraction efficiency and the labeling performance, which impaired system linearity. Further efforts should be performed to deeply investigate the labeling condition and procedure in order to maximize the detection efficiency of the developed assay on complex matrices.

Moreover, the use of the same solvent in the extraction and labeling steps, together with the aforementioned intriguing properties of the MEF substrates, allow for a simple processing procedure.

At the best of our knowledge, these results show the first attempt to detect OTA from complex matrices introducing a unique and versatile protocol of extraction, labeling and sensing on Ag–FON substrates using their powerful enhancement properties. As a matter of the fact, Ag–FON based sensor, preserving sensitivity of ELISA kits, presents some considerable improvements when compared to conventional analytical assays. The developed system could allow to quantitatively measure OTA content using a cheap surface sensor, low time consuming, employable by non–specialized staff in not qualified laboratories, that could be integrated in compact devices allowing its employment in on–site OTA detection.

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Table 1:

Table 1: OTA detection on Ag-FON and commercial slides

	Ag–FON		Commercial	
OTA µg/kg	**Fluo 647 – Bkg (A.U.)**	**sd***	**Fluo 647 – Bkg (A.U.)**	**sd***
5	18793.25	1548.46	1672.00	54.76
0.5	***9935.75***	***809.11***	***621.50***	***26.96***
0.05	674.50	97.18	96.5	2.52
0.005	235.75	28.19	49.75	1.71
0	164.25	21.28	47.00	12.53

*sd = standard deviation

Table 2:

Table 2: Methanol labeled OTA detection on Ag-FON substates

OTA µg/kg	**Fluo 647 – Bkg (A.U.)**	**sd***
500	21210.50	1589.78
50	4822.08	478.84
5	2283.00	101.50
0.5	***896.67***	***76.67***
0.05	618.20	102.54
0	283.00	13.78

*sd = stadard deviation

Highilights

- We developed a novel plasmonic based optical biosensor for ochratoxins A detection.
- Our sensor exploits the metal-enhanced fluorescence phenomenon.
- OTA detection up to one order of magnitude lower than E.U. legislation limit.
- Our sensor presents some considerable improvements when compared to of ELISA kit.