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Fast and Accurate Determination of Minute Ochratoxin A Levels in Cereal Flours: Towards Application at the Field †

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Abstract: Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of *Aspergillus* and *Penicillium* species. Ochratoxin A (OTA) is the most toxic member of the group and can be found in a large variety of widely consumed foods, such as coffee, cocoa, wine, and flour. Reliable determination of OTA levels in food samples is therefore indispensable to assure compliance with MRLs set by national/European regulations and minimize health risks for consumers. In the current study, a label-free biosensor based on White Light Reflectance Spectroscopy (WLRS) for rapid and accurate determination of OTA in cereal flour samples is demonstrated. The transducer employed is a Si chip with a 1-μm thick thermal SiO_2 on top transformed to biosensing element through immobilization of an OTA-protein conjugate on the SiO_2 surface. For the assay, a mixture of an in-house developed anti-OTA antibody with the calibrators or the samples is injected over the chip surface followed by reaction with secondary biotinylated antibody and streptavidin for signal amplification. The WLRS biosensing platform allows for the label-free, real-time monitoring of biomolecular interactions carried out onto the SiO_2/Si chip by transforming the shift in the reflected interference spectrum caused by the immunoreaction to effective biomolecular adlayer thickness. After optimization, the sensor was capable of detecting OTA in wheat flour samples at concentrations as low as 60 pg/mL within 25 min. The assay was repeatable with intra- and inter-assay CVs ≤ 5.9% and ≤9.0%, respectively. The excellent assay analytical characteristics and short analysis time in combination with the small size of the device render the proposed WLRS system ideal for the quantitative determination of minute OTA levels at the Point-of-Need.

Keywords: mycotoxins; Ochratoxin A; flour; white light reflectance spectroscopy; label-free immunosensor; Point-of-Need

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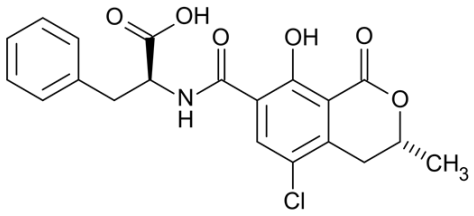
1. Introduction

Ochratoxin A (OTA) is a low molecular weight mycotoxin (403.81 Da; Scheme 1) produced as secondary metabolite mainly by filamentous fungi of the genera *Aspergillus* and *Penicillium* [1]. Due to the colonization of these species in a plethora of food crops during cultivation, harvest and post-harvest procedures, OTA has been reported to be present in a series of highly consumed agricultural products, such as cereals, coffee, cocoa, beer and

wine [2]. OTA is considered harmful for humans and animals, since there is evidence associating its consumption with chronic toxicity (genotoxicity, immunotoxicity, nephrotoxicity, hepatotoxicity, etc.), teratogenicity, mutagenicity and carcinogenicity. In accordance to this, the International Agency for Research on Cancer has classified OTA as a group 2B—possible carcinogen to humans [3]. In order to control and minimize public health risk, the European Union (EU) has established a strict maximum limit of 2 ng/mL for OTA in cereals and cereal flours, which was adopted by several non-EU countries as well [4].



Scheme 1. Chemical structure of Ochratoxin A.

Nowadays, analysis of OTA in foodstuff, including flour, is performed by well-established analytical techniques, mainly high-performance liquid chromatography (HPLC) coupled to fluorescence or mass spectrometry detection [5,6]. These sophisticated techniques are characterized by high reliability, selectivity and sensitivity. However, the high analysis cost, the need for skillful personnel and the bulky instruments, consist major bottlenecks for application of these techniques to routine high-throughput screening and/or point-of-need (PoN) analysis of OTA. In this direction, the last decade(s), biosensors have raised great expectations as an emerging technology with potential for automation, miniaturization and incorporation into portable setups for the rapid and reliable determination of OTA levels in flours across the production line and up to retail shelf [7].

In the current work, we present the development of a sensitive and reliable method for the rapid immunochemical detection of OTA in cereal flours based on a White Light Reflectance Spectroscopy (WLRS) optical sensor. WLRS methodology involves a visible/near infrared light source, a miniaturized USB controlled spectrometer operating in the 400–750 nm spectral range, and a reflection probe of 7 optical fibers from which 6 arranged at the periphery of the probe and one at its center. The white light emitted from the light source is guided through the six fibers at the periphery of the reflection probe to the bio-modified surface and at the same time, the central 7th fiber collects the reflected light and directs it to the spectrometer. The immunochemical reactions take place on top of a 1-μm thick SiO_2 layer grown by thermal oxidation of silicon chips. Thus, the light reflected from the silicon surface passes through the SiO_2 and the biomolecular layer and an interference spectrum is received that shift to higher wavelengths during the course of immunoreactions. The software calculates the effective thickness of the biomolecular layer that caused the spectral shift, and this thickness in nm is the sensor signal. For the determination of OTA, an indirect competitive immunoassay format realized in three steps was implemented. At first mixtures of an in-house developed rabbit anti-OTA antibody with OTA calibrators or samples were passed over an amino-silanized chip biofunctionalized with an OTA-protein conjugate; then a biotinylated secondary anti-rabbit IgG antibody and streptavidin were flown to enhance the signal received by the primary immunoreaction. All assay parameters were optimized in terms of absolute signal, detection sensitivity and total analysis time aiming at a fast method for sensitive and reliable determination of OTA in cereal flours.

2. Materials and Methods

2.1. Reagents and Instrumentation

Ochratoxin A (OTA) and OTA conjugate with ovalbumin (OTA-OVA) were purchased from Aokin AG (Berlin, Germany). Rabbit anti-OTA antibody (primary antibody) was in-house developed. Biotinylated goat anti-rabbit IgG antibody (secondary antibody), streptavidin, and (3-aminopropyl)triethoxysilane (APTES) were obtained from Sigma-Aldrich (Darmstadt, Germany). Bovine serum albumin (BSA) was from Acros Organics (Geel, Belgium). IgG Elution buffer was from Thermo Fisher Scientific Inc. (Waltham, MA). All other chemicals were from Merck KGaA (Darmstadt, Germany). RIDASCREEN® Ochratoxin A 30/15 enzyme immunoassay kit was purchased by R-Biopharm AG (Darmstadt, Germany). Flour samples were provided by Jotis SA industry.

OTA calibrators with concentrations ranging from 0.05–200 ng/mL were prepared from a 2 mg/mL OTA stock solution in absolute ethanol after proper dilution in 1:9 mixture of ethanol with 10 mM phosphate buffer, pH 7.4, 0.9 % (*w*/*v*) NaCl, 0.02 % (*w*/*v*) KCl, 0.2% (*w*/*v*) BSA (assay buffer).

Four-inch Si wafers were purchased from Si-Mat Germany (Kaufering, Germany). The visible/near infrared light source of the sensor is a product of ThetaMetrisis SA (Athens, Greece), the miniaturized USB controlled spectrometer and the reflection probe were obtained from Ocean Insight (Duiven, The Netherlands).

2.2. Chip Biofunctionalization and Assay Protocol

For chip biofunctionalization, a OTA conjugate with OVA was deposited on APTES-modified chips [8] and incubated overnight at RT. The next day, after proper washing with phosphate buffer (washing buffer), the chips were blocked in 2% (*w*/*v*) BSA solution in washing buffer for 3 h, washed once more with washing buffer and distilled water, dried under nitrogen flow and used for the assay.

The fluidic module was applied to each biofunctionalized chip, and assay buffer was run to acquire a stable baseline. For the assay, 1:1 *v*/*v* mixtures of calibrators (0.05–200 ng/mL in assay buffer) or flour extracts 2-times diluted with assay buffer, with the rabbit anti-OTA antibody (1 μg/mL in assay buffer) were flown over the chip for 15 min (primary immunoreaction). Next, a biotinylated anti-rabbit IgG solution (secondary immunoreaction, 7 min), and a streptavidin solution (3 min) were introduced. Lastly, the biochip surface was regenerated by passing IgG elution buffer, and re-equilibrated with assay buffer before reused. All steps were performed under 50 μL/min flow rate. To construct the calibration curve, the effective thickness of the built-up biomolecular layer (signal) corresponding to different calibrators (S_x) was expressed as percentage of the zero-calibrator signal-maximum signal (S_0) and plotted against calibrators' concentration.

3. Results and Discussion

3.1. WLRS-Assay Optimization

Many assay parameters had to be optimized to settle a sensitive and rapid determination of OTA with the WLRS sensor. Due to the competitive nature of the immunoassay, at first, titration experiments were carried out employing different concentrations of OTA conjugate (50–500 μg/mL) combined with different concentrations of the anti-OTA antibody (0.5–4 μg/mL). The combination that provided satisfactory analytical signal and assay sensitivity was 200 μg/mL for the OTA conjugate and 1 μg/mL for the anti-OTA antibody. Another parameter to optimize was the duration of the different assay steps with the condition that the total analysis time should be as short as possible. A total assay duration of 25 min was adopted, consisting of 15 min for the primary immunoreaction, 7 min for the secondary immunoreaction and 3 min for the biotinylated secondary antibody-streptavidin reaction (Figure 1).

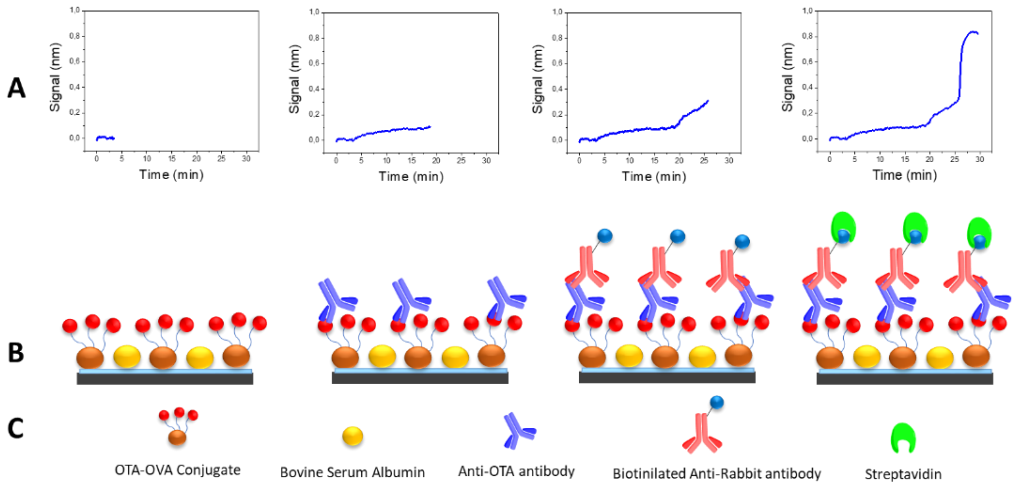


Figure 1. (**A**) Real-time sensor response at each OTA WLRS immunoassay step; (**B**) Schematic representation of the main immunoassay steps; (**C**) main immunoassay reagents.

3.2. WLRS-Assay Characteristics

A representative OTA calibration curve obtained with the WLRS immunosensor and calibrators prepared in assay buffer following the final assay protocol is depicted in Figure 2. It should be noticed that, an identical calibration curve was obtained when extract from a wheat flour that did not contain detectable concentrations of OTA diluted 1:1 *v*/*v* with assay buffer was used as matrix for the preparation of OTA calibrators.

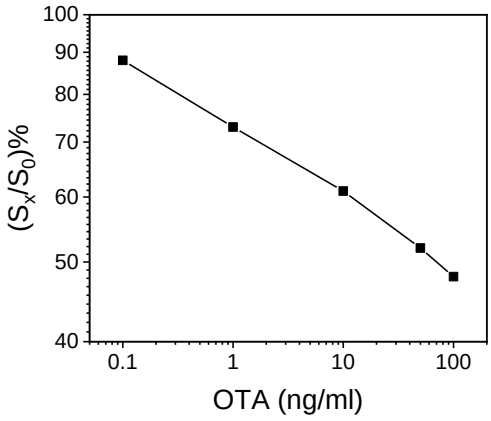


Figure 2. Typical calibration curve obtained with OTA calibrators prepared in assay buffer. Each point represents the mean value of three independent runs ± SD. S_0 = zero-calibrator signal; S_x = calibrator signal.

The analytical sensitivity of the proposed immunosensor, expressed as the assay limit of detection (LoD, and corresponding to mean value-3SD of 15 replicate measurements of zero calibrator) was found to be 30 pg/mL, while the dynamic range extended up to 200 ng/mL. The reproducibility of the assay was determined by running three control samples in triplicate within the same day, and in duplicate in seven different days, in order to calculate the intra- and inter-assay coefficients of variation (CV), respectively. The intra-assay CV values were ≤5.9%, while the inter-assay CVs ≤9.0%.

The OTA-assay developed has been applied to the determination of OTA levels in a small number of cereal flour samples. There was a very good correlation of the OTA con-

centrations determined in those samples with the WLRS assay with the OTA values determined in the same samples using a commercially available immunoassay kit. Evaluation with a larger number of cereal flour samples is under the way.

4. Conclusions

In the current work, a WLRS-based biosensing platform was applied to the label-free and real-time immunochemical determination of OTA. The developed sensor enabled the fast (25 min) and sensitive quantification of OTA levels at concentrations as low as 30 pg/mL. The combination of a series of assets, i.e., the high analytical sensitivity, the short analysis time and the small instrument size, results in a bioanalytical platform ideal for the quantitative determination of minute OTA levels at the Point-of-Need.

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