Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-019-01835-w

RESEARCH PAPER

Immunoassay utilizing imaging surface plasmon resonance for the detection of cyclopiazonic acid (CPA) in maize and cheese

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Received: 31 January 2019 / Revised: 29 March 2019 / Accepted: 5 April 2019

Abstract

α-Cyclopiazonic acid (CPA) is a tremorgenic mycotoxin produced by *Aspergillus* and *Penicillium* fungal species, commonly found on agricultural commodities or fermented food products. A sensitive and rapid imaging surface plasmon resonance (iSPR) assay was developed to detect CPA in maize and cheese by combining an indirect competitive immunoassay and signal amplification based upon a secondary antibody (Ab_2) conjugated with gold nanoparticles. Matrix-matched calibration curves were used to determine CPA content in maize and cheese samples. Recoveries, at two spiking levels in maize and cheese, were 89 to 126%, with standard deviations of repeatability (RSDr) of less than 16%. The limits of detection were 17 and 6 μg/kg in maize and cheese, respectively. To separate the CPA-contaminated samples from uncontaminated samples, a cutoff validation level of 40 μg/kg was introduced. The assay was applied to samples of naturally contaminated maize and was compared with competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA). This is the first report to detect CPA using an immuno-biosensor iSPR format.

Keywords Surface plasmon resonance imaging · Gold nanoparticle · Mycotoxin · Maize · Cutoff · ELISA

Introduction

α-Cyclopiazonic acid (CPA) is an indole-tetramic acid derivative produced by certain species of the fungal genera *Aspergillus* and *Penicillium*. CPA (Fig. 1) was first isolated as a toxic metabolite from a *P. cyclopium* culture during routine screening of toxigenic molds [1] and has been reported in stored maize and maize collected from the field [2]. In addition to maize, this toxin has been found as a natural contaminant in peanuts, sunflower seeds, kodo millet, cheese, and feedstuffs [3]. *Aspergillus flavus*, a potential aflatoxin producer, is also considered as a major CPA producer along with *A. fumigatus* and *A. phoenicis* [4, 5]. CPA may be co-produced with aflatoxins by *A. flavus* in maize and peanuts [2, 6]. A study conducted in the USA after a severe drought showed that CPA was present in 51% of the maize samples, at up to 2.8 mg/kg. Furthermore, 90% of the peanut samples having visible damage to the nutmeats contained up to 2.3 mg/kg [7]. Unlike the accidental growth of molds due to environmental factors, two important molds are intentionally used in the production of fermented foods, namely *Penicillium camemberti* and *A. oryzae* [8]. Le Bars [9] isolated *P. camemberti* from 20 commercial cheese brands. The CPA concentrations found in the crust, which varied greatly with strain and environmental parameters, ranged from 50 to 1500 μg/kg.

Acute toxicosis from CPA has been demonstrated in rats, swine, chickens, turkeys, guinea pigs, and dogs [3, 8]. The effects are weight loss, diarrhea, degeneration and necrosis of muscles and viscera, convulsion, and death [10]. CPA modifies intracellular calcium flux by specific inhibition of the sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA) [3]. SERCA is essential in the muscle contraction-relaxation cycle for calcium re-uptake [11]. Exposure of humans to CPA may occur from ingestion of dairy products such as milk and cheese, as well as eggs, grains, and muscle tissue, especially chicken meat [12–14]. In milk, CPA is stable during storage

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-019-01835-w) contains supplementary material, which is available to authorized users.

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Published online: 25 April 2019

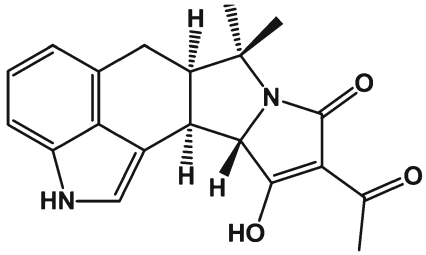

Fig. 1 Chemical structure of cyclopiazonic acid

and commercial processes such as homogenization, pasteurization, and heating [15]. In India, the presence of CPA in kodo millet was suspected to have caused "Koduo poisoning" [16], with symptoms characterized by sleepiness, tremors, and giddiness. According to de Waal [17], the proposed acceptable daily intake for a 60-kg adult would be reached by consuming 50 g of maize/day at a concentration of 140 μg/kg. CPA is a frequent co-contaminant with aflatoxins and may have contributed to Turkey "X" disease in 1960 [18, 19]. A toxicological study conducted by Smith et al. [20] concluded that either aflatoxin or CPA, or their combination, could limit broiler performance and adversely affect broiler health, and that the combination had an additive effect.

CPA contamination of agricultural commodities has been measured by a variety of analytical methods, including chromatographic methods such as thin-layer chromatography, high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), immunochemical methods (immunoassays and immunoaffinity columns), and capillary electrophoresis [3]. Most of the chromatographic methodologies require time-consuming and extensive cleanup procedures. In most cases, halogenated solvents such as chloroform and dichloromethane are also used. Most of the analytical methods are designed for specific matrices [21]. An HPLC-fluorescence detection (FLD) method was reported for the simultaneous detection of aflatoxins and CPA in fungal cultures [22]. Studies of CPA using LC-MS/MS have shown that various commodities caused relatively high matrix effects [23]. Poor recoveries were obtained in pistachios (59%) and maize (19%). The limit of quantification (LOQ) for CPA by LC-MS/MS in foodstuffs ranged from 0.5 to 1 μg/kg [21, 24, 25]. A few studies have used capillary electrophoresis (micellar electrokinetic capillary chromatography) method to measure CPA in milk [26, 27], with a LOQ of 20 ng/mL. In addition to chromatographic and electrophoretic methods, immunochemical methods, such as enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column (IAC) formats, have been reported [28]. Antibodies have been developed against CPA and have been applied in maize, peanuts, and mixed feed by several laboratories [29–32].

In the last decade, biosensor technology has become more widely used in agricultural industries, especially in the food sector. Among the types of biosensors, surface plasmon resonance (SPR) is a powerful technique that can measure biomolecular interactions in real time. The technique has been applied previously to detect a variety of mycotoxins, applications of which have been reviewed [33]. Imaging SPR (iSPR) is a more recent variant of traditional SPR where multiple regions of interest on the sensor surface can be probed simultaneously, allowing for easier multiplexing [34]. iSPR sensors measure changes in the refractive index at the surface of chips having a thin metallic (usually gold) surface. Biosensors have several potential advantages, including reuse of biochips, reduced solvent consumption, and the ability to monitor multiple antigen-antibody reactions in a single test. To the best of our knowledge, there is no previous report on the use of iSPR for the detection of CPA in agricultural commodities. To explore the potential advantages of iSPR for detecting CPA in commodities, a simple, sensitive, and reproducible iSPR biosensor assay was developed to detect this toxin in maize and Camembert cheese.

Materials and methods

Chemicals and reagents

CPA analytical standard was purchased from MP Biochemicals, LLC (Solon, OH, USA). Stock solution was prepared at a concentration of 100 μg/mL by dissolving toxin in LC-grade acetonitrile from Fisher Chemical (NJ, USA). Fresh standards were made daily for calibration over the range of 0.1 to 5 ng/mL. Phosphate-buffered saline (PBS) was prepared as 0.01 M sodium phosphate and 0.15 M sodium chloride in water, with pH adjusted to 7.2. Primary anti-CPA antibody (Ab_1) and primary aflatoxin antibody (AFM1 No. 1-23) were produced at Envigo (Madison, WI, USA) using cell lines previously developed by the USDA-NCAUR (Peoria, IL, USA). Antibody production was in accordance with guidelines established by the National Institutes of Health-Office of Laboratory Animal Welfare. Details of the immunization of animals and isolation of the monoclonal antibody–producing cell lines were described previously [32]. CPA-bovine lactoglobulin (CPA-BLG) antigen was produced at the USDA-NCAUR [32]. AFM_1-BSA (aflatoxin antigen) was purchased from Sigma Aldrich. Gold nanoparticles were produced under conditions reported previously to yield particles approximately 40 nm in diameter [35]. Secondary (anti-mouse) antibody (Ab_2) conjugated with gold nanoparticles (Ab_2-AuNPs) was produced as described previously [36]. Stable isotope ($^{13}C_{20}$-labeled) CPA was purchased from Romer Labs, Inc. (Newark, DE, USA). Gold biochips were purchased from HORIBA Ltd., Japan. The biochips were activated with EDC [1-ethyl-3(3-

dimethylaminopropyl)carbodiimide hydrochloride] and Sulfo-NHS (Sulfo-N-hydroxysuccinimide) from Thermo Scientific (USA) as described previously [36]. Deionized water was prepared with a nano-pure water purification system (Thermo Scientific). Certain of the maize samples, including the control maize, were collected from a local Peoria market and were stored at − 20 °C. Because of the reported co-occurrence of CPA with aflatoxins, reference maize samples containing known levels of aflatoxins were also purchased for evaluation (Trilogy, Washington, MO, USA). Camembert cheese samples from seven different companies were obtained from Igourmet (PA, USA) and stored at − 20 °C until use. Bovine serum albumin (BSA) was purchased from Fisher Scientific, USA. LC-grade methanol was purchased from EMD Millipore Corporation (MA, USA). All other chemicals and reagents were of reagent grade and purchased from major suppliers.

Sample preparation for iSPR assay

Maize samples were milled using a coffee grinder to the consistency of flour. The method for sample extraction was modified from a method used previously with ELISA [32]. Two grams of ground maize was extracted with 10 mL of methanol:water (80:20, *v*/*v*) and 0.4 g of sodium chloride. The mixed sample was shaken for 2 h on a wrist-action shaker at ambient temperature. The mixture was then centrifuged at 4000 rpm (3005×*g*) for 10 min (Thermo Scientific, Sorvall X1R, IL). Before centrifugation, an additional de-fatting step was included for cheese samples by mixing the extract 1 + 1 (*v*/*v*) with hexane, shaking for 1 min using a separatory funnel, and the methanolic phase was collected. The sample extract was diluted fourfold with 10 mM PBS at pH 7.2 and then centrifuged at 12,000 rpm (15,700×*g*) for 10 min. The supernatant was collected and diluted equally with anti-CPA or anti-aflatoxin primary antibody (Ab_1 1:20,000; 100 ng/mL). If the samples were opaque, then they were passed through a 0.2-μm nylon filter (Fisherbrand, Ireland) before injection into the iSPR system.

Biosensor (iSPR) instrumentation

To follow the biomolecular interactions of the Ab_1 and Ab_2-AuNPs with CPA, iSPR analyses were performed with an OpenPlex model iSPR instrument (HORIBA Jobin-Yvon SAS, France). The instrument has three parts. The first contains the optical-mechanical components, consisting of a light source, polarizer, mirror, a primary optical setup, iSPR biochips, a secondary optical setup, and a charge-coupled device (CCD) camera. The second part consists of the fluidic system (degasser, peristaltic pump, and tubing), while the third consists of a personal computer for data acquisition and analysis. An injection valve with a 200-μL loop was used for sample introduction.

Sensor surface modification

Toxin-protein conjugates (antigens) were covalently attached to the sensor surface. To accomplish, this required modifying the "bare" gold surface so that it would contain carboxylate groups and then linking the carboxylate groups to free amino groups on the antigens. In order to generate a surface with free carboxylate groups, the gold surface of the biochips was treated with monothiol reagents containing a terminal carboxylate group [36]. The free carboxylates were activated with an equal mixture of 0.1 M Sulfo-NHS and 0.3 M EDC in deionized water for 1 h at ambient temperature [36]. After washing with deionized water and drying with nitrogen, the antigens (CPA-BLG and AFM_1-BSA) were immobilized by manually spotting 0.65 μL of protein solution at 230, 460, or 920 μg/mL onto the chip surface. The amount applied was therefore 0.15, 0.30, and 0.6 μg per spot. Spots were applied in a grid pattern with four replicate spots for each antigen (see Electronic Supplementary Material (ESM) Fig. S1). Within the spots, 500-μm diameter regions of interest (ROI) were selected from which data were collected. Two types of controls were also included on the chip. The first was BSA immobilized in the same fashion as the antigens. The second consisted of ROI selected from un-spotted areas of the chip (i.e., neither antigen nor BSA immobilized). For efficient immobilization, the chip with spotted solutions was kept in a humid chamber at 4 °C overnight. After washing with deionized water and drying with nitrogen, the surface was blocked with 1 M ethanolamine in 50 mM phosphate buffer (pH 8.5) for 30 min at ambient temperature. After washing with water and drying with nitrogen, the biochip was ready for use. Biochips were stored in an airtight container for future use.

Assay conditions

A biochip was inserted into the iSPR instrument, and running buffer (10 mM PBS with 0.1% BSA) was degassed and injected at a continuous flow rate of 100 μL/min. Before any data were collected, the response from the instrument was calibrated using a sucrose solution prepared at 3 mg/mL in a running buffer. The sucrose solution, with an established refractive index, caused a change in reflectivity that was used by the instrument software (SPRi-Analysis Software, Horiba) as a standard against which the measured reflectivities of the individual spots were compared. The reflectivities seen with each spot were used to calculate calibration coefficients that were used to normalize the responses from the spots during assay conditions. This type of calibration was only needed when a new biochip was installed. Because the sensor chips were re-used, assays were conducted in cycles of use. Each cycle of use started with an equilibration step, followed by an association step with Ab_1, an amplification step with Ab_2-AuNPs, and a series of regeneration steps. A complete iSPR

assay cycle is shown in Fig. S2 (see ESM). The optimal dilution for Ab_1 was 1:20,000, which was equal to 100 ng/mL. After equilibration of the sensor surface with run buffer, a solution containing a mixture of Ab_1 and an extract of sample (maize or cheese) prepared by mixing equal volumes of each was loaded in a 200-μL injection loop and injected. At 3 min, amplification was initiated by the introduction of the labeled Ab_2-AuNPs, diluted 1:9. The kinetics of the binding during association and amplification were monitored in real time. Following amplification, the surface was regenerated using three regeneration solutions (RS). RS1 was 0.05% Tween in water. RS2 was 50 mM sodium dodecyl sulfate and 20 mM NaOH in water pH at 12.0. RS3 was 0.1 M glycine in water mixed with dimethylformamide, 9 + 1, *v*/*v* pH at 3. At the completion of data collection, the iSPR sensograms were analyzed using SPRi-Analysis Software Ver. 2.3 (Horiba Ltd).

The change in response resulting from binding of reagents to the chip surface was calculated by subtracting the signal observed at the equilibration point (i.e., before injection of sample) from the signal observed at the plateau of the response after the Ab_2-AuNPs injection (i.e., the point of maximum specific response). The multiplex nature of iSPR allowed for collection from multiple spots simultaneously. Four spots were averaged for each assay. Further calculations from raw data were done using Microsoft Excel 2007. With ELISA data, the raw data are often normalized to account for the variation in color development between microtiter plates. A similar type of calculation was performed with the iSPR data. The iSPR data were normalized to the signal from a toxin-free sample using (*B*/Bo)%, where *B* was the response from the test sample and Bo was the response from a toxin-free control. To calculate the concentration of CPA in samples, non-linear calibration curves were constructed using a four parameter logistic dose-response equation [37]. For determination of CPA in samples, matrix-matched calibration curves were constructed over the range of 0 to 5 ng/mL equivalent to 0 to 200 μg/kg in maize and cheese. Assays were conducted in triplicate. General assays were conducted by testing the standards (6 cycles) and the samples (8 to 10 cycles) on a single biochip and in a single experiment (i.e., sensorgram or "run"). For recovery studies, CPA was spiked into blank maize and cheese samples at two levels (20 and 40 μg/kg), with assays conducted in triplicate. To compare the iSPR assay and efficacy of cutoff level, natural contaminated maize samples were also analyzed by CI-ELISA as described previously [32].

In-house validation of the iSPR assay

Validation of the iSPR assay as a screening method for CPA was based on the guidance of semi-quantitative screening methods for individual mycotoxins, described in the European Commission (EC) Regulation [38]. In general, screening methods are used for the selection of those samples that have a given certainty of containing mycotoxins at levels which exceed a selected target concentration (STC). A 95% certainty is considered "fit for purpose" in a screening method. This level of certainty allows high sample throughput and increases the ability to find incidents related to high exposure and health risks to consumers. The aim of this validation was to determine if the iSPR method was able to differentiate samples contaminated at the STC, or higher, from blank samples. Because there is no official regulatory limit for CPA, the concentration of interest (STC) for the detection of CPA was set at 40 μg/kg. For single laboratory validation, an experiment was carried out with a set of 20 homogenous blank maize samples and 20 samples of maize spiked with CPA at the STC. The fitness of purpose of the screening method was determined from calculated cutoff levels and the rates of false suspect/negative results. According to the EC Regulation [38], the cutoff value can be calculated as follows:

$$\text{Cutoff} = R_{\text{STC}} + t\ \text{value}_{(0.05)} \times \text{SD}_{\text{STC}} \quad (1)$$

where R_{STC} is the mean concentration of CPA observed in the 20 samples spiked at the STC. The t $\text{value}_{(0.05)}$ is the one-tailed t value for a rate of false negatives of 5%. SD_{STC} is the standard deviation observed from the samples spiked at the STC.

Results and discussion

Biosensors based upon iSPR have the potential to permit the rapid determination of mycotoxins in foods, using a regenerable sensor surface. Given the potential for human exposure to CPA, we undertook development of an iSPR biosensor for this toxin in maize and in Camembert cheese. The sensor used a competitive immunoassay format wherein a CPA-protein conjugate was immobilized on the sensor surface. Extracts of samples were mixed with CPA antibody (Ab_1) and introduced into the sensor. The amount of antibody that bound to the sensor was inversely proportional to the amount of toxin present. Detection of the bound primary (anti-CPA) antibody was improved by the addition of a secondary antibody labeled with gold nanoparticles (Ab_2-AuNPs) [36]. The concept is shown schematically in Fig. 2. To develop the assay required evaluating assay parameters, including the CPA antibody, the impact of added Ab_2-AuNP, the sensitivity of the assay, and the durability of the sensor chips. The developed assay was validated through recovery studies, determination of cutoff values, and application to samples of naturally contaminated maize.

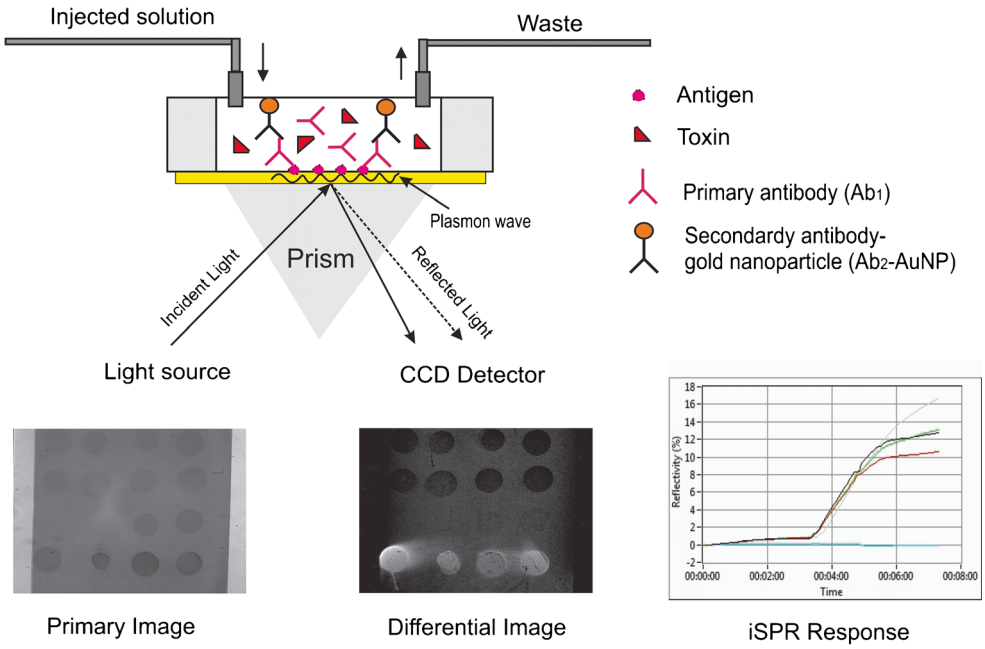

Fig. 2 Schematic diagram of iSPR assay for CPA detection

Optimization of reagents

In immunoassay development, it is important to use the best antibody and antigen combination, wherein the analyte can disrupt the antibody-antigen interaction proportionately and at relevant concentrations. It is also important to maximize the signal strength. In the case of the iSPR technique as used here, that meant optimizing the individual steps of the assay so that the signal and response at the last step (i.e., binding of the Ab_2-AuNPs) were optimized. Two CPA monoclonal antibodies (mAbs) were compared. The antibodies, named mAbs 1231 and 1418, were developed previously for use in competitive indirect ELISA [32]. When Ab_2-AuNP was not used for amplification, mAb 1231 gave a greater iSPR signal than mAb 1418 (0.97 vs 0.28). The optimum dilution determined for mAb 1231 was 1:20k, equal to 100 ng/assay. To determine the optimum level of the immobilized CPA antigen (CPA-BLG), immobilization at three levels was investigated (0.15, 0.3, and 0.6 μg). The percentage of iSPR reflectivity that was observed was higher when 0.6 μg was immobilized (5.55) than when 0.3 μg (2.18) or 0.15 μg (1.94) was immobilized and therefore the highest level was selected. Small compounds like mycotoxins are generally not able to produce significant iSPR signals and therefore they are often measured indirectly by measuring the inhibition of binding of a higher molecular weight material, such as an antibody. Even when using an antibody, the binding of the antibody alone (Ab_1 here) may produce only minor changes in the iSPR signal (ESM Fig. S2). However, following Ab_1 with a labeled secondary antibody (Ab_2-AuNPs) can increase the total mass bound and increase the signal observed. Because the two CPA mAbs could potentially be amplified to different extents by the Ab_2-AuNPs, they were compared again, this time following Ab_2-AuNP amplification. The percentage of reflectivity with Ab_2-AuNPs was 9.53 ± 0.49 ($n = 4$) for mAb 1231 compared with 5.22 ± 0.51 for mAb 1418. This demonstrated the significance of including the Ab_2-AuNP for amplifying the signal. Furthermore, because the mAb 1231 yielded a better signal both with and without amplification, it was chosen for further assay development.

The response of the mAb 1231/CPA-BLG combination to free CPA was evaluated in blank maize matrix. Separately, the response of the aflatoxin mAb 1-23/AFM_1-BSA combination to free AFB_1 was also evaluated. In each case, the combinations were tested with toxin concentrations of up to 5 ng/mL (ESM Table S1). With the CPA antibody and antigen, the response decreased proportionately as the concentration of CPA increased, with 5 ng/mL of CPA reducing the signal to 4% of the original value. With the aflatoxin antibody and antigen, the response also decreased in proportion to the concentration of AFB_1; however, the decrease was less dramatic, with 5 ng/mL AFB_1 only reducing the signal to 57% of the original value. This suggested that the aflatoxin antibody had an affinity for AFM_1-BSA that was greater than its affinity for free AFB_1. This also precluded development of an assay that would simultaneously detect both sets of toxins (i.e., AFB_1 and CPA). No significant binding was observed of the CPA antibody to the aflatoxin antigen or of the aflatoxin antibody with the CPA antigen. This demonstrated there was no cross-reactivity of the CPA assay for AFB_1.

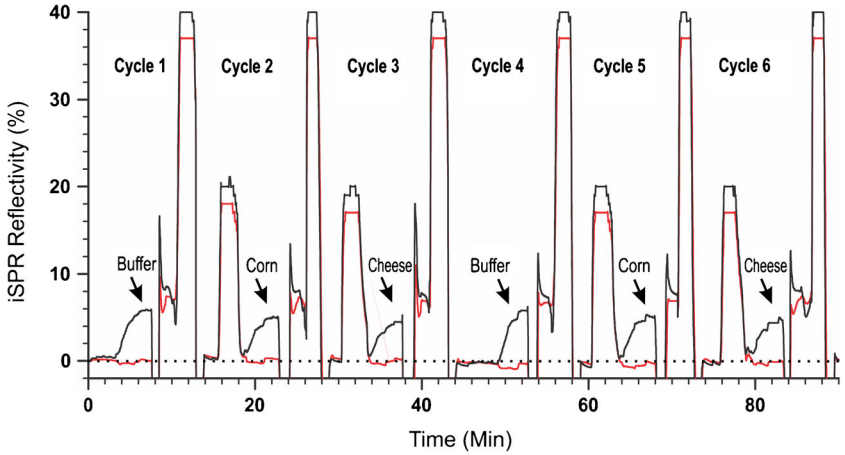

Fig. 3 Effect of maize and cheese matrices on the binding of CPA mAb 1231 to CPA-BLG. A sensorgram of the test spot (black line) is presented over six assay cycles. In addition, a control spot with BSA immobilized is shown (red line). Cycles 1 and 4 used mAb in buffer, while cycles 2 and 5 used mAb in maize matrix and cycles 3 and 6 used mAb in cheese matrix. For comparisons, the signals were evaluated in the plateau region following Ab_2-AuNP addition and before regeneration, as indicated by the arrows

Durability of biochips

An advantage of the SPR platform is the potential for re-usability, which could help to reduce the cost per analysis. For this reason, it was important to evaluate the durability of the biochips over multiple cycles of regeneration. To evaluate the durability of the CPA-BLG-coated biochips, the response of the chips to mAb 1231 amplified with Ab_2-AuNP was investigated over 25 cycles. No significant changes were observed in baseline and antibody binding capacity or carryover after effective regeneration. The result showed that the percentage reflectivity over 25 cycles changed only $7 \pm 2\%$ from the 1st cycle.

Assay performance

Matrix effects

To determine the effect of matrix on assay performance, buffer- and matrix-matched calibration (blank maize/cheese) curves were made using six concentrations of CPA over the range of 0–5 ng/mL (equivalent to 0–200 μg/kg in maize or cheese). A sensorgram showing the effects of maize and cheese matrices relative to buffer is shown in Fig. 3. Also included in that figure is a sensorgram of a control: the response obtained with BSA immobilized instead of CPA-BLG. The figure shows responses over 6 assay cycles. Two of these (cycles 1 and 4) were with mAb in buffer. Two were with mAb in blank maize extract (cycles 2 and 5), and two were with mAb in an extract of Camembert cheese (cycles 3 and 6). To determine the effect of matrix, the signals obtained at the plateau region were compared. This was the region in each cycle after the addition of Ab_2-AuNP and before the regeneration, indicated by the arrows in the figure. The standards prepared in matrix showed lower reflectivity than the standards prepared in buffer. This indicated that binding of the CPA antibody to the CPA-BLG was slightly reduced in the presence of matrix relative to the binding in buffer. Furthermore, the IC_{50} of the CPA calibration curve in maize matrix was 0.84 ng/mL compared with 0.45 ng/mL in buffer.

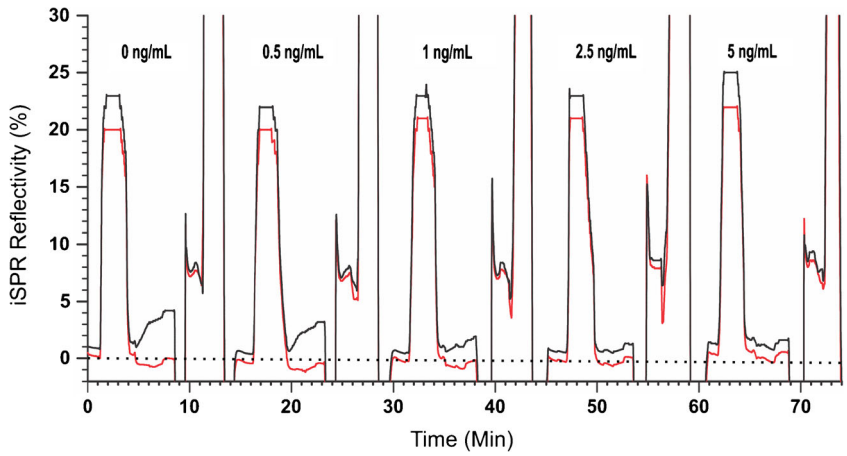

Fig. 4 Sensorgram of five cycles with increasing concentrations of CPA added to blank maize matrix (black line). Also shown is a sensorgram of a control that had BSA immobilized (red line). The region corresponding to the plateau following Ab_2-AuNP addition decreases in magnitude as the concentration of CPA increases

Table 1 Recovery (%) of CPA from spiked maize and cheese ($n = 3$)

Spiking levels (μg/kg)	Maize Recovery (%) ± RSDr	Cheese Recovery (%) ± RSDr
20	89 ± 3	126 ± 7
40	75 ± 10	76 ± 16

The type of matrix had a significant impact upon the shape of the calibration curves as well as the IC_{50} (ESM Fig. S3). In buffer and in maize matrix, the response curves had steep slopes (ESM Fig. S3). A steep slope has also been observed with this antibody in an ELISA format (data not shown). The steep response curves make quantification difficult, since samples need to be diluted to fit within the rather limited range. However, the steep response has the potential benefit in a screening assay where a qualitative result is desired. The response curve in cheese matrix was much more gradual, indicating a substantial effect of cheese upon the assay response. Because of this, the selection of the appropriate matrix for use in matrix matching will be important. Although we have no data to this effect, it would not be surprising if different food brands or different varieties of commodity might affect the shape of the calibration curve and therefore quantification. Therefore, selection of a control matrix as close to that of the test matrix as possible is recommended.

The mechanisms whereby the matrix components inhibit binding to the CPA-BLG (reducing signal) and inhibit binding to CPA itself (suppressing response to toxin) are unknown. Despite this, the sensitivity of the assay, in terms of IC_{50}, was sufficient to suggest that iSPR might be applicable to detecting CPA in maize and in Camembert cheese. Results shown in Fig. 3 also illustrate the effective regeneration of the sensor surface with each assay cycle. If the primary antibody and Ab_2-AuNP were not effectively removed by the regeneration solutions, then the baseline signal (the signal seen during the equilibration step) would increase with each cycle. This type of upward "baseline drift" was not observed, indicating the regeneration solutions were effective at removing the bound primary antibody and AuNPs. Conversely, if the immobilized antigen were being removed during regeneration, the baseline signal would decrease after each cycle. While Fig. 3 shows data over 6 cycles, we did not see a significant drop in baseline over 10 cycles (data not shown), indicating the immobilized antigen was stable over this number of cycles of regeneration.

Limit of detection, limit of quantification

A sensogram containing five assay cycles, each at a different toxin concentration of CPA in maize matrix, is shown in Fig. 4. Also shown is a sensorgram of a control with BSA immobilized instead of CPA-BLG. The limits of detection (LODs), defined as the concentrations of CPA causing 10% inhibition, were 0.42 and 0.15 ng/mL, equivalent to 16.8 and 6 μg/kg in maize and cheese respectively. When defined as the concentrations causing a response 3 standard deviations from that of the blanks, the LODs were 0.45 and 0.29 ng/mL, equivalent to 18 and 12 μg/kg in maize and cheese respectively. The IC_{50}s of CPA in spiked extracts of maize and cheese were 0.84 and 1.46 ng/mL respectively. The coefficients of determination (r^2) for the calibration curves of CPA in maize and cheese were 0.99. The IC_{50}s observed were equivalent to levels of 33.6 μg/kg in maize and 58.4 μg/kg in cheese. In a previous study using mAb 1418 in an ELISA format, the IC_{50} was reported to be 0.58 ng/mL in maize extract [32]. In that case, the presence of maize matrix resulted in a slight improvement in response relative to buffer. Direct comparisons with the previous work are difficult, because the mAb selected for the ELISAs of maize (mAb 1418) was different than the mAb selected for use in the present work (1231). The previous article did, however, report the response for CPA in 20% methanol, the same methanol concentration as used in the present iSPR method. Under those conditions, sensitivity in the iSPR format was slightly better than that in the ELISA

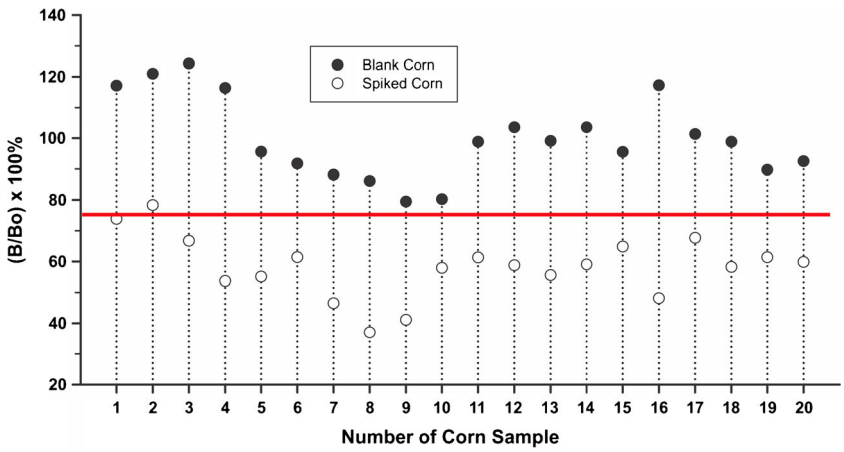

Fig. 5 Cutoff level validation for separating blank and spiked maize samples. White circles represent normalized (*B*/Bo) responses from spiked maize, while black circles represent normalized responses from unspiked maize. The calculated cutoff level is shown as a horizontal red line. Bo is the average of the blank maize samples

Table 2 CPA found in unspiked samples of maize and cheese, as measured by iSPR

Maize sample number	Concentration found (μg/kg)	Qualitative result (+/-)[a]	Camembert cheese sample number	Concentration found (μg/kg)	Qualitative result (+/-)
1	125	+	1	-	-
2	24	-	2	-	-
3	45	+	3	-	-
4	36	-	4	-	-
5	33	-	5	-	-
6	23	-	6	-	-
7	19	-	7	-	-
8	15	-			
9	84	+			
10	14	-			
11	120	+			
12	286	+			

[a] Qualitative results set as positive (+) or negative (-) based on a cutoff level of 40 μg/kg

format (IC_{50} = 0.84 ng/mL vs 1.4 ng/mL, respectively). The sensitivity of the iSPR method for CPA compares favorably with the sensitivities reported in a multiplex iSPR method for six mycotoxins: deoxynivalenol, zearalenone, T-2 toxin, ochtratoxin A (OTA), fumonisin B_1, and aflatoxin B_1 (AFB_1) [34]. In that publication, the LODs ranged from 10 μg/kg (for AFB_1) to 160 μg/kg (for OTA). Comparing the expense, operation cost, and handling of the instrument, the iSPR method was inexpensive, in part because the iSPR biochips are reusable. The iSPR method was also easy to use and sensitive enough to monitor CPA in grains.

Recovery

CPA was spiked into blank maize or Camembert cheese at two levels (20 and 40 μg/kg) in triplicate. For maize, the mean recoveries ranged from 75 to 89% with relative standard deviations of repeatability (RSDr) of 10% or less. For cheese, the mean recoveries ranged from 76 to 126%, with RSDr of 16% or less (Table 1). Results suggested that recoveries of CPA from maize and cheese were acceptable at 20 and 40 μg/kg. Although CPA is not a regulated mycotoxin, such a range would appear to be appropriate given the known toxicity and occurrence of this compound. The detection range falls between those of the highly carcinogenic aflatoxins (regulatory levels generally in the low μg/kg range) and those of the acutely toxic trichothecenes (regulatory levels generally in the low mg/kg range).

Validation as a screening assay and application to naturally contaminated samples

Calculation of a cutoff level is a useful exercise because it describes the concentration at which an assay can effectively determine whether a sample is "suspect" and therefore if it should undergo further testing. To determine the cutoff level, a set of blank maize samples and maize samples spiked at the target concentration of CPA (STC = 40 μg/kg) were analyzed. As we observed in Fig. 5, there was separation between the blank and spiked samples except 1 spiked sample (sample no. 2) out of 20. According to the EC Regulation [38], 5% of the spiked samples (1 out of 20) are acceptable to be false negative. Next, the method was applied to naturally contaminated samples of maize (n = 12) and Camembert cheese (n = 7). As shown in Table 2, 5 samples out of 12 were considered as suspected samples based on the cutoff level of maize of

Table 3 Comparison of CI-ELISA and iSPR methods for measuring CPA in naturally contaminated maize samples

Sample number	CPA (μg/kg)	
	CI-ELISA[a]	iSPR[b]
1	433	125
2	ND	24
3	81.7	45
4	11.6	36
5	130	33
6	8.1	23
7	ND	(19)
8	ND	(15)
9	116	84
10	ND	(14)
11	235	120
12	521	286

[a] LOQ = 5 ng/g

[b] LOQ = 20 ng/g based on IC_{20}

ND, not detected

Numbers in parentheses indicate the value was below the LOQ

40 μg/kg CPA. For cheese, all of the samples analyzed were found as negative, that is, below the cutoff level. The maize samples ($n = 12$) were also analyzed by CI-ELISA for comparison and to see the validation efficacy of the cutoff level (Table 3). The results showed qualitative agreement between the two methods with a Pearson correlation of 0.92. In general, the ELISA tended to report higher levels than the iSPR. Of the 12 samples, 5 were classified as positive by iSPR (cutoff 40 μg/kg). The same 5 samples also contained greater than 40 μg/kg CPA when tested by ELISA. One sample (no. 5) contained 33 μg/kg CPA by iSPR and therefore was classified as non-suspect yet contained 130 μg/kg CPA by CI-ELISA. Assuming the ELISA method was accurate, this was a false negative by iSPR. This sample was examined further with an LC-HRMS method combining the extraction and sample preparation described by Ansari and Haubl [24] with the MS detection described by Permingo et al. [39]. Under most reverse phase LC conditions, CPA remains anionic, which can lead to broad chromatographic peaks unless additives, which can potentially influence ionization in the mass spectrometer, are used in the mobile phase. We were not confident enough in the LC-HRMS method to use it for routine quantification. However, we used it to examine this sample which was considered "borderline" positive. The level of CPA found by LC-HRMS, 45 μg/kg, confirmed that this was a positive sample (i.e., above the 40 μg/kg cutoff). Because the value found by iSPR (33 μg/kg) was below the cutoff, this was a true false negative by iSPR.

Conclusion

A novel iSPR biosensor assay was developed for the detection of CPA in maize and Camembert cheese. No cross-reactivity was seen with the potential co-contaminant, aflatoxin B_1. Sensitivity, precision, and cutoff level were used to characterize the performance of the assay. The developed method was able to detect as low as 17 μg/kg CPA in maize and 6 μg/kg in cheese. Recoveries were acceptable, with RSDr of less than 16%. The iSPR method and CI-ELISA method were applied to determine CPA content in naturally contaminated maize samples. The iSPR method reported herein is rapid and sensitive and can be used for screening of CPA in grains and possibly cheese.

Funding information This work was supported by the United States Department of Agriculture (USDA)-Agricultural Research Service (ARS) project number 5010-42000-0049-00D. This research was supported in part by an appointment to the ARS Research Participation Program, administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the USDA. ORISE is managed by Oak Ridge Associated Universities (ORAU) under DOE contract number DE-SC0014664.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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