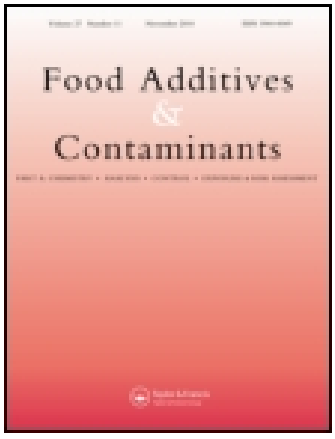

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Signal amplification using colloidal gold in a biolayer interferometry-based immunosensor for the mycotoxin deoxynivalenol

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Signal amplification using colloidal gold in a biolayer interferometry-based immunosensor for the mycotoxin deoxynivalenol

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Deoxynivalenol (DON) is a toxin produced by certain species of *Fusarium* fungi that can infest wheat, barley and corn. The fungi cause diseases in crops worldwide and some of the secondary metabolites, such as DON, can adversely affect animal health and food safety. To monitor DON in wheat rapidly, a biosensor using the principle of biolayer interferometry (BLI) was developed. The signal from the sensor was substantially amplified through the use of a primary antibody–colloidal gold conjugate. The amplification was much greater in the presence of wheat matrix than in buffered solution, suggesting matrix components may have contributed to the enhancement. The improved signal provided by the amplification allowed for the development of rapid qualitative and quantitative assays. The limit of detection of the method was $0.09\,mg\,kg^{-1}$; the limit of quantitation was $0.35\,mg\,kg^{-1}$. Recovery from wheat spiked over the range from 0.2 to $5\,mg\,kg^{-1}$ averaged 103% (RSD = 12%). The quantitative assay compared favourably ($r^2 = 0.9698$) with a reference chromatographic method for 40 naturally contaminated wheats. The qualitative assay was able to classify accurately the same group of 40 samples as either above or below a $0.5\,mg\,kg^{-1}$ threshold. These results suggest that the BLI technique can be used to measure DON in wheat rapidly.

Keywords: screening – immunoassays; screening – biosensor; mycotoxins – trichothecenes; trichothecenes; cereals and grain

Introduction

Certain *Fusarium* fungi can infest small grains causing diseases such as head blight in wheat and barley. The fungi can also produce secondary metabolites, mycotoxins, that can cause disease in animals. Among the mycotoxins that may be produced by *F. graminearum* and *F. culmorum* are a group known as trichothecenes, which share a tetracyclic sesquiterpenoid structure that includes a six member oxygen-containing ring in the 12,13 position and an olefinic bond in the 9,10 position (Betina 1989). Within this group are several toxins important to agriculture, including deoxynivalenol (DON, also known as vomitoxin). Swine are sensitive to DON, and show symptoms that may include reduced feed consumption, abdominal distress and emesis (Rotter et al. 1996). DON has been found worldwide in commodities such as wheat, barley and corn (maize). In order to protect human and animal health many countries have established regulatory or advisory levels for DON in commodities and foods. In Europe a tolerable daily intake of $1\,mg\,kg^{-1}$ body weight was established by the European Commission (Scientific Committee on Food 2002), with limits in cereals and cereal-based foods ranging from 0.2 to $1.75\,mg\,DON\,kg^{-1}$ (European Commission 2006). In the United States the advisory level is $1\,mg\,DON\,kg^{-1}$ (1 ppm) for grain intended for human consumption. Recent reviews have described the potential health effects of DON as well as the occurrence and assessment of exposures (Pestka 2010; Turner 2010).

A concern for human and animal health and the establishment of guidance levels have driven the need to monitor for DON and related trichothecenes. As a result, many methods have been developed to detect these toxins in commodities and foods (summarised in: Krska et al. 2008; Pascale, Lippolis, et al. 2008; Turner et al. 2009; Maragos and Busman 2010; Shephard et al. 2011). All of the widely used chromatographic techniques such as high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), and gas chromatography (GC) have been applied to the detection of DON. Recently, significant attention has been given to multi-toxin methods, including HPLC with mass spectrometric detection (HPLC-MS) (Cavaliere et al. 2007; Sulyok et al. 2010). Immunoassays are also widely used to detect DON, particularly in situations where rapid

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screening is needed. Commercial immunoassay kits are readily available. Recent advances in cleanup and isolation technologies include immuno-ultrafiltration, sol-gel immunoaffinity columns and polymer-based solid-phase extraction (Böhm et al. 2008; Pascale, de Girolamo, et al. 2008; Brenn-Struckhofova et al. 2009). As new technologies have evolved the number and type of assays for DON has continued to grow. This has included the application of technologies such as lateral flow test strips, fluorescence polarisation immunoassay, and biosensors that employ surface plasmon resonance, flow cytometers or array-based devices (summarised in Maragos and Busman 2010). Furthermore, tests that detect the effects of fungal growth upon the acoustic or spectroscopic properties of the commodities have been developed (Juodeikienė et al. 2004; Siuda et al. 2008; de Girolamo et al. 2009).

Recently our group explored the application of a new technology, biolayer interferometry (BLI), to the detection of DON (Maragos 2011). The technique is based on generating an interference pattern that changes as materials bind to the sensor. Light is launched into an optical fibre and when it reaches a polymeric coating near the tip a portion of the light is reflected back (i.e. towards the light source). In a fashion, this reflected light is a 'reference' beam. A small amount of light is also reflected back from the very tip of the fibre at the interface between the fibre and the surrounding solution. The light reflected from this interface and the 'reference' light interfere with one another, establishing a shift in wavelength that can be measured. The magnitude of the shift is related to the thickness of the layer immobilised on the tip of the fibre. Because of this, the instrument measures the increase in thickness as materials bind to the tip of the sensor (Concepcion et al. 2009). This allows the binding of materials to be monitored as they occur. It also avoids the need to separate bound and unbound labels, eliminating the washing steps where such separations are conducted in many other immunoassay formats such as enzyme-linked immunosorbent assay (ELISA). A particular advantage of using fibres, rather than small channels through which reagents flow (a common format for many biosensors), is the avoidance of problems related to the blocking of such channels (Rich and Myszka 2007).

Immunoassay formats that are widely used in biosensors for small analytes are directly analogous to commonly used ELISA formats. These formats involve immobilisation of the toxin (or toxin–protein conjugate) on the surface, or immobilisation of the recognition element (e.g. antibody or receptor) onto the surface. In a previous report a commercial biosensor using BLI was applied to the detection of DON in extracts of spiked whole wheat flour (Maragos 2011). In that report the format used involved immobilising a toxin–protein conjugate onto the surface of the fibre. The increase in the thickness at the sensor tip when the fibre was exposed to toxin-specific antibody was detected. For the detection of DON, assays involved competition between the immobilised conjugate and DON for a limited amount of antibody. In the previous work it was noted that sensors could be reused for several cycles, but that there was a gradual drop-off in sensor response with each cycle. Furthermore, while individual sensors gave reproducible signals, there were obstacles for routine application of the technology. These included the need to pre-condition the sensors before use and the need to control variability between sensors. Reported here are investigations to improve upon the usefulness of the technique. To this end several aspects were investigated here that were not examined in the previous work. These included amplification of the assay signal through the use of labelled reagents (a labelled secondary antibody, or the primary antibody labelled with colloidal gold) and the effects of wheat matrix upon the assay. Furthermore, this work describes procedures to eliminate conditioning of the sensors, and the development of two formats of rapid assays for DON: a quantitative assay and a qualitative assay. The two assay formats were applied to the detection of DON in spiked and naturally contaminated wheats, with comparisons with a chromatographic reference method.

Materials and methods

Chemicals and reagents

Except where noted otherwise, de-ionised water (Barnstead Nanopure, Thermo Scientific, Waltham, MA, USA) was used in the preparation of all reagents. Dimethylformamide (DMF) was ACS reagent grade. The murine monoclonal antibody 'DON Mab #1' was developed at the United States Department of Agriculture National Center for Agricultural Utilization Research (USDA-NCAUR, Peoria, IL, USA) and was described previously (Maragos and McCormick 2000; Maragos et al. 2012). The covalent linkage of DON to bovine serum albumin (BSA) was also described previously (Maragos and McCormick 2000). Deoxynivalenol stock solution was prepared at a nominal concentration of 1.2 mg ml^{-1} by dissolving solid toxin (Sigma Chemical, St. Louis, MO, USA, lot 24H4038) in acetonitrile. The actual concentration of toxin was determined from the ultraviolet spectrum of dilutions of the stock and using the extinction coefficient of 6805 at 219 nm (Krska et al. 2007). Spiking solution (250 μg ml^{-1}) was prepared by dilution of the stock solution with acetonitrile. Casein was from MP Biomedicals (Solon, OH, USA). Solutions of 0.1% (w/v) casein were prepared in 10 mM phosphate buffered saline (C-PBS, pH 7.2). Ag(III) chloride hydrate

and BSA were purchased from Sigma-Aldrich. Goat anti-mouse IgG conjugated to horseradish peroxidase (i.e. GAMP) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). All other chemicals were reagent grade or better and purchased from major suppliers.

Preparation of antibody–colloidal gold

DON Mab #1 was non-covalently attached to colloidal gold using the procedure of Shim et al. (2006). In brief, gold colloid was produced from Ag(III) chloride by heating and the addition of sodium citrate (Frens 1973) at a level reported to yield 40 nm particles (Shim et al. 2006). The colloid was passed through a Whatman #1 filter and the pH was adjusted to 9 with 0.1 M K_2CO_3. To 120 ml of colloid, 2.7 mg of antibody were added in 8 ml of 2 mM sodium borate buffer, pH 8.8. This solution was stirred for 1 h at ambient temperature and 12.4 ml of 10% (w/v) BSA was added. The solution was held an additional 1 h at ambient temperature then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant solution was discarded and the pellet was suspended in 80 ml of 2 mM sodium borate, pH 7.2. The suspension was centrifuged once more under the same conditions. The supernatant solution was discarded, and the pellet was brought to 20 ml with storage buffer (2 mM sodium borate, 1% BSA, 1% sucrose and 0.05% sodium azide). This preparation, Ab-Au, was stored at 4°C and used within 1 month.

Spiking and extraction of wheat flour for the BLI assay

Whole wheat flour was obtained from a local distributor (Detweiler's Bulk Foods, Albany, WI, USA), and determined to contain less than 0.05 mg kg^{-1} DON by HPLC-UV. This material was used for spiking/recovery studies and for the preparation of a control (toxin-free) extract. Control extract was used for matrix matching of the calibration solutions with the samples. Samples of wheat flour (25 g) were spiked with DON in quadruplicate by addition of an appropriate volume of the DON spiking solution (250 μg ml^{-1}). Samples were allowed to dry overnight at ambient temperature prior to extraction. Samples were extracted with 175 ml of 0.02 M phosphoric acid by blending for 3 min. Phosphoric acid was used because previous experiments had shown that it reduced matrix interferences relative to extraction with water alone (Maragos 2011). The crude extracts were filtered (Whatman 2 V, Clifton, NJ, USA) and diluted 1 + 3 (v/v) with 10 mM PBS containing 0.005% (v/v) Tween-20 (i.e. PBS-T), then tested by BLI using either a qualitative or a quantitative assay. For the quantitative assay, samples found to contain high levels of DON (i.e. greater than 5 mg kg^{-1}) received an additional dilution in order to bring the toxin concentration into the more linear portion of the calibration curve. Samples of naturally contaminated wheat were also used to assess performance. The naturally contaminated samples were obtained over several different crop years and were not randomly collected. As such they are not indicative of the DON contamination from any particular region or crop year.

Preparation of sensors

Sensors with an aminopropylsilane (APS) surface coating were purchased from ForteBio (Menlo Park, CA, USA). DON−BSA was non-covalently immobilised to the sensor surface using 25 μg ml^{-1} solution, then blocked with 0.1% C-PBS as described previously (Maragos 2011). The coated and blocked sensors were incubated for 3 min in a solution of 15% (w/v) sucrose, dried and stored at ambient temperature for up to 6 months. Sensors were used without further preparation.

Signal amplification from GAMP and Ab-Au

To study the possibility of signal amplification in BLI the effects of including either a secondary antibody (GAMP) or labelling the primary antibody with colloidal gold (Ab-Au) were investigated. Data were collected with an Octet Red biosensor, which measures the signal from up to eight channels simultaneously (ForteBio). Each assay consisted of repeated cycles having three steps: incubation with buffer to establish a baseline signal (equilibration), incubation with the test mixture containing DON-specific antibody (association), and incubation with a buffer to remove the antibody in preparation for the next cycle (regeneration). Using the system software the signal at the end of the association step was calculated for each standard and sample. The basic form of the assay, without the use of signal amplification, was described previously (Maragos 2011). The effects of adding GAMP, or replacing the unlabelled antibody with Ab-Au, were tested side by side using either: (1) antibody without an amplification agent ('Ab alone'), (2) antibody with added GAMP ('Ab + GAMP'), or (3) antibody–colloidal gold conjugate ('Ab-Au'). Because the magnitude of the signal from the sensor depends upon antibody concentration, an attempt was made to test the three preparations at equivalent antibody concentrations. For the 'antibody alone' preparation antibody was simply diluted to 35 μg ml^{-1} in C-PBS. For the 'antibody + GAMP' preparation the antibody and GAMP were added so that the final level of antibody was 35 μg ml^{-1} and the final level of GAMP was a 1:100 dilution of GAMP stock in C-PBS. Determining

the level of antibody in the Ab-Au preparation was less straightforward, because the gold also has significant absorbance in the UV region (Xiulan et al. 2005). For this reason the antibody level in the Ab-Au preparation was estimated from the amount of antibody used in the synthesis (2.7 mg) and the final volume of the stock solution (20 ml), as 135 μg ml^{-1}. This assumed that all of the antibody was conjugated to the colloid. Based on this assumption the Ab-Au stock was diluted in C-PBS to give a preparation with nominally 35 μg antibody per ml. In this fashion the Ab-Au could be more directly compared with the Ab alone and Ab + GAMP treatments.

To the wells of a black microtitre plate (Greiner Bio-One, Dresden, Germany) 200 μl of C-PBS were added for equilibrating sensors. To separate wells, 200 μl of the Gly/DMF solution were added for regenerating sensors. Test solutions were prepared by adding 100 μl of DON standard in C-PBS and 100 μl of diluted antibody preparation (either antibody alone, antibody + GAMP, or Ab-Au, as described above) to unoccupied wells of the same microtitre plate. Before starting an assay the plate was equilibrated to 30°C. DON standards were tested over the concentration range of 0.35–750 ng ml^{-1}. Each assay cycle consisted of a 30 s equilibration step with 0.1% C-PBS, a 120 s association step with the test mixture, and a 60 s regeneration step with Gly/DMF. Each cycle lasted 210 s. Using the system software the signal at the end of the association step was calculated for each standard and sample. To determine whether wheat matrix would influence the possible amplification caused by GAMP or colloidal gold, the experiments were repeated in the presence of wheat matrix. In these experiments the DON standards were prepared in a 1 + 3 dilution of control (toxin-free) wheat extract prepared as described above, rather than in C-PBS.

Quantitative assay for DON in spiked or naturally contaminated wheat

For quantitative assays Ab-Au, rather than Ab alone or Ab + GAMP, was chosen to provide signal amplification. A calibration curve was prepared by spiking DON into control wheat extract (obtained from toxin-free whole wheat flour), diluted 1 + 3 with PBS-T as described above. The concentrations of the standard solutions ranged from 1.07 to 714 ng ml^{-1}, equivalent to 0.03–20 mg kg^{-1} DON in wheat flour. Each standard solution was tested between those of control extract (e.g. control, standard, control). This was done to improve quantitation, because the signal from the sample (or standard) was calculated relative to the toxin-free control. An assay was initiated by conducting an equilibration with C-PBS for 30 s. Then the association step (60 s) was conducted in a test mixture comprised of a 1 + 1 mixture of sample extract and antibody–gold diluted 1 + 4.4 in C-PBS. Lastly, the regeneration step (30 s) was conducted in a mixture of aqueous glycine and dimethylformamide (Gly/DMF: 0.1 M glycine pH 3, mixed 4 + 1 with dimethylformamide). Each assay cycle took approximately 2 min. Therefore to test a sample (or standard) relative to controls (tested before and after the sample) took approximately 6 min. To adjust for different maximal signal intensities between sensors, the signal from a given sample was represented as a percentage of the average signal from the toxin-free controls tested immediately before and after it (e.g. as a percentage of the control, or maximal, signal). This type of transformation to the percentage of the maximal signal is analogous to representing ELISA data as the percentage of maximal absorbance. Because the device has eight channels, the time to test eight samples (or standards) was the same as the time to test one sample. In this fashion one to eight samples could be tested in 6 min, nine to 16 samples in 12 min, etc. Concentrations of DON in the spiked or naturally contaminated samples were determined by comparing the (transformed) response of the sample with the (transformed) responses of the calibration standards. The calibration curve was generated using a four-parameter logistic dose–response equation (TableCurve, SPSS Science, Chicago, IL, USA). Samples having responses above that of the 357 ng ml^{-1} DON standard (a level equivalent to 10 mg kg^{-1} in wheat) were diluted further with control wheat extract and re-assayed.

Qualitative assay for DON in naturally contaminated wheat

Further increases in the speed of the assays were explored by adapting the quantitative test to a qualitative format. The major differences between the qualitative and quantitative assays were the use of a single control sample (rather than bracketing the test sample between control samples), and the use of a single spiked sample ('threshold sample') rather than a calibration curve. Qualitative assays were conducted with two cycles: one cycle for the toxin-free control and another for the sample. The equilibration was with 200 μl C-PBS for 30 s. The association was with a mixture of 100 μl of diluted sample extract and 100 μl of Ab-Au (at 1 + 4.4 in C-PBS) for 60 s. The regeneration was with 200 μl Gly/DMF buffer for 30 s. Each assay cycle took approximately 2 min. The time to test both sample and control was approximately 4 min. The signal from the test or unknown sample was transformed by representing it as a percentage of the control (toxin-free) sample. The threshold sample, a sample of toxin-free wheat spiked with 0.5 mg kg^{-1} DON, was

assayed concurrently with the test samples. Samples with a transformed signal below that of the threshold sample were deemed 'positive', those with a transformed signal above that of the threshold sample were deemed 'negative'. Using this assay format one to seven unknown samples could be tested in 4 min (the eighth channel being used for the threshold sample), eight to 14 samples in 8 min, etc.

HPLC-UV of naturally contaminated wheat

Wheat was tested by HPLC with ultraviolet detection (HPLC-UV) essentially by the method of Trucksess et al. (1998) with modifications reported previously (Plattner and Maragos 2003). In brief, 25 g samples were extracted with 100 ml of acetonitrile/water (84 + 16, v/v) by shaking for 1 h at ambient temperature, then filtered (Whatman 2 V). A 4.5 ml aliquot of the filtrate was passed through a Romer #225 column (Romer Laboratories, Union, MO, USA). A total of 4 ml of the eluate was transferred to a silane treated vial and dried under a stream of nitrogen gas at 50°C for 1 h. The extract was dissolved in 0.3 ml water/methanol (4 + 1 v/v), passed through a 0.2 micron syringe filter, and 20 µl injected for separation by reverse-phase HPLC with detection at 220 nm. The HPLC system consisted of a SpectraSystem P4000 pump (Thermo Separations Products, San Jose, CA, USA), a Rheodyne 9125 injector with a 200 µl loop, a reversed-phase guard column (Applied Biosystems, Inc., Foster City, CA, USA, NewGuard RP-18 3 mm × 15 mm, 7 µm), a reversed-phase analytical column (TosoHaas, Montgomeryville, PA, USA, ODS-120 T, 4.6 mm × 25 cm, 2 µm), and a Spectra System UV2000 detector (Thermo Separations). Data acquisition used AllChrom Plus software (Alltech Associates, Inc., Waukegan, IL, USA).

Results and discussion

Signal amplification

In previous work, a BLI-based method was developed for the detection of DON in spiked wheat. In that report the variability between sensors was noted. It was also noted that while sensors could be reused for many cycles, there was a gradual drop-off in sensor response with each cycle (Maragos 2011). The current report describes efforts to overcome these obstacles by investigating amplification of the assay signal and the manipulations of the sensors before their use (sensor conditioning). Because the sensor indirectly measures the thickness of materials bound to the tip, reagents that increase the thickness of this layer may potentially increase assay signal and perhaps sensitivity. To determine whether signal amplification could be accomplished, and if it would be useful, two means for amplification were tested. The first of these involved adding a secondary antibody with a label attached. The antibody chosen was a goat anti-mouse IgG to which the enzyme horseradish peroxidase (HRP) had been conjugated (i.e. GAMP). It is important to note that the enzymatic activity of the GAMP was not related to its function as a signal amplification reagent. The amplification, if any, would come from the additional thickness to the tip of the fibre that would result from binding of the secondary antibody–enzyme conjugate. The second reagent investigated was the primary (anti-DON) antibody to which colloidal gold had been conjugated (i.e. Ab-Au). The gold colloid adds significant size to the primary antibody which, in theory, might result in increased thickness at the sensor tip upon binding of the antibody to the immobilised DON. These aspects are illustrated in Figure 1. Colloidal gold has been used for signal amplification in other immunoassay formats such as lateral flow test strips and surface plasmon resonance biosensors (Xiulan et al. 2005; Shim et al. 2006; Yuan et al. 2009).

Initial experiments to determine if there was potential for signal amplification by GAMP or Ab-Au were performed with the reagents in buffered solutions (casein-PBS). Because the signal obtained in BLI is also dependent on the antibody concentration used, comparisons were made at equivalent antibody levels. The effects of adding GAMP along with the primary antibody or of replacing the primary antibody with Ab-Au are illustrated in Figure 2(A). Error bars represent ±1 SD (standard deviation) from the mean. Results suggested that addition of GAMP did not improve the magnitude of the signal from the BLI and

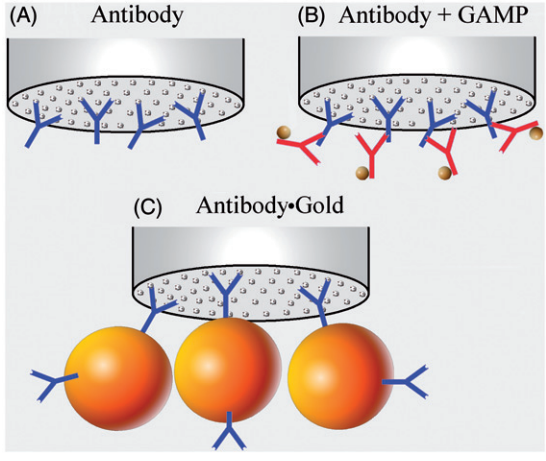

Figure 1. Schematic diagram of the mechanisms for amplifying the signal in biolayer interferometry: (A) binding of the primary antibody to DON-BSA immobilized on the sensor tip (e.g. unamplified); (B) binding of the antibody and amplification with a secondary antibody–HRP conjugate (GAMP); and (C) binding of the primary antibody conjugated to colloidal gold. The drawing is not to scale.

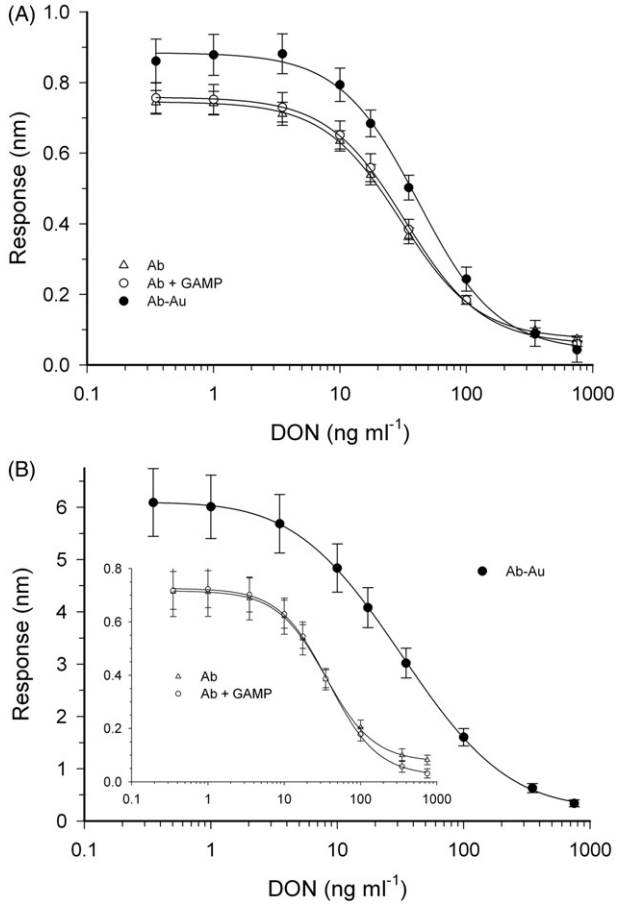

Figure 2. Comparison of the reagents for amplification of the BLI signal and effects of the wheat matrix: (A) responses of the assay with DON in a buffered solution: antibody alone (Δ), antibody with GAMP (○) or antibody with colloidal gold (●); and (B) responses of the assay with DON in a diluted wheat extract.

did not affect assay sensitivity. However, the Ab-Au was observed to increase the magnitude of the signal, an indication that a small degree of amplification might be occurring. Because food matrix components often affect immunoassay performance the potential for GAMP or Ab-Au to amplify the BLI signal was also investigated in the presence of wheat matrix. In Figure 2(B) the DON standards were added in diluted wheat extract rather than buffer. Results suggested that, as with the experiments in buffer, GAMP did not cause an increase in the magnitude of the signal. The results for Ab-Au, however, were dramatic. The presence of the wheat matrix resulted in an approximately six-fold increase in assay signal (note the ordinate scales in Figure 2B). This suggested that Ab-Au might be very useful for signal amplification in the DON BLI assay. The reason for the much greater amplification in the presence of wheat matrix is unclear. We speculate that the gold colloid is also interacting with matrix components, such that when the Ab-Au binds to the sensor tip additional components from the wheat matrix are also attached, substantially increasing the thickness of the layer at the sensor tip and therefore the signal. Preliminary experiments indicated that further dilution of the wheat extract, beyond the 1 + 3 dilution shown in Figure 2, attenuated the increase in signal attributed to the presence of wheat extract. The impact of different wheat varieties, presumably yielding extracts with different matrices, has yet to be determined.

Quantitative assay for DON in spiked or naturally contaminated wheat

The signal amplification from Ab-Au was important because it allowed the assay times to be shortened. This is because, with amplification, a certain level of Ab binding could be detected more rapidly. As a result the cycle times were reduced from 210 s to 120 s. However, previous research had shown that the signal collected from sensors decreased by approximately 1.5% with each assay cycle (Maragos 2011). This effect was speculated to be due to a slow loss of the immobilised antigen (DON-BSA) with each assay cycle. To allow for reuse of the sensor for quantitation of DON therefore required a means for controlling this effect. Attempts to attach the test antigen covalently to the sensor tip did not reduce the effect (data not shown). Therefore to permit reuse of the sensors and to allow quantitation, additional steps were taken to control for the slow drift in sensor response. For quantitative assay the solution was to assay a toxin-free extract before and after each test sample. The response of the test sample was then expressed relative to the response from the toxin-free controls and compared with a calibration curve prepared in the same manner. Because essentially three tests were conducted for each assay (e.g. blank, sample, blank), the assay times became 6 min rather than 2 min. While this lengthened the assay times (6 min for the first sample, 4 min for successive samples on the same plate), the quantitation was improved. An average calibration curve for DON in spiked wheat extract is shown in Figure 3. Note that the error associated with the measurements was substantially reduced relative to experiments where such adjustments were not made (e.g. Figure 2B). The impact of adjusting for the sensor drift can also be illustrated by the effect of the assay changes on the relative standard deviations (RSD) of the calibration curves. The average RSD for the calibration curve using Ab-Au over the range from 1 to 350 ng ml^{-1} (i.e. Figure 2B) was 10.4%. Over a similar concentration range, 0.03–10 ppm equivalent in wheat, the average RSD for the calibration curve (i.e. Figure 3) was 6.4%. Thus the additional steps of testing multiple toxin-free controls, and data normalisation relative to those

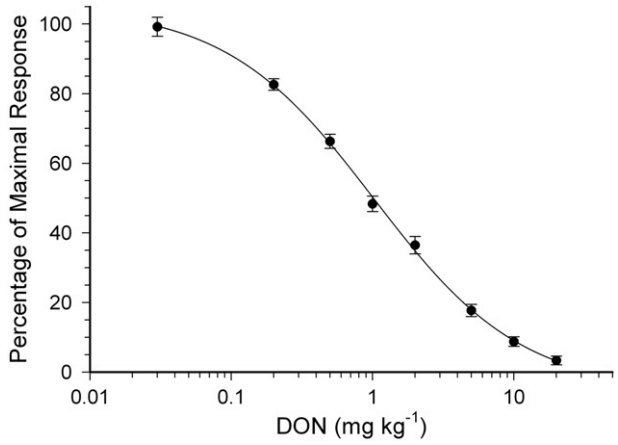

Figure 3. Calibration curve of DON in spiked wheat extract at levels equivalent to 0.03–20 mg kg^{-1} wheat. Data points represent six separate experiments with quadruplicate replicates per experiment (e.g. $n = 24$ per point). Error bars are ± 1 SD (standard deviation). Data were fit to a four-parameter logistic dose–response equation.

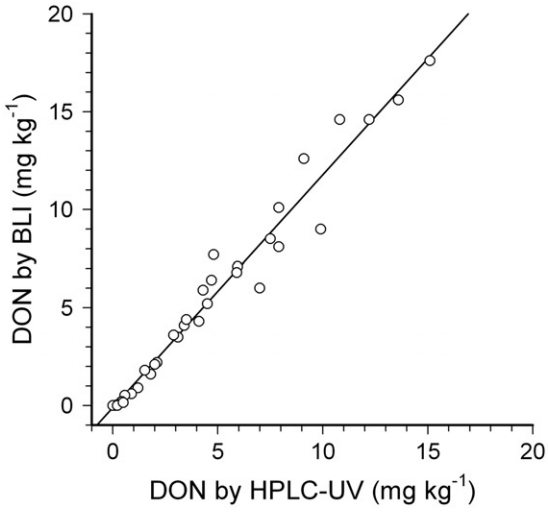

Figure 4. Comparison of DON in naturally contaminated wheat measured by the BLI assay and by an HPLC-UV method.

Table 1. Recovery of DON from spiked whole wheat flour using biolayer interferometry (BLI).

Spiking level (mg kg^{-1})[a]	Recovery (mean ±1 SD)
0.5	117 ± 10
1	114 ± 10
2	101 ± 5
5	98.2 ± 4.9
10	90.9 ± 1.2
All levels[b]	104 ± 12

Notes: [a]Four replicate samples at each level, with each replicate assayed four times.
[b]Average for all five spiking levels.

controls, helped reduce measurement variability. Each sensor was used for a total of 12 assay cycles, a number at which our previous work suggested the maximum sensor response would be expected to decrease by 10–20%. Although it may be possible to reuse the sensors more than 12 times, we did not explore this aspect. Certainly the gradual loss of signal with each cycle will place limits on the number of times that the sensors can be reused and, at this point, we do not recommend using the sensors for more than 12 cycles without further validation.

The limit of detection (LOD), calculated as the DON level required to give a signal 3 SDs below that of the toxin-free control, was 0.09 mg kg^{-1}. The limit of quantitation (LOQ), calculated as the DON level required to give a signal 10 SDs below that of the toxin-free control, was 0.35 mg kg^{-1}. The quantitative assay format was applied to spiked and naturally contaminated wheat. Wheat was spiked in quadruplicate at six levels over the range from 0.2 to 10 mg kg^{-1}. Recoveries ranged from 83% to 128%, with an overall average of 103% ± 12% (Table 1). A group of 40 wheat samples containing various levels of DON was also tested by the BLI technique and a reference HPLC-UV method. There was a good correlation between the BLI and HPLC methods ($r^2 = 9698$). However, the BLI method tended to overestimate the DON content, as evidenced by a slope of 1.188 for the linear regression of the 40 samples (Figure 4). Overestimation has been reported for commercial DON immunoassays as well (Zachariasova et al. 2008). While the cause may vary between immunoassays, a common speculation has been the presence of cross-reacting analogues in the naturally contaminated samples. The cross-reactivity can exist to related toxin congeners such as various acetylated forms of DON (Abouzied et al. 1991; Nicol et al. 1993; Sinha et al. 1995; Maragos and McCormick 2000; Baumgartner et al. 2010). Cross-reactivity can also exist to conjugated toxins such as the glucosides of DON (Ruprich and Ostrỳ 2008; Zachariasova et al. 2008). With the HPLC-UV reference method the potential presence of 3-acetyl-DON or 15-acetyl-DON was assessed. However, neither congener was detected in the naturally contaminated samples, eliminating this as the source of the bias. Potential bias due to the presence of conjugated toxins could not be excluded, as the glucosides were not detectable with the HPLC-UV method used. It is also possible that there is some other, as yet unknown, mechanism operating. While the bias was clearly present, the extent (e.g. approximate 19% overestimation) was not considered a major detriment to use of the assay.

Our previously developed assay for DON in spiked wheat flour had certain performance parameters similar to the quantitative assay developed here, namely a similar LOD (0.10 mg kg^{-1} versus 0.09 mg kg^{-1}), quantitative range, average recoveries (101.4% versus 103%), and RSDs of the recoveries (13.2% versus 12%). The advantages of the method reported here reside primarily in increased speed and ease of use. In the previous work the sensors were conditioned by treating them with two mock 'tests' before using them on real samples. While the conditioning was done outside the instrument, it took significant time, about 90 min. In the present work the sensors were prepared in advance and stored dry. Dried sensors could then be stored for extended periods at ambient temperature, and were used without further conditioning. This significantly improved the ease of use of the method. The second advantage was the increased speed of the assays. Previously the assay used cycles of 7 min duration and therefore quantitation took either 7 min (if the response from an unknown sample was compared to a control sample tested concurrently), or 14 min (if the response from an unknown sample was compared with a control sample tested using the same sensor). In the current report the cycles were shortened to 2 min. This increase in speed was due to the use of the colloidal gold, which amplified the signal. Since the assays were composed of three cycles (control, test sample, control), they took 6 min to complete. This time included the time needed for a comparison with two control samples using the same sensor. This helped to accommodate the slow decrease in signal due to sensor drift while still improving the speed of the assays.

Qualitative assay for DON in naturally contaminated wheat

While the quantitative assay was rapid, it was apparent that further increases in the speed of the assays might be achieved (1) by using a single negative control sample for each test sample (e.g. rather than bracketing the test sample between negative controls), and (2) by using a positive control sample (threshold sample) rather than constructing a calibration curve. In order to reduce variability the response of the test sample was expressed relative to the control (toxin-free) sample, similar to the quantitative format. With the qualitative format samples were deemed 'positive' if they gave a relative response below that of the threshold sample (e.g. contained greater than 0.5 mg kg^{-1} DON) or negative if they gave a signal above that of the threshold sample. The threshold level of 0.5 mg kg^{-1} was selected because it is often used as a practical limit during screening to ensure that samples do not exceed the (US) regulatory limit of 1 mg kg^{-1}. Qualitative assays were completed in approximately 4 min, excluding the sample extraction, filtration and dilution steps. The set of 40 naturally contaminated wheat samples was retested using the qualitative assay and compared with results from the HPLC reference

Table 2. Qualitative assay of 40 naturally contaminated wheats using biolayer interferometry (BLI) and high-performance liquid chromatography (HPLC).

Sample	HPLC result (mg kg^{-1})[a]	BLI result[b]	Sample	HPLC result (mg kg^{-1})	BLI result
WH-1	5.9	+	WH-21	1.8	+
WH-2	15.1	+	WH-22	4.3	+
WH-3	2.1	+	WH-23	3.4	+
WH-4	4.7	+	WH-24	n.d.	−
WH-5	7.0	+	WH-25	2.9	+
WH-6	9.9	+	WH-26	1.2	+
WH-7	12.2	+	WH-27	0.9	+
WH-8	7.9	+	WH-28	0.4	−
WH-9	13.6	+	WH-29	0.6	+
WH-10	4.8	+	WH-30	3.5	+
WH-11	7.5	+	WH-31	0.4	−
WH-12	4.5	+	WH-32	4.1	+
WH-13	0.1	−	WH-33	0.1	−
WH-14	n.d.	−	WH-34	2.0	+
WH-15	10.8	+	WH-35	n.d.	−
WH-16	9.1	+	WH-36	1.5	+
WH-17	7.9	+	WH-37	0.2	−
WH-18	5.9	+	WH-38	n.d.	−
WH-19	3.5	+	WH-39	0.2	−
WH-20	3.1	+	WH-40	0.5	−

Notes: [a]Obtained by the HPLC-UV method. n.d. = Result below 0.1 mg kg^{-1}.
[b]Result expressed as above (+) or below (−) the threshold sample containing 0.5 mg kg^{-1} DON.

method. The qualitative assay correctly assigned 29 of 29 samples above 0.5 mg kg^{-1} as positive and ten of ten samples below 0.5 mg kg^{-1} as negative (Table 2). One sample that was measured as exactly 0.5 mg kg^{-1} by HPLC was negative by BLI (WH-40; Table 2). The qualitative assay was therefore considered adequate for predicting DON levels above the 0.5 mg kg^{-1} threshold.

Conclusions

A primary antibody–colloidal gold conjugate was found to amplify substantially the signal in a BLI assay for DON in wheat. The effect was much greater in wheat matrix than in buffer, suggesting the matrix components may have been contributing to the enhancement. The increase in signal provided by the amplification afforded the development of more rapid assays for DON in wheat. A quantitative assay requiring 6 min per sample, compared favourably with a reference HPLC method when applied to 40 naturally contaminated samples of wheat, although a bias towards overestimation was noted. A qualitative assay requiring 4 min per sample was able to classify accurately 39 of 40 samples as either above or below a threshold of 0.5 mg kg^{-1}. These results suggest that BLI, in particular with amplification provided by the use of colloidal gold conjugates, can be used to measure DON in wheat rapidly.

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