Fluorescence Polarization (FP) Assays for the Determination of Grain Mycotoxins (Fumonisins, DON Vomitoxin and Aflatoxins)

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Abstract: Successful use of fluorescence polarization assays (FPAs) in human clinical, infectious disease, and drug discovery fields has prompted us to extend its use to the grain mycotoxin field. An antibody specific to a mycotoxin and a mycotoxin-fluorophore conjugate are developed. Free toxin (extracted from the grains with a suitable solvent) competes with the toxin-fluorophore conjugate for the antibody and a change in FP relative to the quantity of free toxin occurs. This change is compared to a standard curve obtained by using known quantities of toxin. The use of FP and toxin-fluorophore conjugates for the quantification of fumonisins, deoxynivalenol and aflatoxins is described. These assays are field portable, simple to perform, rapid and require no washing steps.

INTRODUCTION

Fluorescence polarization (FP) immunoassays have been extensively used in human clinical field to monitor therapeutic drugs and drugs of abuse for more than 20 years [1-6]. However, until recently, this technique has rarely been used in agricultural diagnostics. With the advent of a variety of specialized FP instruments, this technique has found widespread use in other fields such as pesticides [7], animal disease [8] and environmental metal analysis [9]. Our group published the first paper on an FP assay for the determination of antibodies to *Brucella abortus* in 1996 [10]. Since then the field of agricultural diagnostics has been wide open for FP; and our group has published many papers using FP in infectious disease and food pathogen diagnostics including *Brucellosis* [11-12], equine infectious anemia virus [13], *leptospirosis*, *Mycobacterium bovis* [8], *Salmonella* species [14-15] and *E.coli* [16].

We have also extended the use of FP to the quantitative analysis of grain mycotoxins. In this article, we give a brief overview of the use of FP assays for the determination of fumonisins, DON-vomitoxin and aflatoxins in different grains. These FP assays are easier, simpler, and more economical to perform than traditional HPLC or enzyme immunoassays. These are specifically useful for grain elevators where a portable instrument is of a great help. The technique involves homogeneous assays that are single step, requiring no separation of bound and unbound materials, and therefore no multiple washing steps. FP can yield results (qualitative and quantitative) in seconds to minutes without any washing and extensive labor.

Mycotoxins are produced by the growth of commonly occurring filamentous fungi in grains such as corn, wheat, peanuts, sorghum and barley. These mycotoxins can cause common allergies, immunosuppression and cancer in humans and animals. Their presence in human food and animal feed is considered as a public health risk and they can have a huge economic impact on livestock production [17].

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Commonly used methods for the determination of mycotoxins include high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) [18-24]. Chromatographic methods are laborious, slow and are not suitable for field testing [2]. ELISAs have their own limitations due to multiple washing and incubation steps.

Involvement of these toxins in human, animal and grain diseases warrants the need for rapid and easy to perform tests for these mycotoxins. Fluorescence polarization (FP) can easily serve that purpose. Reaction of pure mycotoxin-fluorophore with a specific antibody gives a high polarization value (mP). A specific mycotoxin is extracted from the grains with a suitable solvent and mixed with the antibody solution. After adding the fluorophore-toxin conjugate, its FP value is determined. The FP value is inversely proportional to the quantity of free mycotoxin in the solution. This review will focus on the detailed FP study of these toxins.

PRINCIPLE

The method for the determination of these mycotoxins in grains relies on the common principle of the competition of a fluorophore labeled mycotoxin or its analog, with an unlabeled mycotoxin from a sample for a mycotoxin-specific antibody in a buffer solution (Figure **1**). The sensitivity of the assay depends upon the affinity of the antibody-antigen interaction, the instrument and the fluorophore properties. Small molecules rotate fast and give a low FP value. Larger molecules on the other hand rotate slower and give a higher mP [2]. In general a mycotoxin molecule or its analog is labeled with a suitable fluorophore to form a conjugate. Interaction of this conjugate with an antibody specific for that mycotoxin forms an immunocomplex that is larger than the conjugate and therefore gives a higher mP. Addition of the free antigen to the antibody solution and subsequent addition of the conjugate results in a competition for antibody binding, and a lower polarization value is observed, which is proportional to the amount of free toxin present. A standard curve with known concentrations of the

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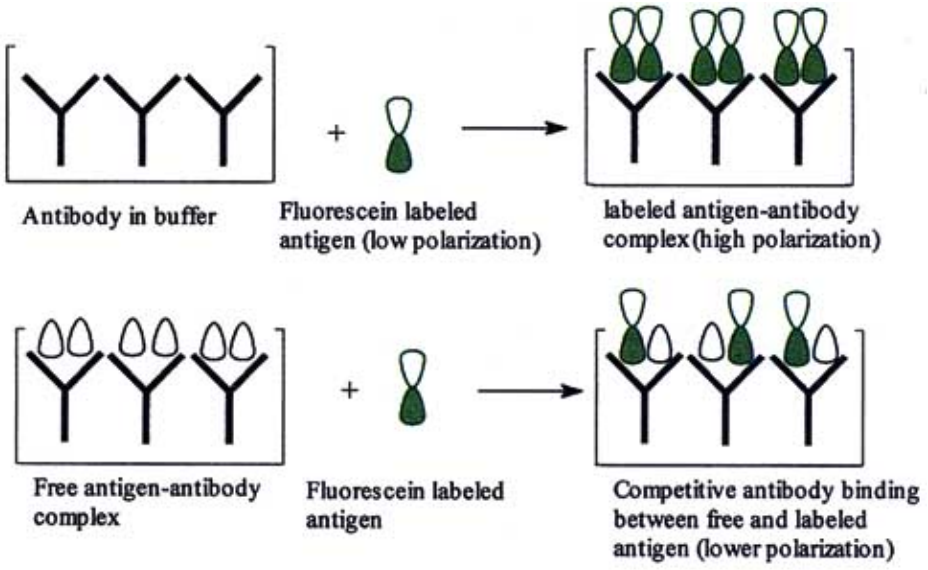

Fig. (1). Principle of small molecule detection based upon fluorophore labeled antigen-antibody interaction.

antigen (mycotoxin) is obtained and the unknown antigen concentration is calculated from the standard curve.

FPA FOR FUMONISINS

Fumonisins are produced by *Fusaria* species in foods and are implicated in various animal diseases including *leukoencephalomalacia* in horses, cancer in rats and pulmonary edema in swine [25-27]. This has prompted the development of various methods for the determination of fumonisins in grains. Since fumonisins are mostly found in corn and its products, we utilized FP to develop a rapid and portable assay for the determination of fumonisins in corn [25]. Fumonisin is historically extracted with a mixture of organic solvent/water (MeOH/water or CH_3CN/water). FP however, works better in pure aqueous media. Most of the antibodies lose their affinity in organic solvents. We therefore extracted fumonisins in aqueous solutions for FP analysis. The extracted fumonisin was filtered, mixed with antibody solution and a specific fumonisin B1-fluorescein fluorophore (FL-FB1) added. The FP value of the conjugate was determined within 1-2 minutes using a portable FP machine (Sentry^{TM}, Diachemix). A limited number of naturally contaminated maize samples (48 samples) gave a good correlation between FP and HPLC with a $R^2 = 0.85$ [25]. The same samples were run in two independent laboratories using slightly different FP protocols and the results from both laboratories correlated nicely with each other, indicating the robustness of the FP assay. The fumonisin detection limit was 0.5 ppm in spiked samples. We have expanded our preliminary work to make this assay more robust, reliable and sensitive. After thorough study, purification of the tracers and refining each and every step, we have increased the sensitivity (Limit of detection [LOD] is improved 5 fold to 0.1 ppm, 0.02 ppm in buffer) in real samples. We further analyzed naturally contaminated corn gluten meal samples (10 samples, analyzed in duplicate)

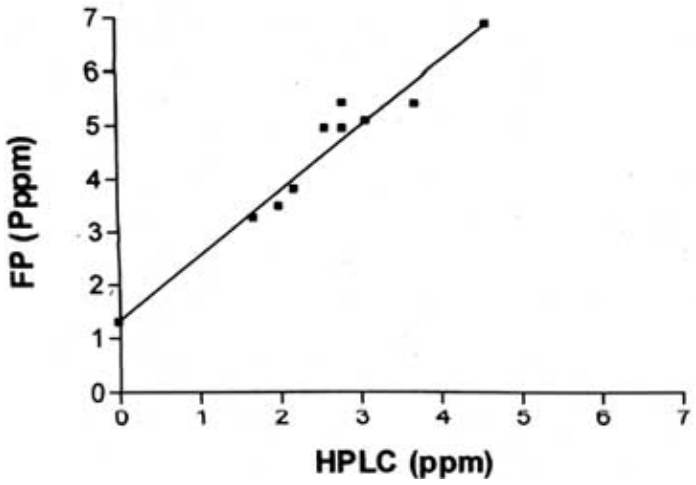

Fig. (2). Fumonisin analysis comparison of FP with HPLC in naturally contaminated corn gluten meal samples. FP samples were run in duplicate of duplicates. HPLC samples were analyzed by Trilogy Analytical Laboratory, Washington, MO.

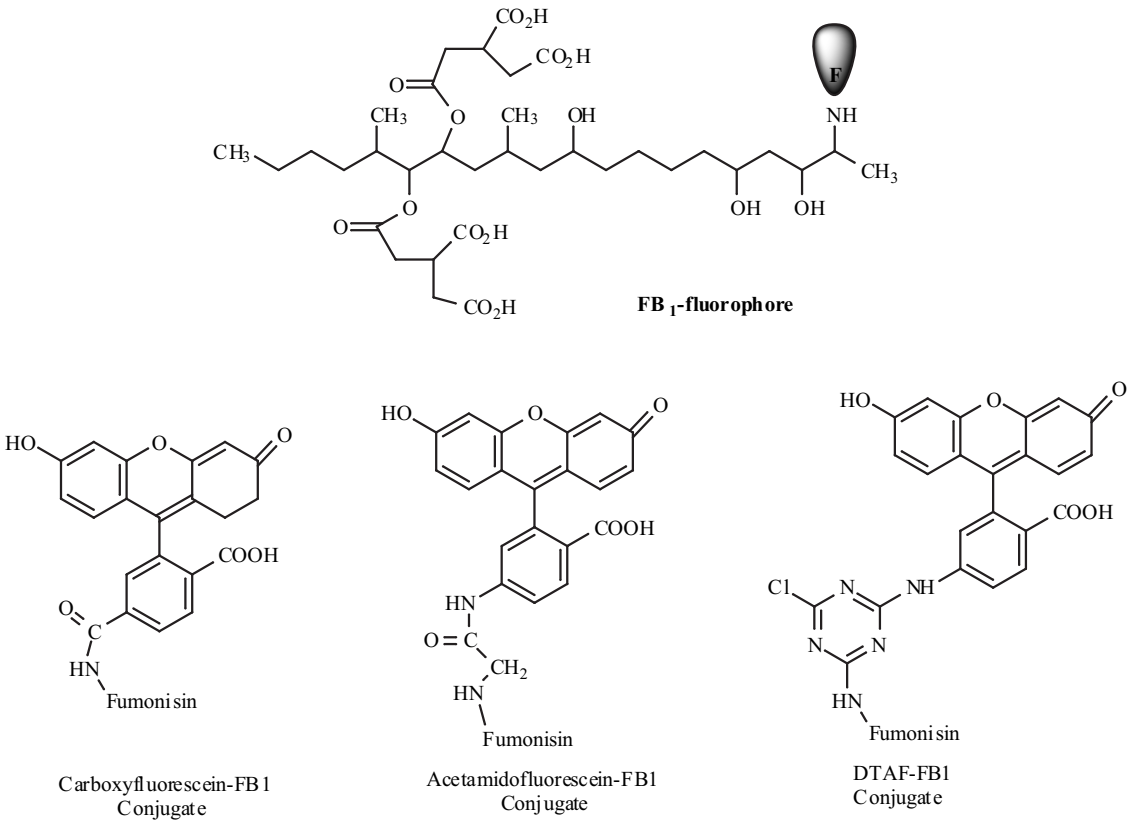

Fig. (3). Structures of various fumonisin-fluorophore conjugates showing the propeller effect. DTAF-FB1 is the most rigid structure having the least propeller effect. Acetamidofluorescein-FB1 is the most flexible structure with most propeller effect.

using FP and compared the results to HPLC. The graph (Figure **2**) again shows a good correlation between two methods (R^2 = 0.95, slope = 1.22, Y intercept = 1.33) similar to the original work. FP however, consistently gave higher values than HPLC. Various fumonisin fluorophore conjugates (FL-FB1) were prepared using commercially available fluorescein derivatives including both isomers of iodoacetamido-fluorescein (IAF), carboxy-fluorescein (FAM) and [4,6-dichlorotriazin-2-yl]amino-fluorescein (DTAF). The chemical structure of these conjugates (Figure **3**), suggests that the most active fluorophore should be the one with the least "propeller effect" (a necessary requirement in the development of effective FP assays [2]). Since DTAF gives the most rigid structure of FL-FB1 with the least flexibility (and thus least propeller effect), this tracer is the best candidate. In deed this was found to be the best tracer with fumonisin antibodies. Iodoacetamido-fluorescein on the other hand, gives the most flexible structure (with the most propeller effect) and least interaction with the antibody. This fumonisin conjugate therefore is essentially non-reactive towards antibody interaction in the FP assay.

FP analysis of fumonisin extraction with a mixture of methanol/water gave inconsistent results. We therefore, investigated the effect of methanol on the fumonisin assay by serial addition of methanol to the antibody solution and found a consistent decay in the antibody activity with the increase in methanol concentration [Nasir, M. S. and Jolley, M. E. in preparation]. We then investigated the efficacy of fumonisin extraction using water. Having previously compared two methods (HPLC vs FP) that use different solvents for extraction (HPLC uses water/methanol as an extraction solvent whereas in FP we use buffer) and found a nice correlation in the results, we investigated water extraction in detail.

When a limited number of naturally contaminated corn samples (already studied using FP and HPLC) were extracted with water at various time intervals, most of the fumonisin was extracted within the first 5 minutes of shaking. We therefore further explored the time-based water extraction and FP determination of fumonisin in spiked as well as naturally contaminated corn samples. The results suggest that a full FP assay for fumonisin determination including the extraction and analysis could take only 10-15 minutes. We further found that the FP assay is so robust that essentially simple water can be used for extraction (buffer, tap water or distilled water) without any interference with the assay. Since there is a drastic effect of pH change on the FP assay [Nasir, M. S. and Jolley, M. E. in preparation], the pH of the buffer prepared for the fumonisin assay (antibody diluted solution, PBS) should be kept close to 7. The small amount of sample (extract, 50 µl in 1 ml of antibody-buffer solution) does not change the overall pH of the solution to any noticeable extent; and therefore does not affect the assay.

The best FL-FB1 tracer was purified using HPLC and found to be very stable for at least 2 years when stored at – 4º C (we have been using the same tracer for all the studies by diluting the stock solution from time to time). Similarly, the diluted solution of the fumonisin monoclonal antibody used in this study was very stable at room

temperature (diluted 1/10,000 in PBSA to make a one liter solution, this solution was used for more than a year). A small variation of starting polarization values (mP) of these diluted solutions is due to the day to day variation of temperature and the instrument settings. Calibrating with a standard set of samples before or during any batch of testing eliminates any variation of that nature. Real field samples however tend to give consistently higher values with FP as compared to HPLC results. This is very similar to most of the immunoassays like ELISAs. However, some of the current ELISAs have a much lower intercept than the previous ones [42]. This suggests that FP assays can be in some cases expected to give a higher fumonisin concentration in the samples that other tests (HPLC) suggest as toxin free (less than 0.1 ppm). The reason for this phenomenon is not yet known. Perhaps FP measures some of the unknown fumonisin analogs and metabolites (that may be equally toxic as fumonisin B1, B2 and B3) different from fumonisins B1, B2 and B3 that HPLC does not pick. Nevertheless, our studies show that with the increased sensitivity, robustness and speed, the FP assay is very useful for screening especially in field and grain elevators.

FPA FOR DEOXYNIVALENOL (DON)

Deoxynivalenol (DON, Vomitoxin) is produced by *Fusarium graminearum* [29] and is found in wheat, barley, oats, rye and corn. The toxin is known to cause many diseases in domestic animals and therefore the US FDA has set advisory levels for DON in various foods. For further discussion of the risk assessments of deoxynivalenol, readers are referred to a recent review [41]. Due to its link with various animal diseases, rapid and simple methods that can be performed in the field for DON analysis are very much needed. We have developed a fluorescence polarization method for the determination of DON in grains [30-31]. Initially we prepared a DON-fluorophore by reacting DON with BODIPY-8-propionic acid (Molecular probes) via the 15-OH position of the DON molecule using regiospecific Steglich esterification method [28]. After purification, this tracer (Figure 4, DON-BODIPY tracer) was used with a DON antibody [32]. Naturally contaminated wheat and barley samples were extracted using water and analyzed using FP. Limited data showed a good correlation with HPLC (Figure 5, $R^2 = 0.97$, Y intercept = 0.70). However, further testing showed that the tracer was unstable and decomposed into smaller products after few days at low temperature, and in a day at room temperature. Therefore this tracer was not further explored.

We recently developed a FP assay for the determination of DON in wheat [30]. DON-fluorophore (DON-FL tracer) was prepared by reacting DON with fluoresceinamine (isomer II) in the presence of 1,1-carbodiimidazole. This fluorophore was tested with three monoclonal antibodies previously developed for ELISA (reference clones #1, #4 and #22 [22]). Surprisingly, the antibody that was most sensitive in an ELISA format (#22) did not affect the polarization value. The other two antibodies (#1and #4)

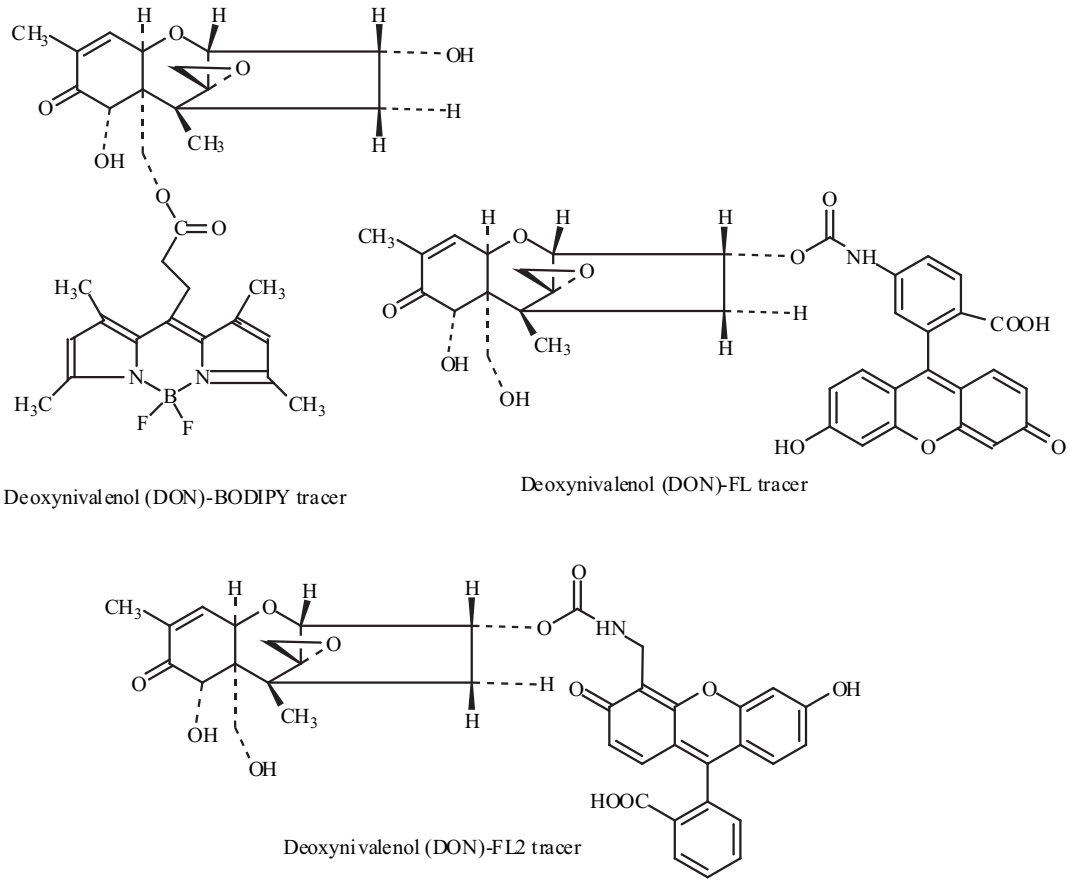

Fig. (4). Chemical structure of DON-Fluorophore conjugates

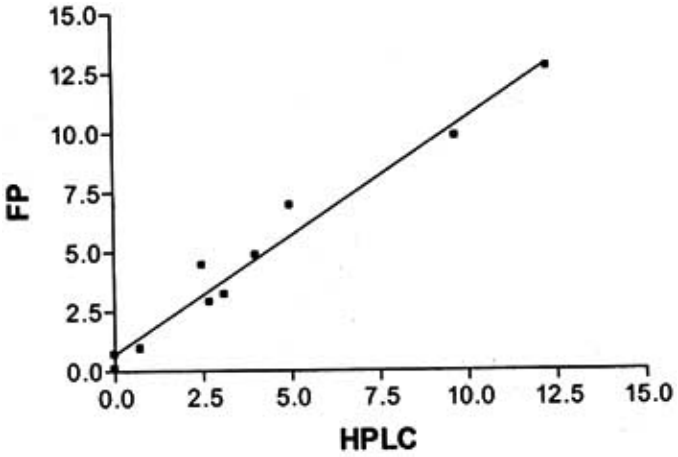

Fig. (5). FP vs. HPLC analysis of Deoxynivalenol in naturally contaminated wheat and barley samples using DON-BODIPY Tracer.

however had high affinities for this tracer. FP assay was very simple to perform only requiring the mixing of a water extract of DON with the tracer (DON-FL) and the antibody. Although the test was very sensitive, it took approximately 10 minutes to equilibrate for the attainment of a stable polarization value (mP). Naturally contaminated wheat samples (34 samples) were analyzed by FP and compared with HPLC. The two methods correlated very well with slight overestimation of the values by FP [30]. This overestimation was postulated due to the higher cross reactivity of the antibody with other DON analogs. Using FP this antibody was tested for its cross reactivity towards all the DON analogs. In addition to DON, only two other analogs cross-reacted to an appreciable extent (HT-2 Toxin reacted 9% with the antibody whereas 15-acetyl-DON reacted 358%). Therefore FP will overestimate any sample containing 15-acetyl-DON. Nevertheless, the test was very robust and good for general DON screening in wheat. This tracer (DON-FL) also gave a good correlation with HPLC in a limited number of barley samples (Figure 6, $R^2 = 0.92$, Y-intercept = 0.42) and further studies are warranted.

In order to increase the assay speed further, a new tracer was prepared. Reaction of DON with another fluorescein analog (4`aminomethyl-fluorescein) produced a new DON-fluorophore (DON-FL2, Figure 4) which rapidly reacted with antibody #22 (the one that did not react with DON-FL tracer but was the most sensitive in ELISA format). Using the antibody #22 and this new tracer (DON-FL2), the speed of the assay was substantially improved. In combination with fast extraction with water (3 minutes only), the assay time was substantially reduced to less than 5 minutes overall [31]. The polarization value did not change in the presence of antibodies #1 and #4. The reason for this behavior of tracers with various antibodies is not yet known. Perhaps the antibodies were prepared through different linkers of the molecule and so were the tracers. As before, the assay was compared with HPLC using naturally contaminated and spiked wheat samples. This assay cross-reacted 339% with 3-acetyl-DON and 8% with 15-acetyl-DON (contrary to our previous report where the assay cross-reacted 9% with 3-acetyl-DON and 358% with 15-acetyl-DON). 34 naturally contaminated wheat and 15 naturally contaminated maize samples were analyzed and compared with HPLC. Although the FP correlated nicely with HPLC for wheat samples, in maize samples the FP overestimated the values substantially [31]. Therefore the overestimation of DON contents with FP may not solely be due to the higher cross reactivity of antibodies with acetyl-DON derivatives. The reason for this

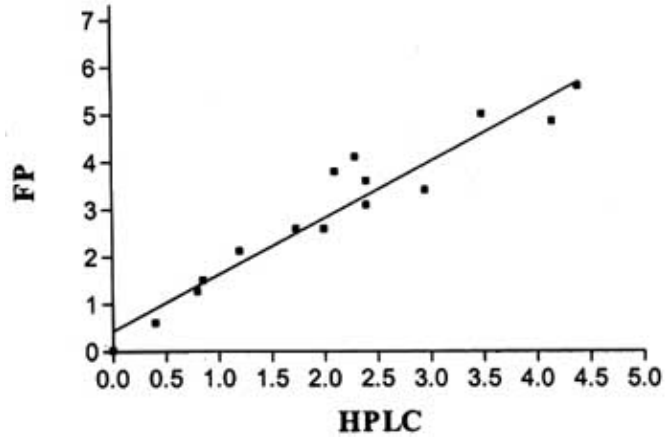

Fig. (6). FP vs. HPLC analysis of Deoxynivalenol in naturally contaminated barley samples using DON-FL Tracer.

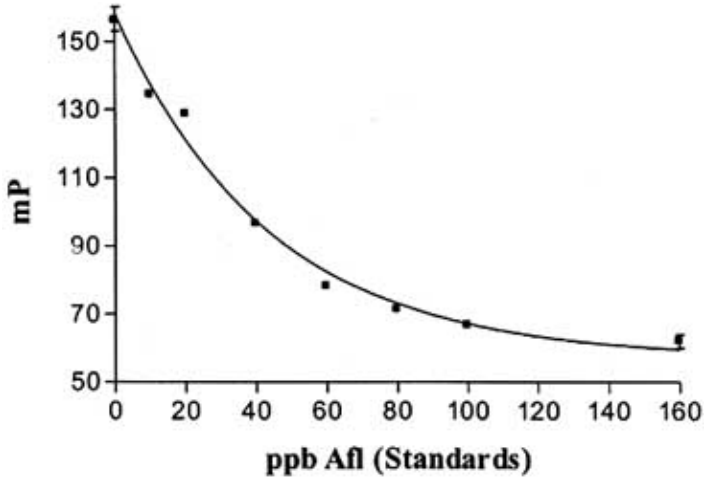

Fig. (7). A typical FP standard curve for aflatoxin analysis using aflatoxin standards in MeOH/water (70/30). A solvent resistant aflatoxin specific antibody was used.

effect is unknown and is very typical of most immunoassays. Although work is still in progress to overcome this effect, the test in its present form is very useful for screening purposes.

FPA FOR AFLATOXINS

Aflatoxins produced by *Aspergillus flavus* are known carcinogens. These are found in foods including grains, cereals, sorghum, soy seeds and peanut products. These toxins were first found in 1960s [33]. Since then, various analytical methods have been developed for their analysis in grains.

Widely used methods for aflatoxin determination include chromatographic methods (HPLC, TLC) and enzyme linked immunosorbent methods (ELISAs) [19-20, 23-24, 34-36]. Chromatographic [38] and ELISA [39] methods have been recently reviewed. These methods require extended cleanup procedures and are rather slow to perform. ELISA methods are faster, but require enzymatic reactions and multiple washing steps to separate bound and free labels. Some novel detection methods (especially biosensors) for aflatoxin detection for research purposes have been reviewed recently [23]. Different formats for biosensors, including surface plasmon resonance, fiber optic probes and microbead-based assays were discussed.

Recently we reported a FP based assay for the aflatoxin determination in various grains [37]). We analyzed spiked Corn, naturally contaminated Corn, peanut butter, peanut paste and sorghum samples using FP. Similar to other mycotoxins (fumonisins and DON-vomitoxin) described above, in FP the signal for the aflatoxin determination is measured without any additional steps after extraction. Only in very colored samples, which interfere with the absorption of fluorescein (dark paprika), one has to use a simple cleaning column (unpublished data).

Since the aflatoxin molecule does not have an active functional group to react directly with a fluorophore, we first prepared an aflatoxin B1-oxime [40] that reacted with an amine derivative of fluorescein to give the aflatoxin tracer. Due to the high affinity of the antibody, it takes approximately 15 minutes to completely equilibrate the final FP values. To control this phenomenon, a batch mode was used where all the samples and standards were first mixed with 1 ml of a pre-diluted antibody solution in buffer in separate test tubes. After adding the tracer to each tube, all the samples were incubated at room temperature for 15 minutes before reading. Figure 7 shows a typical aflatoxin standard curve from which the aflatoxin concentration in unknown samples can be calculated. Results correlated very nicely with spiked as well as naturally contaminated sample results analyzed with HPLC. Since other analogs of aflatoxin B1 cross-reacted with the antibody with only approximately 30% efficiency, FP slightly underestimates the samples that contain some of aflatoxins B2, G1 and G2.

We originally used a commercially available aflatoxin antibody that reacted with the specific aflatoxin tracer with a very high affinity. This antibody however, was not stable in organic solvents. Since aflatoxins can only be extracted using a mixture of organic/aqueous solvent, this antibody gave unreliable results and therefore could not be used for analysis. We used another antibody (provided by Dr. Chris Maragos of USDA-ARS, NCAUR, Peoria, IL) that was organic solvent resistant. The interaction of aflatoxin-fluorophore with this solvent-resistant antibody gave nice results where one did not even have to filter the extracts before analysis [37]. It takes approximately 15-20 minutes to extract and analyze the aflatoxin samples. The simplicity, portability and minimum handling steps make this assay a novel method for screening purposes.

CONCLUSION

After successfully utilizing FP in animal disease diagnostics and food pathogen detection, we have extended the use of FP to the determination of various mycotoxins in grains. The technique has many advantages over ELISA,

RIA and chromatographic methods. The absence of washing and cleanup steps in FP makes this technique very attractive for mycotoxin analysis. It is our belief that in coming years, the grain community will start using FP for routine analysis especially for rapid screening purposes. Since FP can work in oily, milky and colored solutions, no special cleaning reagents or columns are needed. In addition, the tests could be performed in the field, in the central laboratory or in the elevators. Although the currently available FP instruments could be used routinely, smaller and cheaper instruments will definitely accelerate the use of FP by grain producers and other testing facilities. Nevertheless, the use of FP has been extended to virtually all fields of diagnostics.

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