Chapter 11

The Aflatoxin Quicktest™—A Practical Tool for Ensuring Safety in Agricultural Produce

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Additional information is available at the end of the chapter

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Abstract

Contamination of corn, peanuts, milk and dairy products with aflatoxins is a worldwide problem, particularly in subtropical regions where the climatic conditions are ideal for the growth of *Aspergillus flavus*, the fungi that produces these toxins. Developing countries have major difficulties in marketing these products abroad due to the stringent international regulations concerning this carcinogenic toxin. Adding to the problem is the analytical cost involved in monitoring the produce, which require sophisticated instrumentation and qualified personnel, neither of which are available for field testing. The development of a rapid Aflatoxin Quicktest™ provides an effective, reliable and cheaper option for screening levels of aflatoxin above the regulatory thresholds in such produce. The test consists of a lateral flow device (LFD) coated with antibodies specific to aflatoxin B1, although it detects other aflatoxins (i.e. G and M) with high cross-reactivity. Its high sensitivity allows analysis of these toxins in the range 2–40 μg/kg of sample in 15 minutes, plus the time for extraction, which varies among different products. Quantification of the test results is done using a Quick Reader, by comparing the readings of individual tests against a standard curve of the analytes in the same manner as it is done with any other analytical equipment. A validation study was carried out using peanuts from Australia and peanuts and corn from Timor-Leste to assess the performance of the Aflatoxin Quicktest™. Results obtained with the LFD showed a good correlation with the standard analytical measurements by HPLC-fluorescence (r^2 above 0.90 for all cases), indicating the Aflatoxin Quicktest™ is capable of measuring levels of aflatoxins accurately and reliably. Given their ease of use, low cost and fast processing time, the Aflatoxin Quicktest™ can be used for screening agricultural produce in countries that cannot afford the costly alternative of using specialised personnel and equipment.

Keywords: mycotoxins, food, analysis, lateral flow devices, peanuts, maize

1. Introduction

Aflatoxins constitute a major group of mycotoxins produced by certain fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus*, which grow in soil, decaying vegetation, hay and grains. These fungi can infect various crops — frequently peanuts, maize, other cereals as well as tree nuts — either prior to harvest or under moist conditions in stored agricultural produce, leading to their contamination with aflatoxins [1–3]. Contamination levels can sometimes exceed thousands of parts per billion (ppb, either μg/kg or μg/L) in individual kernels of peanuts or other grains, but given the uneven distribution within a batch of produce an intensive, strategic sampling is required to assess the extent of the contamination [4].

Four main types of aflatoxins are recognised, namely B1, B2, G1 and G2, with aflatoxin B1 and G1 as well as their metabolic product M1 being the most commonly found in agricultural produce (**Figure 1**). In terms of acute toxicity, the oral lethal dose (LD50) of aflatoxin B1 for monkeys is 1.75 mg/kg, and for ducks 1.70–2.45 mg/kg. However, these toxins are also carcinogenic because once ingested they are metabolised by the liver to a reactive epoxide intermediate; as a result, chronic ingestion of small amounts of aflatoxins (i.e. in the ppb range) typically produce liver cancer, so they are classified as strong carcinogens by the International Agency for Research on Cancer (IARC). For example, it has been estimated that 12% of cancer occurrence in Indonesia is in the liver, which is linked to consumption of aflatoxin contaminated food [5]. Livestock animals suffer the same effects, with pigs and chicken being particularly susceptible to these mycotoxins. However, animals can also transform aflatoxins B and G into the M metabolites by hydroxylation in the liver, and these transformation products can appear in eggs as well as in poultry meat [6]. Both aflatoxin M1 and M2 are even more toxic, with LD50 for ducks in the range 0.28–0.32 mg/kg. Ruminant animals fed contaminated fodder are more tolerant, but can also pass M aflatoxins into milk and other dairy products [7–9].

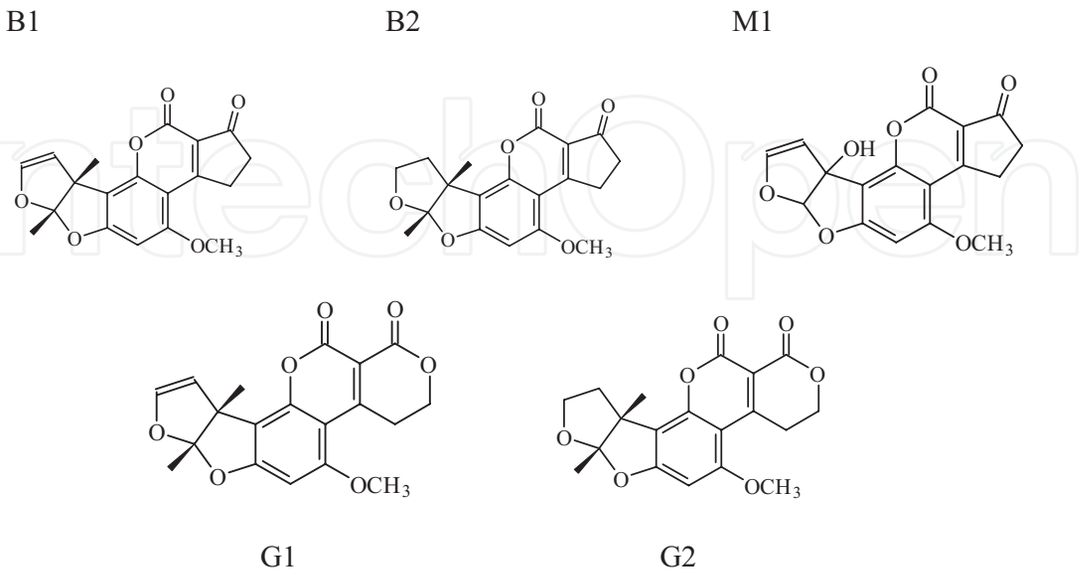

Figure 1. Chemical structures of common aflatoxins found in nuts and grains (B1, B2, G1, G2) or milk (M1) as a by-product.

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Contamination of the food supply with aflatoxins poses a serious problem not only for the health effects it causes in people and livestock, but also for trade. Strict regulations have been enacted by the World Health Organisation to prevent trading aflatoxin contaminated produce among countries. The maximum residue limits (MRLs) in grain for human consumption are in the range 2–20 ppb, and for animal feed in the range 20–300 ppb, depending on the country, whereas for milk they can be as low as 0.05 μg/L [10]. In order to meet the international regulations on aflatoxin, countries have to adopt expensive monitoring programs in order to screen their agricultural produce. Screening to detect aflatoxin contamination often relies on fluorescence and has been achieved by reference standards using thin layer chromatography, adsorption on minicolumns [11], high-performance liquid chromatography (HPLC) or liquid chromatography coupled to mass spectrometry (LC-MS) [12, 13]. Unfortunately, many developing countries lack the infrastructure necessary to comply with such regulations even though they often recognise the problem [14]. For example, aflatoxin has been identified as a threat to human health in Timor-Leste [15], and yet some of their agricultural produce could not be marketed in recent years for lack of compliance.

Specific antibodies to aflatoxins have provided an alternative means to conduct ELISA immunoassays [16], which can accurately measure the levels of contamination in grains and nuts. ELISA technologies are more affordable than instrumental analysis such as HPLC-fluorescence or LC-MS, but require a level of analytical skills that may not be found in many developing countries, where the aflatoxin problem is most prevalent [14, 17]. Based on the same immunoassays principles, lateral flow devices (LFD) employing gold nanoparticles have been recently developed [18–20] to be used as rapid methods for screening aflatoxins in food commodities.

One such device [19], the Aflatoxin QuickTest™, provides an effective, reliable and low cost option for screening levels of aflatoxins and meets the regulatory thresholds of agricultural produce. Quantification, as described here, is achieved using a suitable reader and standard curve of the analytes in the same manner as it is done with other analytical equipment. A validation study for quantification using peanuts and maize from Australia and Timor-Leste was carried out in order to assess the performance of the Aflatoxin QuickTest™ and it is presented here for the first time.

This chapter reviews the past research that led to the development of the current Aflatoxin QuickTest™, its use and applications. But before describing how it works, a description of this novel technology is required.

2. Development of antibodies for detection of aflatoxin

2.1. Antibodies for aflatoxin

A polyclonal antibody specific to aflatoxin B1 (AFB1) was developed by Lee et al. [16] using a conjugate of aflatoxin B1-bovine serum albumin (BSA) as antigen. The antibodies were specific to aflatoxin B1, detecting this compound in a mixture of four aflatoxins (B1, B2, G1 and G2),

but showed significant cross-reaction with aflatoxin G1 (57–61%) when an individual compound was tested (**Table 1**). This is fortunate, as both aflatoxin B1 and G1 are the two most common aflatoxins found in contaminated produce. Sensitivity of short competitive ELISA assays (15 minutes) showed median inhibition concentration (IC50) values of 21.6 ± 2.7 ppb after a 5-fold dilution of the sample extract – a necessary step to minimise the negative effect of solvent on the antibodies – and a detection range from 4.2 to 99.9 μg/kg sample. This ELISA was able to detect and quantify levels of aflatoxins in peanut, corn, soybean and pistachio samples without significant matrix effects [16].

Furthermore, a validation of the SUNQuik ELISA, which uses the AFB1 antibodies, was carried out using 12 peanut samples that were also analysed by standard HPLC-fluorescence. Levels of total aflatoxins measured by the two analytical methods showed an excellent correlation ($r^2 = 0.938$) over a concentration range 0–1200 μg/kg sample, with no false negatives [21].

2.2. Lateral flow devices for aflatoxins

Whilst the ability of the polyclonal AFB1 antibodies to quantify levels of aflatoxins in grains and nuts was demonstrated, the application of ELISA assays to monitoring surveys requires certain laboratory conditions and appropriate skills by qualified personnel. A simpler method was needed that could be used in field settings by less skilled operators.

In recent years, more convenient procedures based on immunochemistry have been sought for organic contaminants in food and the environment. The lateral flow device (LFD) has been most popular because of its simplicity in design and its automatic function, each LFD unit requiring only a few drops of sample solution for operation. Comprehensive reviews [22–24] have described the lateral flow assay system in depth, covering a broad range of immunoassay procedures and including nucleic acid applications. Many successful ELISAs can be readily converted to LFDs using the same or similar immuno-reagents; important properties established for ELISAs such as the plots of concentration giving 50% inhibition (IC50) perform in a similar manner in LFDs. In essence, a competitive LFD consists of an impervious nitrocellulose strip coated transversal with two lines that contain either a particular target analyte (test line, T) or a general antibody (control line, C). In addition, a sample pad contains colloidal gold

Compound	IC50* (ppb)	%CR
Aflatoxin B1	0.8–6.5	100.0
Aflatoxin G1	1.4–10.8	57.1–60.5
Aflatoxin B2	13.5–55.0	5.9–11.8
Aflatoxin G2	50.1–83.0	1.6–7.8
Aflatoxin P1	>250	<0.3–1.0
Aflatoxin M1	>250	<0.3 to <2.6

*IC50 = median inhibition concentration.

Table 1. Cross reactivity of aflatoxins and metabolites in polyclonal AFB1 assays (after Lee et al. [16]).

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nanoparticles conjugated to the specific antibodies of the target analyte (AuNP-IgG); at the other end of the strip there is an absorbent pad (**Figure 2**). The strips are contained within small plastic cassettes that have a well for placing drops of the sample solution and a window to visualise the T and C lines. Conjugates and antibodies on the LFD are usually stable for months when the strips are kept under dry and dark conditions at room temperature, enabling easy transport and storage for use at a later time.

Some authors have successfully described LFDs for aflatoxins that work well with corn [18], grains and feedstuffs [25, 26] and milk [27], but require strip treatment before use. A new LFD for aflatoxins that did not require special pre-treatment was developed by Masinde et al. [19] and has been commercialised as the Aflatoxin Quicktest™ by QuickTest Technologies. The T line in the Aflatoxin Quicktest™ contains aflatoxin-conjugate (AFB1-C) and the C line a non-specific goat anti-rabbit antibody (G-IgG); the sample pad contains gold nanoparticles (~10–15 micron) conjugated to the specific antibodies (AuNP-IgG) developed by Lee et al. for aflatoxin (AFB1).

For running a test, two drops of sample extract are placed over the sample pad, dissolving the AuNP-IgG nanoparticles, which run laterally over the strip towards the absorbent pad. Any aflatoxin present in the sample extract will compete with the AuNP-IgG particles at the T line, where the excess conjugated antigen will bind to them and produce a coloured line. The remaining AuNP-IgG particles will continue moving towards the other end and will bind to the G-IgG at the C line, also producing colour. The time for the competing targets, aflatoxin and AuNP-IgG, to reach an equilibrium is about 15 minutes, although 5 minutes may be sufficient for initial visual detection [19].

The interpretation of the assay is straightforward: an absence of colour at the T line indicates a high concentration of aflatoxins in the sample extract, as it has outcompeted the gold nanoparticles, whereas a full coloured T line indicates the absence of aflatoxins in the sample. A faded line indicates the presence of some aflatoxin in such a way that the less the colour development, the more aflatoxin is present. The coloured C line confirms that the test is valid, that is, when no colour appears at the C line, the test is invalid or the strip is faulty.

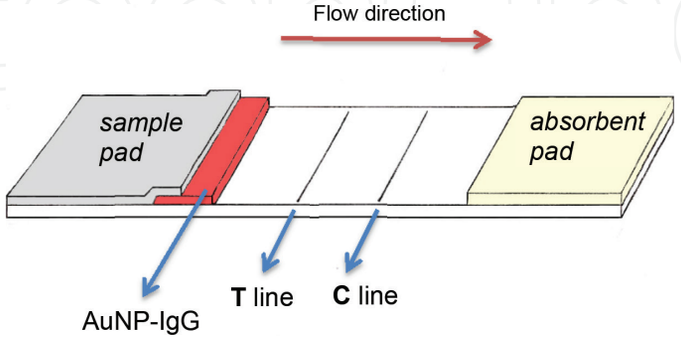

Figure 2. Schematic diagram of a lateral flow device (LFD). A nitrocellulose strip is coated with a solution of a particular target analyte (T line) and a general antibody (C line). The sample pad contains gold nanoparticles conjugated to the specific antibodies of the analyte (AuNP-IgG). The absorbent pad at the other end captures the excess solution flowing across the strip.

The Aflatoxin Quicktest™ can detect aflatoxins at 0.1 μg/L in water or liquid samples that do not require dilution, that is, milk. For solid samples that require extraction (e.g. grain, nuts) with methanol or ethanol, a 10-fold dilution must be applied to avoid serious inactivation of the antibodies; in this case, the limit of detection is at least 1 μg/L or more, depending on the extraction method.

3. Quantification of aflatoxin by the Aflatoxin Quicktest™

The conjugated AuNPs-IgG in the LFD compete with the sample analyte for the same target at the T line in a similar way as in a competitive ELISA assay [21]. Therefore, the amount of immuno-gold attached to the T line is inversely proportional to the level of analyte in the sample and this simple relationship can be used to estimate the analyte concentration when an optical reader that measures reflectance of immuno-gold is available. Reading is typically done for one LFD unit at a time, but instruments with multiple slots for reading test devices are also available, allowing greater sample throughput speed. In practice, readers operate by estimating the ratio of the area under the peaks corresponding to the test and the control line (T/C), because no two strips contain exactly the same amount of gold nanoparticles. A ratio around 1 indicates absence of the analyte, whereas lower values indicate its presence and zero values indicate levels of analyte above the range of detection.

Given the operation of the law of mass action in binding of analytes by specific antibodies, the volume or number of drops added to the sample pad of the LFD is not critical for analysis as the reaction with AuNP-IgG nanoparticles is concentration dependent; the majority of the analyte molecules remain in solution, given the small number of antibody molecules. However, it is preferable to standardise the number of drops to induce reliable lateral flow, with 2 drops being optimal for the Aflatoxin Quicktest™.

As with any other analytical technique, the ratio readings must be compared to a standard curve established beforehand using known concentrations of the target analyte. An example for the Aflatoxin Quicktest™ is presented in **Figure 3**. It should be noted that the shape of the curve is best described by an exponential function, which becomes sigmoidal (with a straight section between two bend ends) when plotted against the logarithm of the aflatoxin concentrations. As in ELISA assays, extreme ratio values either at near zero or at some maximum of the range in LFDs must also be rejected, since accurate estimates can only be made in the straight region of the curve, which is usually found between ratio values of 0.15 and 0.85. Consequently, the working range of Aflatoxin Quicktest™ is in the 0.1–2.0 ppb region for direct sample analysis, but 2.0–40 ppb for solid samples requiring solvent extraction and dilution 10-fold. Repeated measurements of the standard solutions using the Aflatoxin Quicktest™ show good reliability of the assays, with a coefficient of variation of 6.4% within this working range.

Samples that contain aflatoxin levels above the range of detection are diluted and reanalysed. More than one dilution may be needed when the contamination levels are very high. Care is needed to extract only well-mixed grain samples given the extremely uneven distribution of aflatoxin among kernels.

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B)

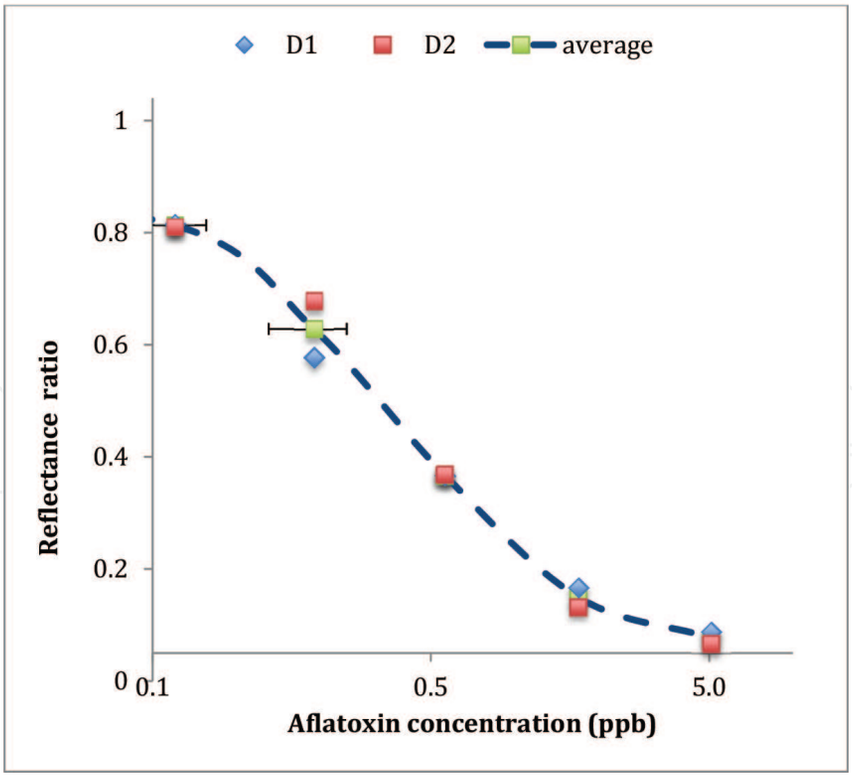

Figure 3. Establishment of standard curves using LFDs. (A) Aflatoxin QuickTest™ cassette strips used at different concentrations of an aflatoxin mixture; (B) standard curve from the reflectance ratio of test- and control-lines. Error bars indicate the 95% confidence intervals of duplicate measurements (D1 and D2) by the optical reader.

4. Validation of the Aflatoxin Quicktest™

Two separate studies were carried out to validate the performance of the Aflatoxin Quicktest™ in analysing peanuts and maize samples for aflatoxin contamination. The first study comprised peanuts samples from Australian growers in Queensland, which were collected during the 2015 and 2016 growing seasons and maize kernels infected with *A. flavus* in a laboratory trial. The second study involved a comprehensive survey of peanuts and maize from markets in Timor-Leste, carried out during 2014 and 2015. In both studies, extracts of the raw samples were analysed using the Aflatoxin Quicktest™ as well as the standard methods of analytical laboratories, that is, HPLC with fluorescence detector or LC-MS/MS, so their results could be compared.

4.1. Australian validation study

From late April 2015, from every load of peanuts delivered into the Kingaroy intake of the Peanut Company of Australia (PCA), duplicate 80% methanol extracts were collected as part of the normal aflatoxin mini-column test conducted at intake. For each load, a separate extraction was performed, such that a duplicate methanol extract was collected for both HPLC (15 ml) and Aflatoxin Quicktest™ (5 ml) aflatoxin analysis. For the 2015 season, about 170 extracts were collected and stored in the fridge (5°C) until the validation study commenced in June of that year.

All 15 ml extracts were analysed by HPLC-fluorescence at the PCA Technical Centre using the company's standard method accredited by National Association of Testing Authorities (NATA, Australia), which included addition of 5% Holaday salt solution to the methanol extract. A selected subset of 13 positive and 12 negative samples randomly chosen was then used in the validation study.

The corresponding subset of 5 ml extracts (n = 25) were analysed in June by the Aflatoxin Quicktest™. For this analysis, 200 μl were taken into an Eppendorf tube and 1.8 ml of phosphate buffer solution (PBS – 50 mM, pH 7.4) added so as to reduce the concentration of methanol to less than 10%. Two drops of the solution were placed on the strip well and left to develop colour for 15 minutes on the laboratory bench, after which time the strips were immediately read using an LFD Quick Reader (Tianjin Jiuding Diagnostics Ltd., China). Samples that produced results above the detection range (2–40 ppb) were diluted further in PBS and reanalysed until their readings fell within this range; all results were calculated taking into account the dilutions factors used for each sample.

Results from the 2015 peanut validation study are shown in **Figure 4A**, where it can be seen the excellent correlation between the HPLC and Aflatoxin Quicktest™ analyses for total aflatoxins ($r^2 = 0.934$ on the logarithmic transformed data).

The same procedure was repeated in 2016, but this time 45 peanut extracts were used in the validation study, which was conducted in October of that year. The results for 2016 (**Figure 4B**) showed also a good correlation between the two analyses ($r^2 = 0.956$), even though a number of samples were well above of the detection range. The results of the second year confirm the validity of the Aflatoxin Quicktest™ as a tool for detecting and measuring total aflatoxin levels in peanuts.

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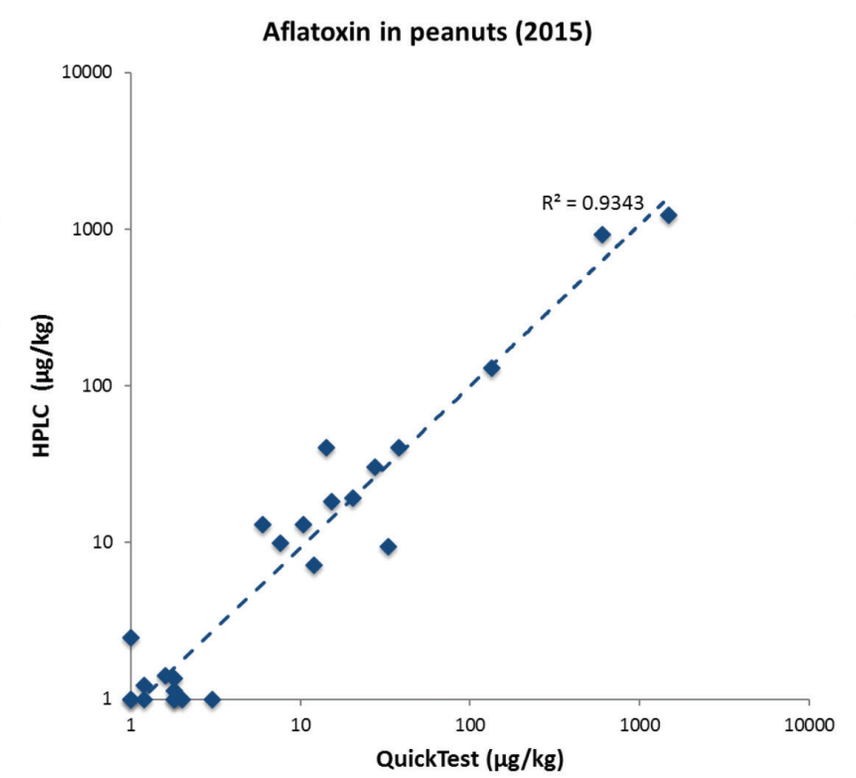

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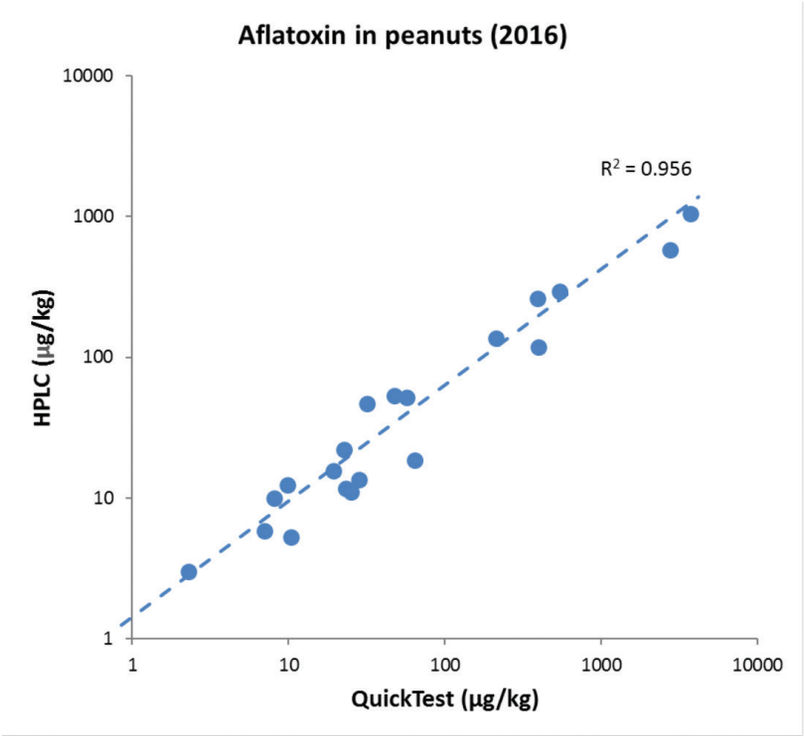

Figure 4. Validation of the Aflatoxin QuickTest™ for peanut samples collected by the Peanut Company of Australia in 2015 (A) and 2016 (B).

In addition to the peanut extracts, 16 samples of maize kernels that had been infected with *A. flavus* in the laboratory were also used for validating the Aflatoxin Quicktest™. The kernels were extracted with a mixture of 80% methanol and 4% Holaday salt solution and analysed first by HPLC-fluorescence at the PCA Technical Centre. The levels of aflatoxins in the kernels were sometimes very high, with the highest reaching 111 mg/kg (ppm). Most of the aflatoxin was found to be G1 (63 ± 27%) and B1 (26 ± 23%), whereas both G2 and B1 were usually below 3%.

Aliquots of the extracts (100 µl) were taken into 900 µl of PBS solution for direct analysis by Aflatoxin Quicktest™, and diluted further in PBS if the readings were above the detection range.

In spite of the high levels of contamination in the maize kernels, which required dilutions of the extracts up to 60,000-fold, a comparison of results by the two analytical methods showed an acceptable correlation for all the aflatoxins in the samples (**Figure 5**, $r^2 = 0.89$). This correlation improved when the results of the Aflatoxin Quicktest™ were compared to the levels of aflatoxin B1 and B2 ($r^2 = 0.97$ and 0.93, respectively) as determined by HPLC, whereas those of aflatoxins G1 and G2 showed lower correlations ($r^2 = 0.86$ and 0.76, respectively). This is in agreement with the differential sensitivity of the antibodies present in the commercial Aflatoxin Quicktest™, which are 100% specific to aflatoxin B1 but less specific to the other forms (see **Table 1**). Aflatoxins B1 and B2 are commonly found in tropical and subtropical regions of the world, whereas aflatoxins G1 and G2 are commonly produced by *A. parasiticus*, a soil species that is rare or absent in South East Asia [28].

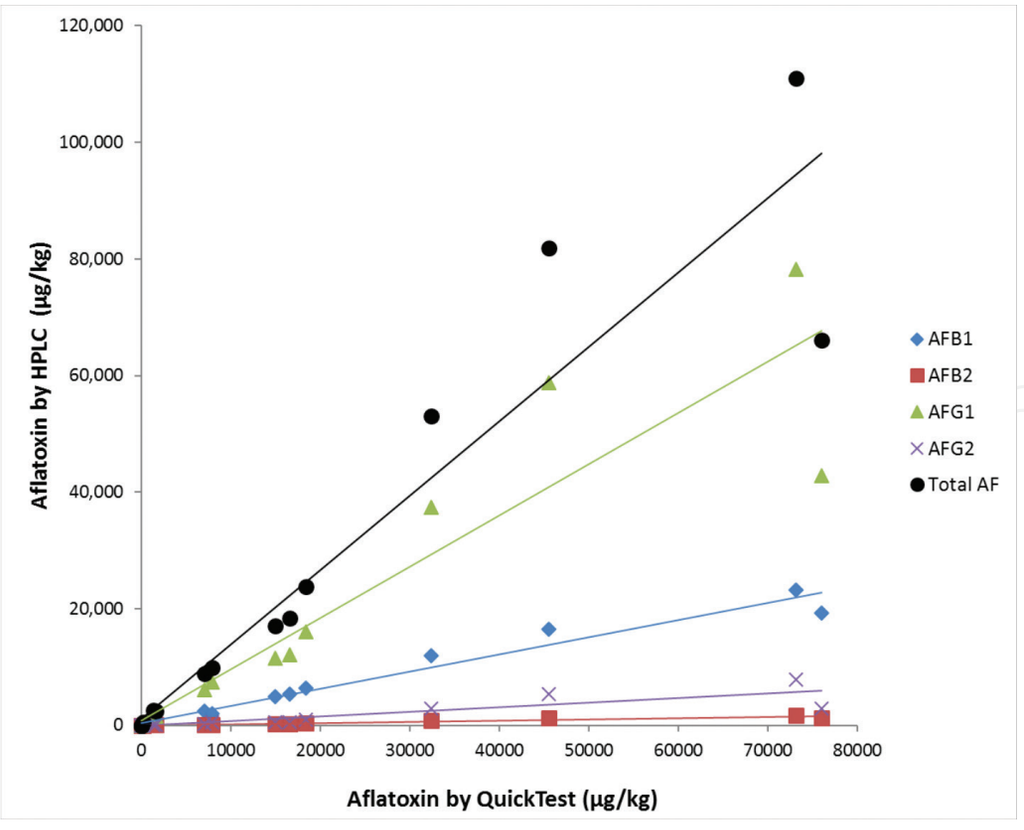

Figure 5. Validation of the Aflatoxin QuickTest™ for maize kernels infected with high levels of aflatoxins: AFB1, AFB2, AFG1, AFG2 and total aflatoxins (AF).

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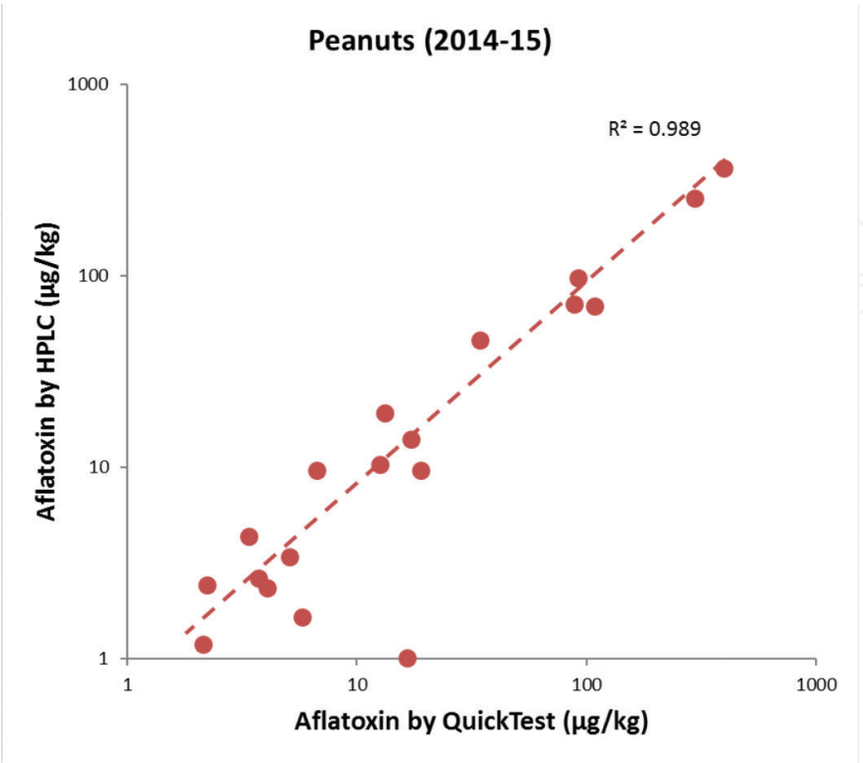
A)



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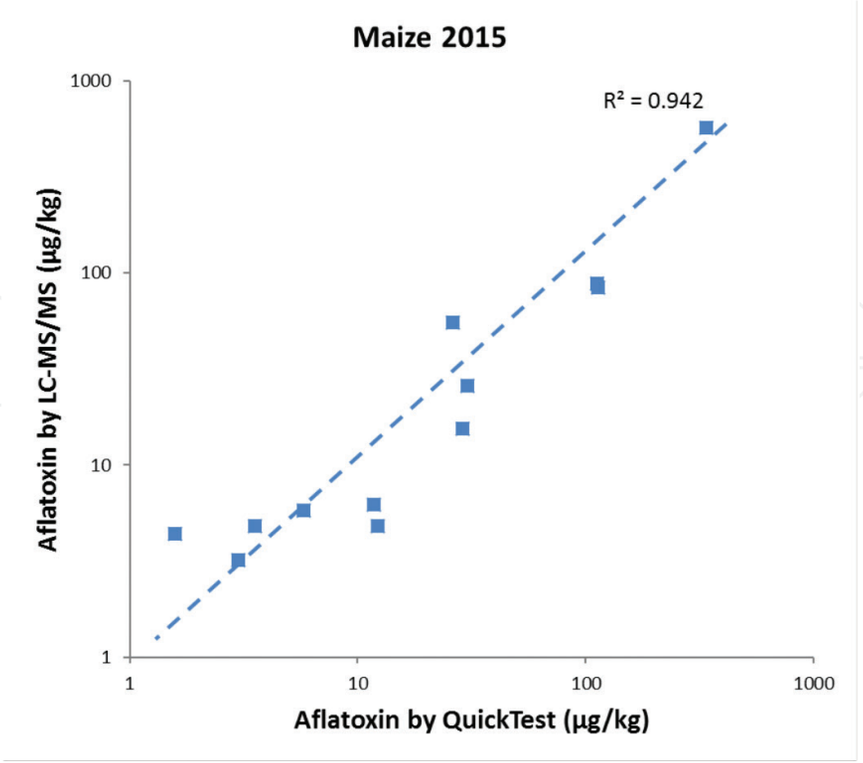

Figure 6. Validation of the Aflatoxin QuickTest™ for peanut (A) and maize (B) samples from Timor-Leste market surveys.

4.2. Timor-Leste validation study

Surveys were conducted in 2013, 2014 and 2015 to collect maize and peanut kernels from markets, seed producers and households in 42 districts of Timor-Leste. A small subset of 33 peanut and 30 maize samples from the two latter years were used for the validation study.

Samples of well-mixed kernels (100 g) were ground using a commercial blender and the meal thus obtained was extracted with 200 ml of 80% methanol containing 4% NaCl in a blender for 3 minutes. The extracts were filtered and 4 ml of supernatant collected for subsequent analysis by both Aflatoxin Quicktest™ and standard analytical methods. All peanut samples were analysed by HPLC-fluorescence at the PCA analytical facilities (Kingaroy, Queensland), whereas only 15 maize samples were analysed by LC-MS/MS at the National Measurement Institute (Sydney, Australia).

Aliquot of the extracts (200 μl) were diluted in phosphate buffer solution (ratio 1:10) to make it ready for Aflatoxin Quicktest™ analysis. Two drops of this solution were added to each strip and allowed to develop colour in 15 minutes. The strips were then read using the Quick Reader, and the results printed and recorded. For readings above 40 ppb, the sample extracts were further diluted and reanalysed again.

The results by both analytical methods were compared in order to validate the Aflatoxin Quicktest™ procedure. The majority of samples showed levels of total aflatoxin below 100 μg/kg. Regression analysis on the sets of peanut (n = 33) and maize samples (n = 15) showed coefficients of determination (r^2) of 0.989 for peanuts (71% aflatoxin B1) and 0.942 for maize (91% aflatoxin B1) (**Figure 6A** and **B**).

In summary, both validation studies were successful, demonstrating the accuracy of the Aflatoxin Quicktest™, which renders results comparable to those obtained by the standard analytical methods in certified laboratories.

5. Advantages of the Aflatoxin QuickTest™

Advantages of the LFD technology are the ease of use, rapid development time, no need for dangerous chemicals, straightforward reading of test results and low cost of the strips. Moreover, very little and inexpensive equipment is required other than a Quick Reader and solvents for the extractions.

This technology is designed to help primary producers screen their produce before selling it in the market. Because the test is very simple and easy to understand, users only require a basic training to become proficient. Thus, local co-operatives, small companies and even farmers can learn it and apply it in their own facilities.

Based on the demonstrated performance of the Aflatoxin QuickTest™ in evaluating aflatoxin contamination in maize and peanuts, the government of Timor-Leste is supporting its use for screening these and other agricultural produce that may be contaminated with this toxin. Local companies may now use this technology to meet international food safety standards for the export market, instead of resorting to the expensive alternative of sending samples to

certified laboratories in Indonesia or Australia. Moreover, they can now check the raw produce on site without having to wait weeks until they receive the results from the laboratories. No doubt, this potentially gives them significant competitive advantage in the markets. It is anticipated that application of this technology may allow rapid, accurate and low cost screening of Timorese agricultural produce.

By measuring the levels of aflatoxin in their produce farmers can now manage the problem, whereas lack of awareness of this contamination will only spread the contamination with these toxins among the local population [29], leading to unpleasant health consequences for consumers [5], and probably to a rejection of the products in the market place. Only what can be measured can be managed. In this regard, monitoring of agricultural produce should be followed by practical training sessions where farmers are instructed how to avoid aflatoxin contamination in the first place [1, 30, 31].

So far only grain and nuts have been tested using the Aflatoxin QuickTest™, but it is obvious that other agricultural commodities such as milk and dairy products can also be analysed using this technology. In the case of milk, no extraction may be required, so samples can be used directly for detecting levels of this contaminant in the range 0.1–2.0 μg/L or higher, after diluting by a given factor.

The current Aflatoxin QuickTest™ discussed here uses polyclonal antibodies specific for aflatoxin B1 and G1 (**Table 1**). Different antibodies have also been developed that have variable sensitivities towards other forms of aflatoxin and can be marketed in the near future in accordance with specific market needs.

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