

A SENSITIVE CHEMILUMINESCENT ASSAY FOR AFLATOXIN B₁

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ABSTRACT

In this study a chemiluminescent label was substituted for an enzyme marker used in AFB₁ ELISA and the minimum detection limits of the direct/modified indirect competitive chemiluminescence immunoassay (CIA), the direct competitive CIA (2 step method), the direct competitive CIA (1 step, 30 min method), and the direct competitive CIA (1 step, 5 min method) were shown to be 10.0, 5.1, 5.6, and 6.8 pg ml⁻¹ respectively. In a direct comparison between different labeling methods but using the same antibodies, the direct competitive CIA (1 step, 5 min method) is superior in sensitivity to the AFB₁ ELISA (detection limit 200 pg ml⁻¹). In addition to an increase in sensitivity, the assay time was also reduced. The sensitivity of the assay was not affected by 6.25% (v/v) methanol and required a 1:8 dilution of the peanut extract [using 80% (v/v) methanol as extracting solvent] was required to avoid matrix interference.

Key words: aflatoxin B₁, chemiluminescent label, enzyme marker, immunoassay

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1. INTRODUCTION

Aflatoxin B₁ (AFB₁), a secondary metabolite produced by *Aspergillus flavus* and *A. parasiticus* [1], is classified as a Group I, known human carcinogen by the International Agency for Research on Cancer [2] and the Joint FAO/WHO Expert Committee on Food Additives [3] due to its known carcinogenic effect on experimental animals and the strong, persuasive, circumstantial evidence associating AFB₁ exposure with human liver cancer.

To date no practical method is available for the prevention of AFB₁ contamination of foods and feeds. Similarly, the elimination of AFB₁ from human and animal diets has proved to be difficult and no threshold of exposure below which there is no risk of neoplasia has yet been determined. Therefore, efforts have focused on the development of more rapid, sensitive and specific methods for aflatoxin detection.

Detection methods for AFB₁ can be divided into 2 groups: 1) physicochemical methods e.g. TLC and HPLC which tend to be time consuming and laborious, and 2) biological methods which are generally less precise, non-specific and less sensitive, with the exception of the immunoassays e.g. RIA and ELISA, which are highly precise, specific and sensitive.

In immunoassays the sensitivity is dependent on three factors, namely antibody (Ab) affinity, the specific activity of the label, and non-specific binding [4]. The main limiting factor in inhibition assays is Ab affinity, since the smallest amount of analyte detectable is ultimately limited by the affinity of the Ab employed. However, the use of high specific activity labels may improve the detection limits of the inhibition assays [5]. Therefore, unless the limit of detection of the assay is already restricted by the immunochemistry of the method, direct replacement of labels may increase both detection limits and speed of assay. There have been few reports on comparisons between different labeling techniques using the same Abs. Alkan *et al.* [6] reported that when chemiluminescence was used instead of horseradish peroxidase (HRP) the sensitivity of the assay for detecting human γ interferon increased by a factor of 75. In view of the potential advantages to be gained, namely greater sensitivity and the speed of assay, an acridinium ester label, which produces a rapid light reaction with a low

background [7],[8] was used in the analysis of AFB₁. The results derived from this study when compared with the ELISA of Candlish [9] represent a direct comparison between different labels but using the same Abs. Since the sample extracts (using methanol/water as extracting solvent) were assayed directly without cleaning up, the effects of methanol and sample matrix on the developed chemiluminescence immunoassay (CIA) were also investigated.

2. MATERIALS AND METHODS

Apparatus

A Protein A column was purchased from Bio-Rad Laboratories Ltd. (Hertfordshire, UK); Superdex[®] 200 HR 10/30 column from Pharmacia Biotech (Uppsala, Sweden) and Lucy[®] luminometer from Rosys Anthos (Lagerhausstrasse, Austria).

Materials

4-(2-succinimidylloxycarbonyl)ethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulphonate, labeling buffer and quenching buffer were purchased from Molecular Light Technology Research (Cardiff, UK); bovine serum albumin (BSA), AFB₁, AFB₁-BSA, Tris and Tween 20 from Sigma Chemical Co. (Dorset, UK); CH₃OH, HCl, NaH₂PO₄, NaN₃, NaCl from BDH (Dorset, UK); H₂O₂ (0.5% v/v) in 0.1 M HNO₃ and NaOH (0.25 M) containing surfactant from Ciba-Corning Diagnostics (Essex, UK); opaque white microtiter plates from Dynex Technologies (Middlesex, UK); L-Glutamine, Penicillin/Streptomycin and RPMI-1640 from Life Technologies Ltd. (Paiseley, UK); fetal calf serum (FCS) from Harlan Sera-Lab Ltd. (Leicestershire, UK) and peanuts from KP Foods UK (Leicestershire, UK).

Methods

Anti-AFB₁ monoclonal antibody

The hybridoma cell line 4E1 producing specific monoclonal antibodies for AFB₁ was developed by Candlish [10]. The monoclonal Ab named McAb 4E1 was separated from cell culture media [1% (v/v) 5,000 IU penicillin, 1% (v/v) 5,000 µg ml⁻¹ streptomycin, 1% (v/v) 200 mM L-Glutamine and 5% (v/v) FCS in RPMI-1640 medium] using a protein A column.

This Ab has previously been shown to have cross reactivities of 100, 13, 14 and 1% against AFB₁, AFB₂, AFG₁ and AFG₂ respectively using various concentrations of the cross reacting metabolites as competitors in ELISA [9].

Anti-AFB₁ McAb -acridinium ester conjugate

The McAb 4E1 was labeled with 4-(2-succinimidylloxycarbonyl)ethyl-phenyl-10-methylacridinium-9-carboxylate fluorosulphonate according to the manufacturer's instructions. Antibodies (100 µg) in 200 µl of labeling buffer (0.1 M NaH₂PO₄/Na₂HPO₄, pH 8.0) were incubated at room temperature (RT) with 5 µg of the label. After 15 min in the dark, the reaction was quenched with 100 µl quenching buffer (labeling buffer containing 5 mg ml⁻¹ lysine monohydrochloride) at RT for 5 min in the dark and the resulting conjugate was purified on a Superdex[®] 200 HR 10/30 column.

AFB₁-BSA-acridinium ester conjugate

The preparation and purification of AFB₁-BSA-acridinium ester conjugate were as described above except that AFB₁-BSA was substituted for the antibodies.

Chemiluminescence immunoassay (CIA) protocols

a) Direct/modified indirect competitive CIA

In this study a direct competitive assay was employed by conjugating Ab directly to the label instead of using a second labeled Ab [10],[11].

Opaque white microtiter plates were coated with AFB₁-BSA (1 µg ml⁻¹ of AFB₁) in coating buffer (0.02 M Tris/HCl, pH 9.0; 100 µl per well) at 37°C for 1 h. The plates were washed 3 times with wash buffer (0.2 M Tris, 0.2 M NaCl and 0.05% v/v Tween 20; pH 7.4) and blocked with 0.2% (w/v) BSA in PBS (200 µl per well) at RT for 30 min, before washing again. Aflatoxin B₁ standards in PBS were then added (100 µl per well), together with 4 µl of the acridinium labeled anti-AFB₁ Ab at a dilution of 1:200 in assay buffer (0.01 M NaH₂PO₄, 0.15 M NaCl, 0.1%w/v NaN₃ and 0.25% w/v BSA; pH 7.4). The plates were incubated at RT for 30 min before being washed. Hydrogen peroxide (0.5% v/v) in 0.1 M HNO₃ (10 µl) was

then added, followed by 0.25 M NaOH containing surfactant (20 μ l) to initiate light emission.

A luminometer (Lucy[®]) was used to measure chemiluminescence expressed as photon counts (light units) for 2 seconds. The assays were performed 4 times, each in duplicate.

b) Direct competitive CIA (2 step method)

Opaque white microtiter plates were coated with anti-AFB₁ Ab (1 μ g ml⁻¹) in coating buffer (100 μ l per well) at 37°C for 1 h. The plates were washed 3 times with wash buffer and blocked with 0.2 % (w/v) BSA in PBS (200 μ l per well) at RT for 30 min. The plates were washed 3 times following which AFB₁ standards in PBS were added (100 μ l per well) and incubated at RT for 30 min. Acridinium labeled AFB₁-BSA conjugate at a dilution of 1:50 in assay buffer was then added (4 μ l per well) and the plates were incubated at RT for 30 min. The plates were then washed 3 times and chemiluminescence was measured as described previously. The assays were performed 4 times, each in duplicate.

c) Direct competitive CIA (1 step, 30 min method)

The assays were performed as described for the 2 step method except that after free AFB₁ was added, acridinium labeled AFB₁-BSA was added sequentially and incubated for 30 min. The assays were carried out 5 times, each in duplicate.

d) Direct competitive CIA (1 step, 5 min method)

The assays were performed as described for the 1 step, 30 min method except that the incubation time of the free AFB₁ with the acridinium labeled AFB₁-BSA conjugate was reduced to 5 min. The assays were carried out 3 times, each in duplicate.

Effect of methanol on CIA

The effect of methanol on the standard dose-response curve for AFB₁ present in 6.25, 12.5 and 25% v/v methanol was determined by the direct competitive 1 step, 30 min method. The assays were carried out 3 times, each in duplicate.

Effect of sample matrix on CIA

Peanut extract was prepared by mixing finely ground peanuts (10 g) with 40 ml extraction solvent [methanol:water (80:20)] for 2 min in a Waring blender. The extract was left to stand at RT for 30 min before being filtered through a Whatman® No. 1 filter paper.

The effect of peanut extract on the standard dose-response curve for AFB₁ present in peanut extract diluted in PBS (1:2, 1:4 and 1:8 dilutions) was determined by the direct competitive 1 step, 30 min method. The assays were carried out 3 times, each in duplicate.

3. RESULTS

Sensitivity of CIA

Different assay formats were investigated to determine which format would produce the highest sensitivity and the standard dose-response curves of the 3 formats are shown in Figure 1. When the AFB₁-free solution was set at 100% binding, the 50% inhibition (I_{50}) of the dose-response curve from the direct/modified indirect competitive CIA was 41 pg ml⁻¹. The minimum detection limit, defined as the minimum amount of AFB₁ that could be significantly distinguished from zero (i.e. mean binding at zero dose minus 2 SD), was 10 pg ml⁻¹ with the working range up to 400 pg ml⁻¹. Intra-assay coefficients of variation (CV) (%) (n=2) were 1.8, 3.2 and 5.2 at 10, 40 and 100 pg ml⁻¹ of AFB₁ respectively. At the same concentrations of AFB₁, inter-assay CV (%) (n=4) were 5.1, 8.8 and 8.9 respectively. The I_{50} of the 2 step and 1 step, 30 min direct competitive CIA methods were 14.7 and 16.5 pg ml⁻¹ respectively and both methods had a working range up to 100 pg ml⁻¹. When compared to the direct/modified indirect CIA the sensitivity of the assay improved to 5.1 pg ml⁻¹ for the 2 step direct CIA and 5.6 pg ml⁻¹ for the 1 step, 30 min direct CIA. The intra-assay CV (n=2) at 10, 40 and 100 pg ml⁻¹ of AFB₁ were 2.8, 5.0 and 5.4% for the 2 step method and 2.7, 5.7 and 2.0% for the 1 step, 30 min direct CIA. At the same concentrations of AFB₁, the inter-assay CV (n=4) of the 2 step method were 3.1, 5.6 and 7.9% respectively, whereas the inter-assay CV (n=5) were 9.6, 10.0 and 4.6% respectively for the 1 step, 30 min direct CIA.

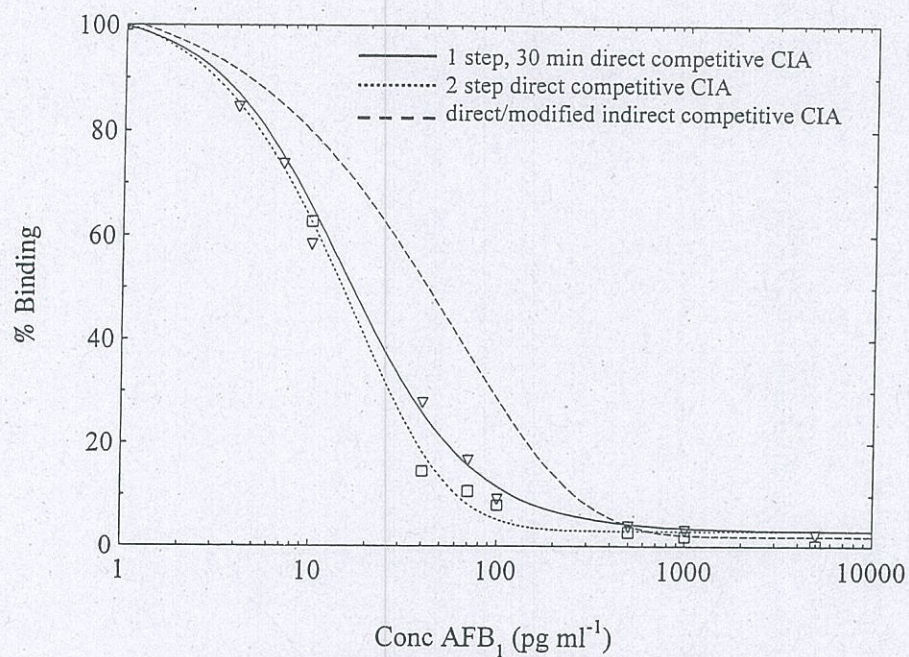


Figure 1 Dose response curve for AFB₁.

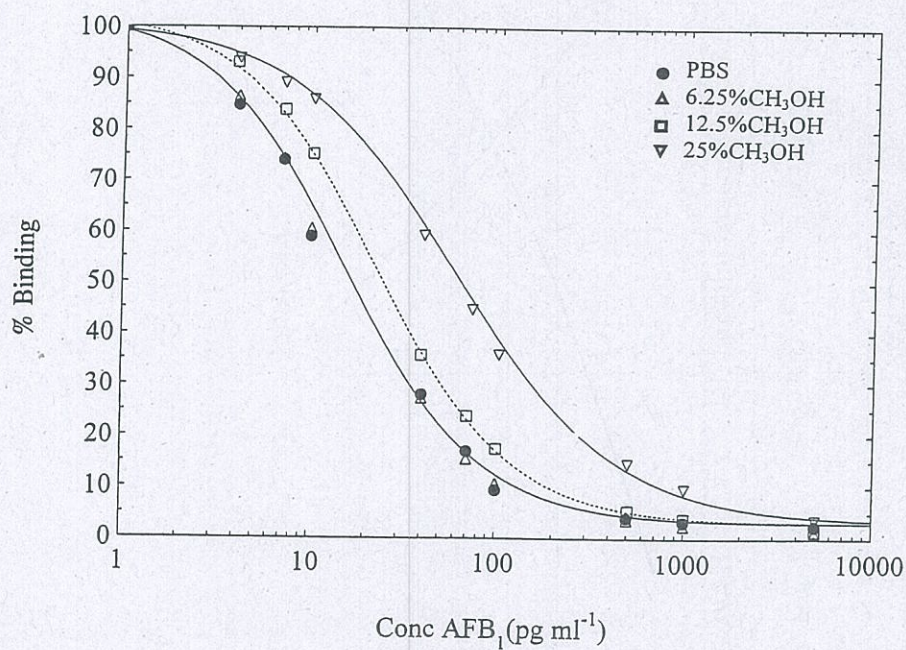


Figure 2 Effect of methanol on the dose-response curve for AFB₁ by direct competitive CIA (1 step, 30 min method).

Interference of CIA by methanol

As shown in Figure 2, in the direct competitive CIA (1 step, 30 min), the dose-response curve derived from AFB₁ in 6.25% (v/v) methanol was identical with that of AFB₁ in PBS. However, with increasing concentrations of methanol the dose-response curves for AFB₁ became less sensitive in proportion to the concentrations of methanol added. Consequently the I_{50} values varied to 21.4 and 52.7 pg ml⁻¹ in the presence of 12.5 and 25% (v/v) methanol, respectively. The intra-assay CV (n=2) at 10, 40 and 100 pg ml⁻¹ of AFB₁ were 3.9, 5.1 and 7.0% in 6.25% (v/v) methanol; 3.2, 5.1 and 8.1% in 12.5% (v/v) methanol and 4.6, 4.2 and 6.0% in 25% (v/v) methanol respectively. At the same concentrations of AFB₁, the inter-assay CV (n=3) were 6.0, 6.7 and 10.9% in 6.25% (v/v) methanol; 7.0, 6.6 and 9.8% in 12.5% (v/v) methanol and 6.4, 10.5 and 6.5% in 25% (v/v) methanol respectively.

Matrix effect on CIA

Figure 3 shows the absence of matrix effect on the CIA, when the peanut extract was diluted in PBS buffer to 1:8, as the dose-response curve at this dilution was superimposable on the curve obtained in PBS. The interference from peanut extract was more significant when lower dilutions of extracts were assayed i.e. 1:4 and 1:2, as the dose-response curves were shifted to the right with I_{50} values of 38 and 258 pg ml⁻¹ respectively. At 10, 40 and 100 pg ml⁻¹ of AFB₁, the intra-assay CV (n=2) were 0.6, 17.3 and 7.0% at 1:2 dilution; 3.6, 4.9 and 5.6% at 1:4 dilution and 2.6, 2.2 and 1.8% at 1:8 dilution of peanut extract. The inter-assay CV (n=3) at the same concentrations of AFB₁ were 7.6, 21.2 and 15.7% at 1:2 dilution; 14.3, 7.6 and 9.8% at 1:4 dilution and 2.6, 3.6 and 8.6% at 1:8 dilution of peanut extract.

Direct competitive CIA (1 step, 5 min method)

The effect of sample incubation time on direct competitive CIA is shown in Figure 4. Reducing the incubation time from 30 min to 5 min resulted in some loss of assay sensitivity. The minimum detection limit and I_{50} were increased to 6.8 and 18.3 pg ml⁻¹ respectively while the working range was 6.8 to 100 pg ml⁻¹. Intra-assay CV (n=2) were 7.6, 5.8 and 11.7%

at 10, 40 and 100 pg ml^{-1} of AFB_1 respectively. At the same concentrations of AFB_1 , inter-assay CV ($n=3$) were 8.8, 6.9 and 14.1% respectively.

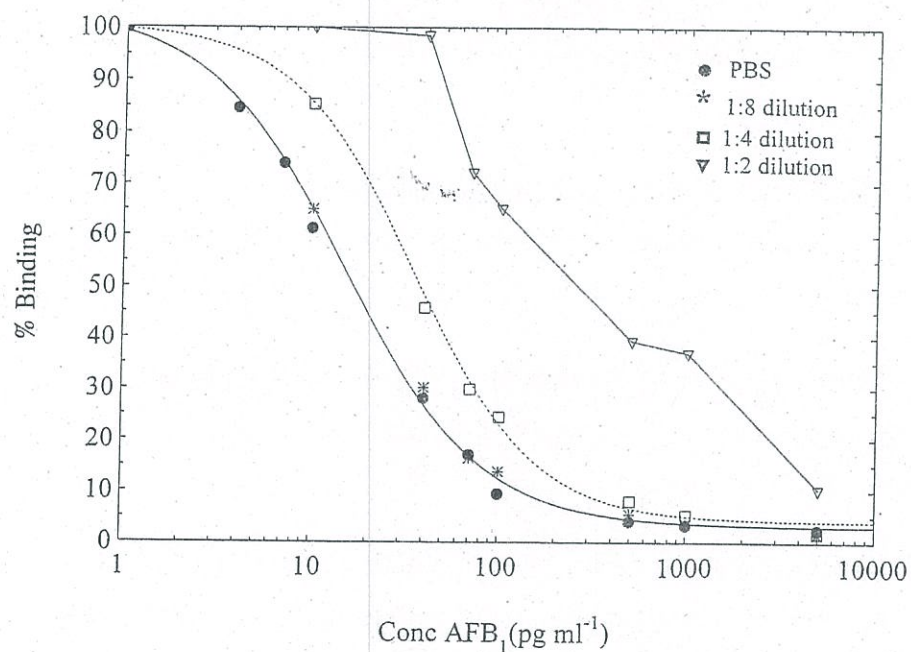


Figure 3 Effect of peanut extract on the dose-response curve for AFB_1 by direct competitive CIA (1 step, 30 min method).

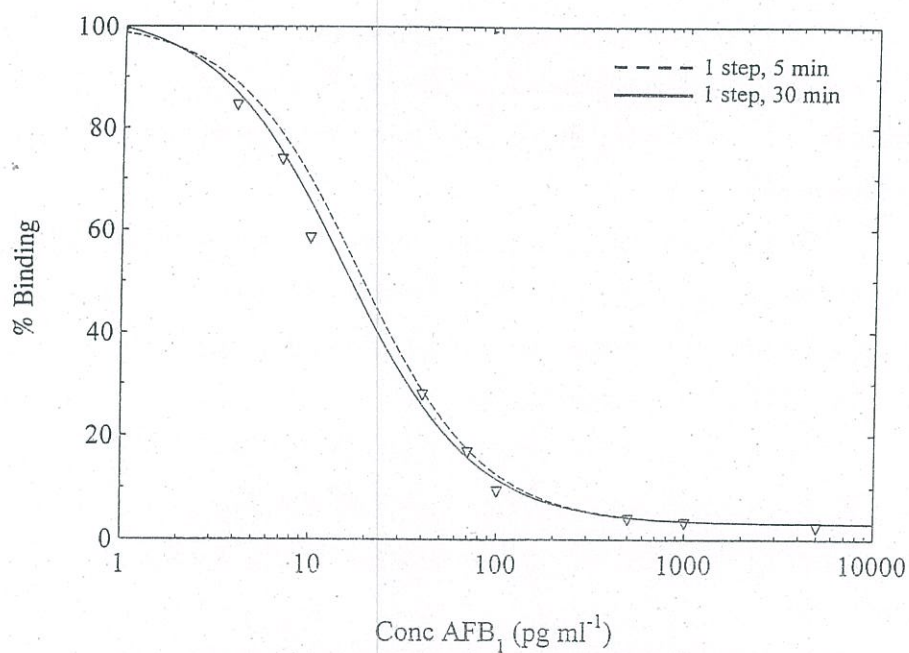


Figure 4 Dose response curve for AFB_1 by direct competitive CIA.

4. DISCUSSION

The immunoassays used for the analysis of aflatoxins in agricultural commodities include RIA and competitive ELISAs (both direct and indirect formats) [12]. The sensitivity of the direct ELISA has been reported to be comparable to or slightly less than that of the indirect ELISA [12]. In addition the direct assays have the disadvantage of requiring exact molar ratios of toxin-enzyme preparation for optimal sensitivity and the instability of such conjugates also constitutes a problem [13]. These problems can be surmounted by the use of acridinium esters as labels, due to their ease of coupling to other molecules and the stability of the labeled conjugates [8],[14]. In this study the use of such labels was applied to the analysis of AFB₁ in both direct and direct/modified indirect competitive formats and the detection limits of these assays were compared with those of previously reported ELISAs using the same [9] or different [15] anti-AFB₁ monoclonal Abs.

In this study the sensitivity or minimum detection limit is defined as the minimum amount of AFB₁ that could be significantly distinguished from zero (mean binding at zero dose minus 2 x SD) [14]. Candlish [9] defined the sensitivity of the AFB₁ ELISA as the smallest amount of toxin which could be distinguished from no toxin with an appropriate degree of probability (95% confidence limit). The 95% limits lie 2 SDs below and above the mean [16]. The McAb 4E1 based direct/modified indirect competitive ELISA for AFB₁ detection described by Candlish [9] had a sensitivity of 200 pg ml⁻¹ and a working range up to 30 ng ml⁻¹ for AFB₁. Using the same assay format but substituting the HRP enzyme label with a chemiluminescent marker, we obtained a minimum detection limit of 10 pg ml⁻¹, representing a 20 fold increase in sensitivity, and a working range up to 400 pg ml⁻¹ (Figure 1).

In the 2 step direct competitive CIA method, in which the immobilized specific McAb 4E1 was incubated first with the free AFB₁ and then with the acridinium labeled AFB₁-BSA, the sensitivity was 5.1 pg ml⁻¹ which is approximately twice as sensitive as the direct/modified indirect competitive CIA and 40 times more sensitive than the ELISA of

Candlish [9]. In a study comparing the sensitivities of the direct and indirect competitive ELISAs with AFB₁, Kawamura *et al.*[15] reported the detection limits of the direct and indirect procedures to be 50 and 100 pg ml⁻¹ respectively. This group of investigators expressed the detection limits as concentrations which gave significant (over 10%) inhibition. In this study the definition of sensitivity when expressed in terms of % inhibition, is nearly equivalent to 20% inhibition (i.e. 80% binding). Although the direct ELISA (detection limit 50 pg ml⁻¹) of Kawamura *et al.*[15] is 4 fold more sensitive than the ELISA of Candlish [9] (detection limit of 200 pg ml⁻¹), the 2 step direct CIA with a detection limit of 5.1 pg ml⁻¹ is superior in sensitivity by a factor of 10.

The 1 step, 30 min direct competitive CIA, in which the immobilized McAb 4E1 was incubated with the free AFB₁ and acridinium labeled AFB₁-BSA simultaneously, resulted in a dose-response curve to the right when compared to the curve of the 2 step method, indicating a loss of sensitivity from 5.1 to 5.6 pg ml⁻¹ (Figure 1). However, this decrease was compensated by the reduction in the number of steps (one less incubation step as well as one less wash step) and hence the assay time. Subsequent experiments to determine the interference caused by methanol and the sample matrix were performed using the 1 step, 30 min direct competitive CIA.

Methanol 55 to 70% (v/v) is commonly used as an extraction solvent for AFB₁ in agricultural products [17],[18]. The interference of ELISA by methanol is thought to be due to the influence of the alcohol on enzyme activity and antigen-antibody interaction [15]. El-Nakib *et al.*[19] reported that the dose-response curves in the presence of 10 and 30% (v/v) methanol were similar to each other in the direct competitive ELISA employed by them. Kawamura *et al.*[15] noted that I₅₀ values were 28 and 73 pg per assay in the presence of 10 and 30% (v/v) methanol respectively for the indirect ELISA, whereas in the case of the direct ELISA the I₅₀ values were 31 and 35 pg per assay in the presence of 10 and 30% (v/v) methanol respectively. Using the McAb 4E1 in the direct/modified indirect ELISA, Candlish [9] reported that methanol did not have a significant effect until 20% (v/v) and that methanol

seemed to cause non-specific binding of conjugate at 100% (v/v). In contrast Ram *et al.*[20] reported that 70% (v/v) methanol did not have a significant effect on the direct competitive ELISA. It is probable that the differences in assay conditions i.e. concentrations of Abs and AFB₁-enzyme conjugates, incubation time and temperature may have been responsible for the different results described above, even though the same solvent was used in the ELISAs. In this study methanol affected the 1 step, 30 min direct CIA at lower concentrations (Figure 2). Although methanol at 6.25% (v/v) had no effect on the 1 step, 30 min direct CIA (I_{50} 16.5 pg ml⁻¹), the dose-response curves of the CIA moved to the right at 12.5% (v/v) methanol (I_{50} 21.4 pg ml⁻¹) and 25% (v/v) methanol (I_{50} 52.7 pg ml⁻¹). The high sensitivity of chemiluminescent analysis is due mainly to low background values.

It is important not to overlook the problem of sample matrix interference since after the toxins are extracted from the commodities with a solvent, the samples are then used directly in the immunoassay after dilution in buffer. Peanut extract [extracting solvent = 80% (v/v) methanol] at 1:8 dilution did not interfere with the 1 step, 30 min direct CIA (Figure 3). Significant interference was detected at lower dilutions (i.e. 1:4 and 1:2) of peanut extracts. Candlish [9] required a 1:8 dilution of sample [55% (v/v) methanol extract of peanut] whereas Kawamura *et al.*[15] reported needing only a 1:2 dilution of sample [60% (v/v) methanol extract of peanut] to avoid matrix interference in their respective ELISAs. Ram *et al.*[20] advised the incorporation of "toxin-free sample matrix extracts" in the preparation of the dose-response curve. However, the disadvantages were an increase in errors and a loss of sensitivity. Chu *et al.*[21] developed an ELISA for AFB₁ and overcame the sample matrix interference problem by diluting the sample extract 1:20. This is encouraging since the 1 step, 30 min direct CIA at 1:8 dilution of the sample extract resulted in a dose-response curve superimposable on the curve obtained from buffer alone.

Increasing the incubation time has been shown to improve sensitivity for the AFB₁ ELISA [9]. Experiments on how the sensitivity of the direct CIA would be affected when the incubation period was reduced from 30 min to 5 min were carried out. A loss of sensitivity

was detected, however, the sensitivity of the 1 step, 5 min direct CIA at 6.8 pg ml^{-1} is still greater than the direct/modified indirect ELISA of Candlish [9] and the direct ELISA of Kawamura *et al.* [15] by factors of 30 and 7 fold respectively.

5. CONCLUSIONS

The results presented here indicate that the acridinium ester is a suitable alternative to HRP for the analysis of aflatoxins in agricultural commodities. However, the effectiveness of the 1 step, 5 min direct competitive CIA protocol needs to be confirmed both by analytical recovery studies and by the analysis of naturally contaminated samples.

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