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Development of a portable field immunoassay for the detection of aflatoxin M_1 in milk

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Abstract

Many methods for AFM_1 detection exist, but most are time consuming, employ expensive equipment and require experienced personnel. To overcome these problems a membrane-based flow-through enzyme immunoassay has been developed (patent pending). The assay comprised a nylon Immunodyne ABC membrane spotted with anti-mouse antibodies, a plastic snap-fit device, absorbent cotton wool, mouse anti-AFM_1 monoclonal antibodies (Mab), and AFB_1–horseradish peroxidase (HRP) conjugate. This assay was coupled to an immunoaffinity column (IAC). The visual detection limit was 0.05 ng/g AFM_1 in milk. Assay time for IAC clean-up was 12 min, and that for the flow-through assay was 18 min, hence the total assay time was 30 min. This method allows for a rapid screening of milk consignments which do not conform to the maximum permissible limits of 0.05 ng/g AFM_1, hence enabling the rejection of such at the farm level. Laboratory validation was done using certified reference materials (CRM) with AFM_1 concentrations of <0.05, 0.09 and 0.76 ng/g. Precision of the assay was high as shown by the high repeatability of the assay results. There were no significant differences in recoveries between standard in buffer and CRM ($P > 0.05$), and assay responses for these two were highly correlated (99.63%).

Keywords: Flow-through; Aflatoxin M_1; Milk; Portable; Field immunoassay

1. Introduction

Fungal invasion of food can result in a marked deterioration in quality and often outright destruction. A more subtle, but even more compelling reason to control such fungi is that some species are capable of producing mycotoxins. The historical record of human and animal mycotoxicoses is a long one (Sibanda et al., 1997). In Africa, for example, aflatoxins, which are produced by *Aspergillus* species, have been implicated in human diseases including liver cancer, Reye's syndrome, Indian childhood cirrhosis, chronic gastritis, kwashiorkor and certain respiratory diseases (Sibanda et al.,

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1997). Naturally occurring aflatoxins are comprised of B_1, B_2, G_1 and G_2.

Ingestion of AFB_1-contaminated feed by dairy cattle leads to the excretion of AFM_1 in their milk. Experimentally established carry-over rates for high and low contaminated feedstuff rations average 1–2%, the percentages varying from animal to animal and from one milking day to another (Van Der Linde et al., 1964; Van Egmond, 1989). AFM_1 appears in milk 12–24 h after ingestion of the first AFB_1-contaminated ration.

Natural contamination levels of AFM_1 are not at a level to cause overt mycotoxicoses, but rather causing chronic symptoms (Smith et al., 1995). It is, therefore, the chronic effects of mycotoxins that are of importance with regards to humans. The frequent detection of AFM_1 in commercial milk samples and dairy products, the high consumption of these products, especially in infant populations and the probable carcinogenicity of AFM_1, led to the establishment of measures to control the contamination of foodstuffs and feedstuffs with AFM_1. The European Commission proposes a maximum permissible level of 0.05 ng/g AFM_1 in milk and milk products. Aflatoxins are relatively heat stable, meaning that once contamination has occurred a considerable percentage of the initial levels will persist to the final product and finally to the human digestive tract. This, therefore, necessitates the screening of raw materials in order to ascertain quality products. The early detection of mycotoxin-contaminated lots is essential because the value of the commodity increases as it moves through the production and marketing systems. Therefore, detection and segregation of mycotoxin-contaminated material at the farm level will greatly reduce costs.

Current aflatoxin detection methods include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS) and enzyme-linked immunosorbent assay (ELISA). These methods are laborious, use expensive equipment, require well-trained personnel and can only be employed in laboratories. In order to screen for contaminated milk at the farms before accepting milk consignments a portable rapid field assay is required. This paper reports on the development of a rapid, portable, field immunoassay for AFM_1 in milk.

2. Materials and methods

2.1. Materials

Rabbit anti-mouse immunoglobulins (No. Z259) were purchased from Dako (Glostrup, Denmark). Plastic snap-fit device (bottom and top members) was obtained from Trosley Equipment (Dover, UK) and the Immunodyne ABC membrane (pore size, 0.45 μm) from Pall BioSupport (Portsmouth, UK). Cotton wool (100%) was obtained from Utermöhlen Medical Care (Belgium). AFB_1–HRP conjugate and anti-AFM_1 monoclonal antibody clones were produced and characterised by the Institute for Animal Sciences, Agricultural Biotechnology Center, Gödöllö, Hungary (Barna-Vetro et al., 1997). Cross-reactivity of the anti-AFM_1 monoclonal antibody clone was 100, 20, 74 and 57% against AFM_1, AFM_2, AFB_1 and AFB_2, respectively. Cross-reactivities against AFB_{2a}, AFG_2 and AFG_{2a} were $< 0.01\%$. Aflaprep® M immunoaffinity columns (IAC) were obtained from Rhône-Diagnostics Technologies (Glasgow, UK). Phosphate-buffered saline (PBS, pH 7.4) was used to make the wash solution (PBS–Tween 0.05%) and assay buffer (PBS–Tween 0.05%–casein 0.1%). The substrate chromogen solution which consists of solution A and B was prepared as described by Dürsch and Meyer (1992). Solution A (pH 5.0) contained H_2O_2 (0.1 g), $Na_2HPO_4 \cdot 2H_2O$ (1.8 g), citric acid.H_2O (1.03 g) and HPLC-grade water (100 ml). Solution B (pH 2.4) contained TMB (50 mg) (Sigma), dimethylsulfoxide (4 ml), citric acid $\cdot H_2O$ (1.03 g) and HPLC-grade water (96 ml). Pure AFM_1 standard was purchased from Sigma.

2.2. Preparation of membranes

The Immunodyne ABC membrane was cut into sections (2 cm^2). Undiluted rabbit anti-mouse immunoglobulins (5 μl; protein content, 3.2 g/l) were manually spotted onto the membrane with an Eppendorf pipette. The membranes were dried at 37°C for 30 min. The remaining protein binding sites were blocked by incubating the spotted membranes in a blocking solution (PBS–casein 2%) for 30 min. The membranes were then dried at 37°C for another 30 min, after which they were vacuum-packaged in

plastic bags and stored at room temperature in the dark.

2.3. Flow-through device

The flow-through apparatus was set-up as described by De Saeger and Van Peteghem (1999) (patent pending), and De Saeger et al. (1997). The snap-fit device (Fig. 1), comprises a bottom and top member, 1.5 g cotton wool, and 2 cm^2 Immunodyne ABC membrane spotted with 5 μl anti-mouse antibodies. The cotton wool functions as an absorbent material for added reagents.

2.4. Detection of AFM_1 using the flow-through assay

The 2-cm^2 membrane was fixed between the cotton wool in the bottom member of the snap-fit device and the top member. AFM_1 was assayed using anti-AFM_1 monoclonal antibody dilution of 1:100 and AFB_1–HRP conjugate dilution of 1:50. Every reagent added sequentially was allowed to filter through completely and any remaining liquid on sides was added onto the membrane using a micropipette.

One hundred μl of anti-AFM_1 monoclonal antibody (1:100 in assay buffer) were added onto the membrane. The membrane was washed with 300 μl of wash buffer (PBS–Tween 0.05%). Six hundred μl of standard or sample were applied onto the membrane. AFB_1–HRP conjugate, 100 μl, (1:50 in assay buffer) was added.

The membrane was then washed with 600 μl of wash buffer. Equal volumes of reagent A and B were mixed prior, then 50 μl of the TMB–H_20_2 solution were applied onto the membrane.

The dot colour intensity was visually compared to that of the negative control which showed the most intense blue colour because toxin concentration is inversely related to colour intensity. The smallest toxin concentration that resulted in no colour development was considered the visual detection limit.

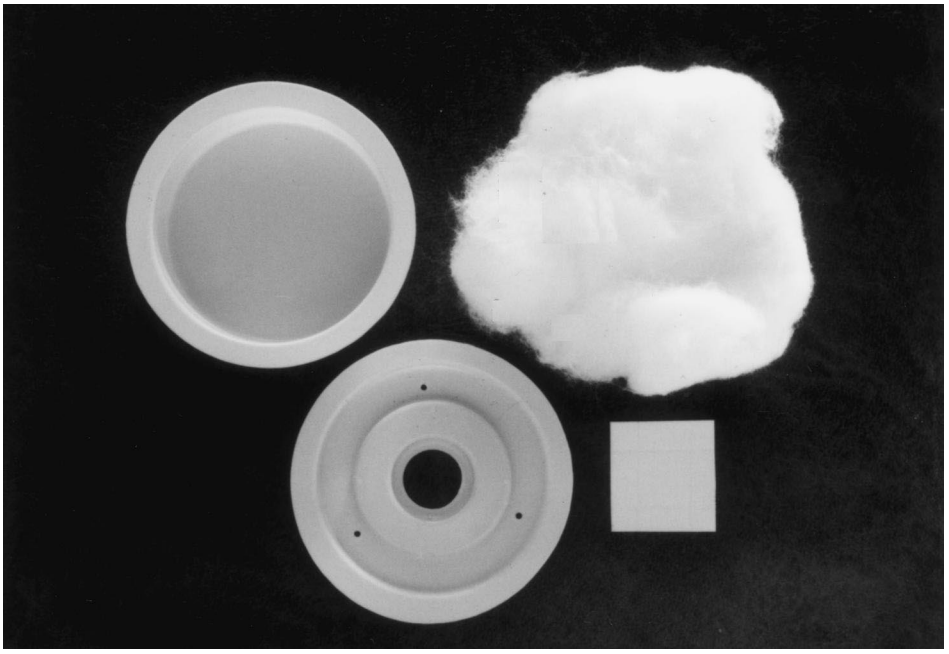

Fig. 1. Components of the flow-through device. Upper row (from left to right): plastic bottom member and absorbent material. Lower row (from left to right): plastic top member and membrane.

2.5. Preparation of milk samples

Due to the high solid content of milk direct analysis of milk by the flow-through is impeded by the blocking action of milk solids on the membrane. A clean-up step had to be coupled to the flow-through assay, and an IAC (Aflaprep® M) clean-up step was chosen. One batch of liquid milk samples was spiked with 0.05, 0.1 and 1.0 ng/ml, and another batch with 0.01, 0.025 and 0.05 ng/ml AFM_1 24 h prior to extraction. Certified reference materials (CRM; European Commission, Joint Research Center, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium) (<0.05, 0.09 and 0.76 ng/g) were used as powdered milk samples. Similar concentrations were prepared in PBS (pH 7.4) and assayed against the milk samples.

2.5.1. Powdered milk

The method used to prepare the powdered milk was that described by the IAC manufacturer. Briefly, 10 g of powdered milk (naturally contaminated/CRM) were dissolved in 80 ml of warm water (40–50°C) by stirring. The liquid milk (80 ml) was transferred to a 100-ml measuring cylinder and made up to the 100 ml mark with warm water.

2.5.2. Liquid milk

Liquid milk (50 ml) was warmed on a magnetic hot-plate stirrer and filtered through a Whatman number 4 filter paper to remove the fat. AFM_1 was then extracted from milk using Aflaprep® M IAC as described by the manufacturer. The only modification made in this procedure was the final washing step of the column after the 1.25 ml methanol extraction step where 1.25 ml PBS (pH 7.4) was used instead of 1.25 ml of HPLC water. The resultant 2.5 ml (50% methanol) was made up to 8.33 ml using PBS (pH 7.4) to adjust the methanol content to 15%.

3. Results and discussion

The negative control gave a blue colour within 60 s, while an AFM_1 concentration of 0.05 ng/ml or more did not produce any colour even after 4 min. In practice results are interpreted as either negative if there is colour development or positive if no colour develops within 60 s of the addition of the colour substrate.

In order to quantify the differences in colour intensities between negative and positive samples, as well as among the different concentrations, a portable colorimeter, Chroma Meter CR-321 (Minolta), was used. This was done by measuring the difference between the colour intensity of a white membrane (as a reference) and the dot colour intensity of the test membrane. This colour difference was expressed as a single numerical value ΔE^*_{ab} (Minolta, 1994).

Initially a Mab dilution of 1:400 and a HRP dilution of 1:50 was used. With these dilutions a standard curve for AFM_1 in buffer was prepared (Fig. 2) at concentrations 0.00, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ng/ml. The detection limit for AFM_1 in a milk sample, 0.05 ng/ml, is assayed as 0.3 ng/ml as a result of the pre-concentration effect of IAC clean-up step. The recovery of AFM_1 from spiked liquid milk and subsequent detection with the flow-through assay was comparable to that for buffers. The comparability was quantified by the analysis of variance of means of colour development values (ΔE^*_{ab}) at the same concentrations, which showed no significant differences ($P > 0.05$). Colour development values were significantly similar and differences between AFM_1 in buffer and spiked milk (0.05, 0.1 and 1.0 ng/ml) were insignificant at 95% significance level.

The colour development (ΔE^*_{ab}) versus concentration shown in Table 1 confirms the similarities. In this assay the limit of detection is defined as the AFM_1 concentration which inhibits colour development for 60 s. The colour difference between the negative and positive samples illustrated in Table 1 shows that the sensitivity of the assay is high. The calculated recoveries ($n = 10$) were 97, 95 and 99% for 0.05, 0.1 and 1.0 ng/ml, respectively. The precision of the assay, that is, the closeness in agreement between the results obtained by applying a given procedure several times under prescribed conditions, was high. Precision was quantified using the standard deviations of colour development values, since precision depends on variability among replicate analyses of the same material. Standard deviations ranged between 0.16 and 0.82 and, from 0.14 to 0.86 colour development values for AFM_1 in buffer and spiked samples, respectively.

The response of the negative control could be

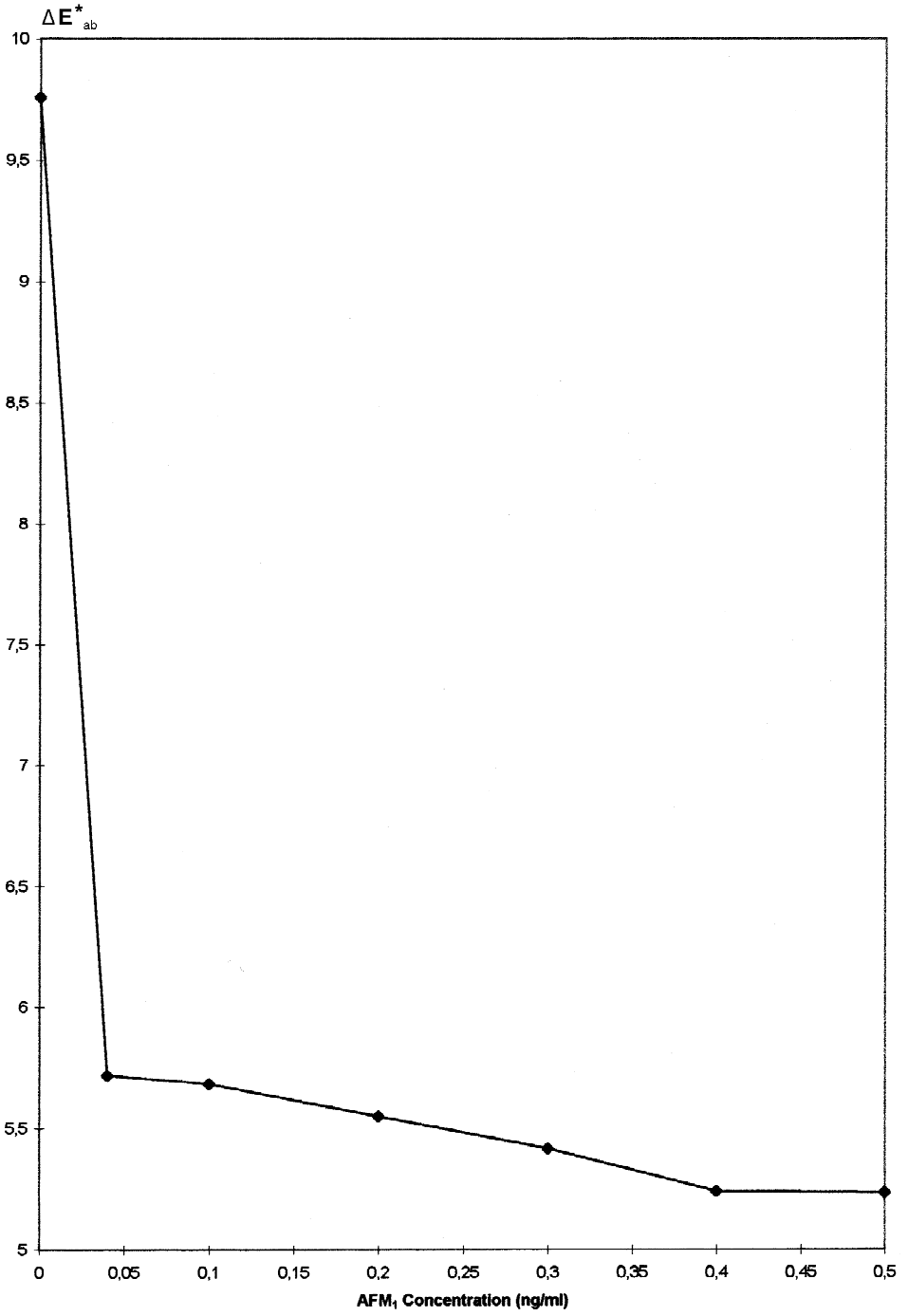

Fig. 2. Standard curve for ΔE^*_{ab} (colour development) against aflatoxin M_1 standard in buffer at concentrations 0.0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ng/ml.

Table 1
Recovery of aflatoxin M_1 from spiked milk compared to aflatoxin M_1 in buffer at concentrations of 0.00, 0.05, 0.1 and 1.0 ng/ml[a]

	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk	AFM_1 in buffer	AFM_1 spiked milk
AFM_1 concentration (ng/ml)	0.0	0.0	0.05	0.05	0.1	0.1	1.0	1.0
$(\bar{x})$ ΔE^*_{ab}[b]	9.13	9.48	4.73	4.87	4.58	4.84	4.56	5.04
SD (±)	0.82	0.33	0.16	0.59	0.20	0.14	0.20	0.86
Sample variance	0.67	0.10	0.03	0.35	0.04	0.02	0.04	0.75

[a] $n = 10$ (number of samples/assays ran for each concentration).
[b] Colour development.

improved by lowering the immunoreagent dilutions. Further, the sensitivity of the assay would not be effected by the lowering of the immunoreagent dilution because the concentrating effect of the IAC enhances the sensitivity of the assay. Therefore, the sensitivity of the assay was tested at lower concentrations (0.05, 0.025 and 0.01 ng/ml) and at a anti-AFM_1 dilution of 1:100 and AFB_1-HRP dilution of 1:50 in assay buffer.

The assay sensitivity proved to be as low as 0.025 ng/ml as colour development was inhibited for more than 4 min (time taken to perform the Chroma meter readings). There were no significant differences ($P > 0.05$) among the ΔE^*_{ab} values of the three concentrations 0.01, 0.025 and 0.05 ng/ml ($\Delta E^*_{ab} = 5.82$, 5.06 and 4.81) and ($\Delta E^*_{ab} = 6.71$, 6.14 and 5.81) for AFM_1 in buffer and spiked milk, respectively. There were no differences in the efficiency of detection, precision and recoveries at these lower concentrations between the spiked milk samples and AFM_1 standard in buffer ($P > 0.05$). The specificity of the assay was also enhanced by the IAC which selectively bound AFM_1 from samples hence eliminating any potential cross-reactivities.

The most important attribute of a measurement process is whether it can be made to run in a state of 'statistical control'. Although repetitive measurements are subject to variability, the achievement of statistical control implies that the statistical properties of this variability are uniform over time and, therefore, a level of confidence can be assigned to be associated with the limits of future measurements. Therefore, AFM_1 detection in buffer and CRM (< 0.05, 0.09 and 0.76 ng/g) were compared (Table 2). The buffer and CRM ΔE^*_{ab} values were highly correlated, 99.63%, and there were no differences in ΔE^*_{ab} values between the CRM and buffer in Table 2 ($P > 0.05$) at 95% significant level. The colour development values (ΔE^*_{ab}) for AFM_1 in buffer and CRM were comparable illustrating the degree of the accuracy of the assay in achieving similar responses in the different sample matrices (Table 2).

Table 2
Summary statistics of the flow-through assay for the detection of AFM_1 in buffer and (CRM)[a]

	Standard in buffer	Zero level CRM No. 282	Standard in buffer	Low level CRM No. 283	Standard in buffer	High level CRM No. 285
AFM_1 concentration (ng g^{-1})	0.00	<0.05	0.09	0.09 (±0.04, 0.02)	0.76	0.76 (±0.05)
$(\bar{x})$ ΔE^*_{ab}[b]	10.44	10.40	5.66	6.01	5.69	5.58
SD (±)	1.08	2.07	0.78	0.70	0.50	0.46
Sample variance	1.16	4.29	0.60	0.48	0.25	0.20

[a] $n = 10$ (number of samples/assays ran for each concentration).
[b] Colour development.

4. Conclusion

A flow-through enzyme-linked immunoassay was developed for the detection of AFM_1 in both liquid and powdered milk. The sensitivity of the assay achieved was 0.05 ng/ml, although lower, 0.025 ng/ml, could be detected with comparable precision. This detection limit is in accordance with most existing maximum permissible limits in fluid milk and powdered milk. The development activities on this flow-through immunoassay have largely been focused on testing the kit performance with an effort to associate objective measures with theoretical or technical considerations known to affect assay attributes, such as precision, sensitivity and specificity. Potentials for matrix interferences were eliminated by the coupling of the Aflaprep® M IAC to the flow-through assay which also enhanced the sensitivity of the assay through its pre-concentrating effect. The flow-through assay functions in kit form and does not cause any environmental hazard given the minute quantities of solvents involved. In any case these are appropriately disposed of since they are contained in the cotton wool within the snap-fit device.

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