Assessment of the Sensitivity and Accuracy of Immunochromatographic Test Strips in the Qualitative Detection of Aflatoxin Contamination in Peanuts

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ABSTRACT

Despite the availability of several mechanical and chemical methods for aflatoxin detection, most are not accessible to many developing countries. In these developing countries, immunochromatographic test strips can be an alternative. Research was conducted to assess the sensitivity and accuracy of immunochromatographic test strips in the qualitative detection of aflatoxin at a 20 ppb cut-off limit as compared to the standard fluorometry method, and to evaluate the effect of continuous high or fluctuating temperatures to the sensitivity and accuracy of the test strips. Results showed significant association ($P<0.0001$) between the results obtained from the test strips (AflaCheck™, Vicam) and the Vicam fluorometric method, producing an overall accuracy of 97%, sensitivity of 94%, and specificity of 100%. When exposed to continuous high (34 C) and fluctuating (34 C for 8 hr, 25 C for 16 hr daily) temperatures, AflaCheck™ and AgraStrip® (Romer Labs) retained their ability to detect aflatoxin levels up to 32 and 47 wk (8 and 12 months), respectively, without loss of efficacy. This documents the reliable use of the test strips in tropical peanut production areas where technologies like the fluorometer for aflatoxin quantification and refrigeration for storage are not readily accessible.

Key Words: Aflatoxin, fluorometry, immunochromatographic test strip, lateral flow test strip.

Aflatoxin has carcinogenic, hepatotoxic, and immunosuppressive properties that have caused high mortality and reduction of productivity in livestock as well as reduced immunity and liver cancer in humans (Chu, 1991, JEFCA, 2001, Swindale, 1989). Due to these risks to human and livestock health, aflatoxin contamination is regularly monitored in peanut (*Arachis hypogaea* L.), corn and cotton (Wilson, 1995). In the United States and many other countries, peanuts with aflatoxin contents lower than 20 ppb are accepted for direct human and livestock consumption, while those with aflatoxin contents with 20 ppb and above are rejected (FAO, 2004, Whitaker *et al.,* 2005).

Several mechanical and chemical methods for the detection, extraction, and quantification of aflatoxin have been developed. These include Fourier transform near-infrared spectroscopy (Tripathi and Mishra, 2009), a fluorometric method (Holbrook *et al*., 2000), high performance liquid chromatography (HPLC) (Manetta *et al.*, 2005), liquid chromatography-tandem mass spectrometry (LC-MS) (Edinboro and Karnes, 2005), and enzyme-linked immunosorbent assay (ELISA) (Li *et al.*, 2009b). These methods are accurate, selective, sensitive, and effective. However, they are usually costly, largely intended for laboratory scientific research, and require special equipment and training (Zhou *et al.*, 2009). In addition, most of these technologies are not accessible to developing countries where aflatoxin is of greater concern (Pitt *et al*., 2012, Waliyar *et al*., 2008). The improvement of standards of living in developing countries demands increased attention to food safety and monitoring of aflatoxin. There is a need in the food and feed sectors of both developed and developing countries to develop rapid aflatoxin testing methods that are low-cost, easy to handle, usable on-site, independent of other instruments, and that can be easily integrated into the production process (Shim *et al*., 2007). Immunochromatographic test strips, also known as lateral flow test strips, have been developed and are now firmly integrated into routine quality-monitoring procedures. These test strips are easily operated following simple manufacturer's procedures, produce immediate results, and do not require expensive instruments (Li, *et al.* 2009a, Zhang *et al*., 2011). In addition, they do not require refrigeration, thus, facilitating use in developing countries.

A list of test strips, including AflaCheck™ (Vicam) and AgraStrip® (Romer Labs) which were used in this study, have been approved by USDA Grain Inspection Packers and Stockyards Administration (GIPSA) for qualitative aflatoxin testing

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(USDA-GIPSA, 2014). These test strips have an expiration date of 1 - 1½ years when kept under the manufacturer-recommended storage temperatures of 15 - 30 C and 2 - 25 C for AflaCheck™ and AgraStrip®, respectively. In many peanut production areas, however, storage within the range of temperatures may not be feasible. Considering these conditions, this study addressed two objectives: (1) To assess the sensitivity and accuracy of immunochromatographic test strips in the qualitative detection of peanut aflatoxin at a 20 ppb cut-off limit as compared to the quantitative fluorometry method; and, (2) To evaluate the effect of continuous high and fluctuating temperatures to the sensitivity and accuracy of the test strips.

Materials and Methods

Performance of the Test Strips in Comparison to the Vicam Fluorometric Method

Sample Collection and Preparation. Peanut samples were collected from different fields during the summer of 2013. Peanut samples were collected from field trials that had been inoculated and others not inoculated with aflatoxigenic fungi in order to acquire a good range of aflatoxin contamination. These samples were subjected to aflatoxin extraction and quantification using the Vicam fluorometry method. Briefly, representative samples (100 g) of shelled peanuts were added with 10 g of NaCl and 200 ml of methanol/water (80:20 v/v), homogenized using a Waring blender at high speed for 1 minute and filtered through Whatman paper. Five ml of the filtrate was diluted with 20 ml HPLC water then re-filtered. Ten ml filtrate was purified with Vicam immunoaffinity columns (Vicam Aflatest, MA) containing aflatoxin-specific (B_1, B_2, G_1 and G_2) monoclonal antibodies and washed with 10 ml HPLC water before the aflatoxin was eluted with 1 ml methanol. The eluted fraction was diluted twice with HPLC water and measured with the Vicam fluorometer (Vicam Series 4EX Fluorometer). All procedures were done according to the manufacturer's instructions. Results of the fluorometer readings showed aflatoxin levels ranging from 1.9 - 1,200 ppb (Table 1).

A total of 108 AflaCheck™ (Vicam, MA) test strips were used to test the aflatoxin level of the same peanut samples quantified using the fluorometer (Table 1). Fifty four test strips were used to test peanut samples with fluorometer readings <20 ppb (1.9 to 19 ppb) and another 54 to test those with ≥20 ppb (20 to 1,200 ppb). The number of test strips used was based on the suggested number of not less than 50 positive and 50 negative samples for qualitative assay studies (CLSI/NCCLS, 2008).

Accuracy, Sensitivity and Specificity Validation. The AflaCheck™ test strip used in this study was designed to qualitatively detect aflatoxin at a cut-off limit of 20 ppb. A positive reaction indicating the detection of aflatoxin level ≥20 ppb is displayed by the production of one visible line (Figure 1). A negative reaction indicating the detection of aflatoxin level <20 ppb is displayed by two visible lines. The absence of any line is indicative of an invalid result. The accuracy, sensitivity and specificity of the test strips to detect aflatoxin levels were evaluated by comparing the observed results to the fluorometer readings. The calculation of accuracy, sensitivity, specificity and Fisher's exact test were completed using the PROC FREQ procedure in SAS ver. 9.3 (SAS Institute, Cary, NC).

Performance of the Immunochromatographic Test Strips when Exposed to Continuous High or Fluctuating Temperatures at Increasing Time Duration

Incubation Setup. Immunochromatographic test strips from two companies, AflaCheck™ (Vicam, MA) and AgraStrip® (Romer Labs, MO), were stored in three temperature regimes: T_0 = room temperature (approximately 25 C); T_1 = high temperature (34 C); and, T_2 = fluctuating at 34 C (8 hours) and room temperature (16 hours) daily. High temperature (34 C) was imposed by warming the test strips inside an incubator. Fluctuating temperatures were imposed by incubating the test strips for 8 hr at 34 C then bringing them out at room temperature for 16 hr overnight until the next day.

Incubation Stability Test. While the test trips were continually incubated under the three temperature regimes, 10 test strips were taken from each treatment at certain time durations. These test strips were used to test the following solutions prepared from calibrated aflatoxin standards: (a) 50 ppb (≥20 ppb), to examine the test strips for positive results; (b) 10 ppb (<20 ppb), to examine the test strips for negative results; and, (c) 0 ppb (distilled water), as blank control.

The test strips were incubated and tested for a maximum duration of 53 wk (~1 year). Any problems such as inability to detect aflatoxin or production of results contrary to what was expected were recorded. The data were analyzed using PROC ANOVA procedure in SAS version 9.3 to compare the performance of AflaCheck™ and AgraStrip®. Means were compared using Fisher LSD at $P \leq 0.05$.

Table 1. Comparison between the fluorometer readings and immunochromatographic test strip reactions in detecting aflatoxin level in peanut samples collected from field trials.

Fluorometer reading (ppb)	Test strip reaction	Fluorometer reading (ppb)	Test strip reaction	Fluorometer reading (ppb)	Test strip reaction	Fluorometer reading (ppb)	Test strip reaction	Fluorometer reading (ppb)	Test strip reaction	Fluorometer reading (ppb)	Test strip reaction
1.9	-	4.5	-	10	-	20	-	67	+	250	+
1.9	-	4.5	-	10	-	20	-	67	+	250	+
1.9	-	4.5	-	10	-	20	-	67	+	250	+
2.2	-	5.1	-	11	-	28	+	89	+	420	+
2.2	-	5.1	-	11	-	28	+	89	+	420	+
2.2	-	5.1	-	11	-	28	+	89	+	420	+
2.6	-	5.8	-	12	-	37	+	110	+	460	+
2.6	-	5.8	-	12	-	37	+	110	+	460	+
2.6	-	5.8	-	12	-	37	+	100	+	460	+
3.6	-	8.0	-	14	-	48	+	120	+	890	+
3.6	-	8.0	-	14	-	48	+	120	+	890	+
3.6	-	8.0	-	14	-	48	+	120	+	890	+
3.7	-	8.8	-	16	-	56	+	220	+	1,100	+
3.7	-	8.8	-	16	-	56	+	220	+	1,100	+
3.7	-	8.8	-	16	-	56	+	220	+	1,100	+
4.2	-	9.3	-	19	-	61	+	240	+	1,200	+
4.2	-	9.3	-	19	-	61	+	240	+	1,200	+
4.2	-	9.3	-	19	-	61	+	240	+	1,200	+

[a]A negative reaction was produced, indicating the detection of aflatoxin level <20 ppb.
[b]A positive reaction was produced, indicating the detection of aflatoxin level ≥20 ppb.

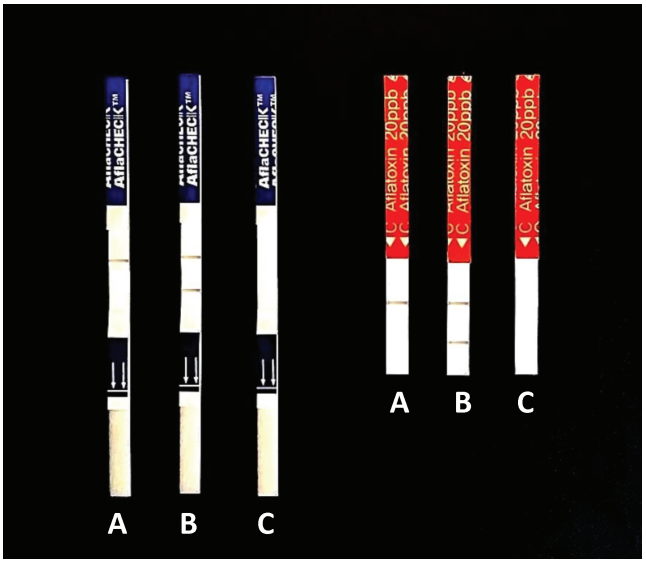

Fig. 1. Detection reactions of the chromatographic test strips (left = AflaCheck™ from Vicam; right = AgraStrip® from Romer Labs). Production of one visible line indicates detection of aflatoxin level ≥20 ppb (A); two visible line indicates detection of aflatoxin level <20 ppb (B); while no line is an invalid result (C).

Results and Discussion

Performance of the Test Strips in Comparison to the Vicam Fluorometry Method

Aflatoxin contamination of peanut is a worldwide concern. Adequate knowledge and several methodologies are currently available to control aflatoxin in food and food products. However, most of these technologies are only readily available in developed countries which have the capability to establish analytical methods to screen for toxins and establish strong regulatory controls. The techniques used in developed countries require sophisticated infrastructure, stable electricity, readily available supplies, and experienced technicians. Most developing countries lack the resources, infrastructure, sustainable supplies, and personnel for efficient regulatory system (Pitt *et al*., 2012, Waliyar *et al*., 2008). The use of relatively affordable simple-to-use materials in these developing countries would be a great advantage.

The aflatoxin content of the peanut samples quantified through the Vicam fluorometry method ranged from 1.9 to 1,200 ppb (Table 1, Figure 2). Samples with aflatoxin contents <20 and ≥20 ppb were separated and tested in triplicates. Results from the assay showed that all samples with aflatoxin contents <20 ppb as read by the fluorometer yielded a negative reaction in the test strips. Similarly, all samples with aflatoxin contents >20 ppb as read by the fluorometer yielded a positive reaction in the test strips. Only three samples with exactly 20 ppb (borderline for negative and positive reaction) yielded negative instead of the expected positive reaction. This gave the test strips an overall accuracy of 97.2%, sensitivity of 94.4% and specificity of 100% ($P<0.0001$, Table 2), indicating that the test strips can be a good option when a fluorometer is not available. Even if the results of the test strips are only qualitative, the negative reaction for aflatoxin detection <20 ppb would be useful in determining that the sampled peanut lot is safe for human consumption. On the other hand, the positive reaction for aflatoxin detection ≥20 ppb will allow for the rejection of the peanut lot for human consumption.

Performance of the Test Strips when Exposed to Continuous High and Fluctuating Temperatures at Increasing Time Duration

The AflaCheck™ (Vicam) test strips correctly detected the aflatoxin levels of the standard solutions calibrated at ≥20, 10 and 0 ppb for up

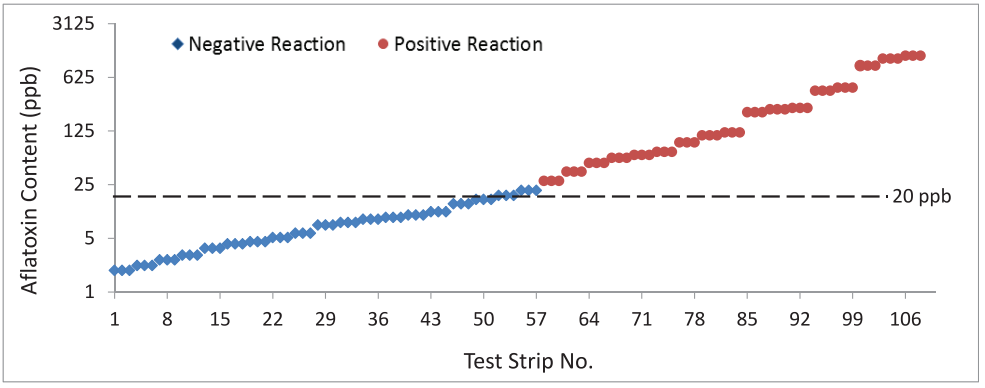

Fig. 2. Results of the immunochromatographic test strip assay. A total of 108 test strips were used (54 each for samples with aflatoxin contents <20 and ≥20 ppb). These were used to test peanut samples containing <20 and ≥20 ppb of aflatoxin as measured using the Vicam fluorometer. Results show that the test strips yielded positive reactions when tested on samples containing <20 ppb while samples with aflatoxin contents >20 ppb had negative reactions. Only three samples containing exactly 20 ppb of aflatoxin content, as quantified by the fluorometer, yielded negative reactions instead of the manufacturer-claimed positive reaction if used with the test strips.

Table 2. Positive predictive value, misclassification rate, negative predictive value, accuracy, sensitivity and specificity of the immunochromatographic test strips in comparison to the fluorometer readings.

	Immunochromatographic test strip results		
Fluorometer readings	≥20 ppb (positive reaction)	<20 ppb (negative reaction)	Total
≥20 ppb	51	3	54
	100[a]	2.8[b]	
<20 ppb	0	54	54
	0[b]	94.7[c]	
Total	51	57	108

Immunochromatographic test strip analysis	
Overall accuracy[d]	97
Overall sensitivity[e]	94
Overall specificity[f]	100
Fisher's exact test	P<0.0001

[a]Positive predictive value = [# of correct positive reactions / (# of correct positive reactions + # of incorrect positive reactions)] x 100

[b]Misclassification rate (MR) = (# of inconsistent reactions / overall # of reactions) x 100

[c]Negative predictive value = [# of correct negative reactions / (# of correct negative reactions + # of incorrect negative reactions)] x 100

[d]Overall accuracy = 100 – MR

[e]Overall sensitivity = [# of correct positive reactions / (# of correct positive reactions + # of incorrect negative reactions)] x 100

[f]Overall specificity = [# of correct negative reactions / (# of correct negative reactions + # of incorrect positive reactions)] x 100

to 32 wks regardless of incubation at room (T_0 = approximately 25 C), continuous high (T_1 = 34 C) or fluctuating (T_2 = 34 C for 8 hours, 25 C for 16 hours daily) temperatures (Table 3, Figure 3). However, starting at wk 35 and 38, invalid results (no bands formed) were obtained from the test strips incubated at continuous high and fluctuating temperatures, respectively. It was also observed that some of these test strips had slower absorbing flow rate of the liquid towards the pad and/or produced blurry pink-dyed pads as the liquid was absorbed. At 47 wk, the test strips incubated under both temperature regimes began to yield results that were contradictory to what were expected. Test strips maintained at room temperature started yielding invalid results in the same week. In comparison, the AgraStrip® (Romer Labs) test strips yielded correct positive and negative results up to 47 wk of incubation under all temperature regimes (Table 4, Figure 3). This indicated a considerably longer shelf life of AgraStrip® as compared to AflaCheck™. The only observed problem occurred at wk 50 when three (1.4% occurrence) invalid results were obtained from test strips incubated at continuous high temperature. Data analysis on the detection reaction of the test strips also showed that AgraStrip® had significantly higher number of test strips producing correct results over the 53 wk of incubation than AflaCheck™ (Table 5).

For both AflaCheck™ and AgraStrip®, room temperature was within the manufacturer-recommended storage temperatures. High temperature was purposely imposed to mimic the usual temperature in a tropical environment. Exposing the test

Table 3. Number of AflaCheck™ (Vicam) test strips that produced correct, incorrect and invalid results after incubation at room, high and fluctuating temperatures over 53 wk.

	Room temperature 25 C			High temperature 34 C			Fluctuating temperatures 34 C (8 hrs), 25 C (16 hrs)		
Week	Correct	Incorrect[a]	Invalid[b]	Correct	Incorrect[a]	Invalid[b]	Correct	Incorrect[a]	Invalid[b]
1-32	150	0	0	150	0	0	150	0	0
35	10	0	0	9	0	1	10	0	0
38	10	0	0	10	0	0	9	0	1
41	10	0	0	8	0	2	9	0	1
44	10	0	0	8	0	2	8	0	2
47	9	0	1	8	2	0	9	1	0
50	9	0	1	9	1	0	9	1	0
53	8	0	2	8	2	0	7	3	0
Total	216	0	4	210	5	5	211	5	4
%	98.18	0.00	1.82	95.45	2.27	2.27	95.91	2.27	1.82

[a]Incorrect indicates a result that is opposite of the expected negative or positive reaction as tested on aflatoxin standards calibrated at ≥20, 10 or 0 (distilled water) ppb.

[b]Invalid indicates no production of any visible line.

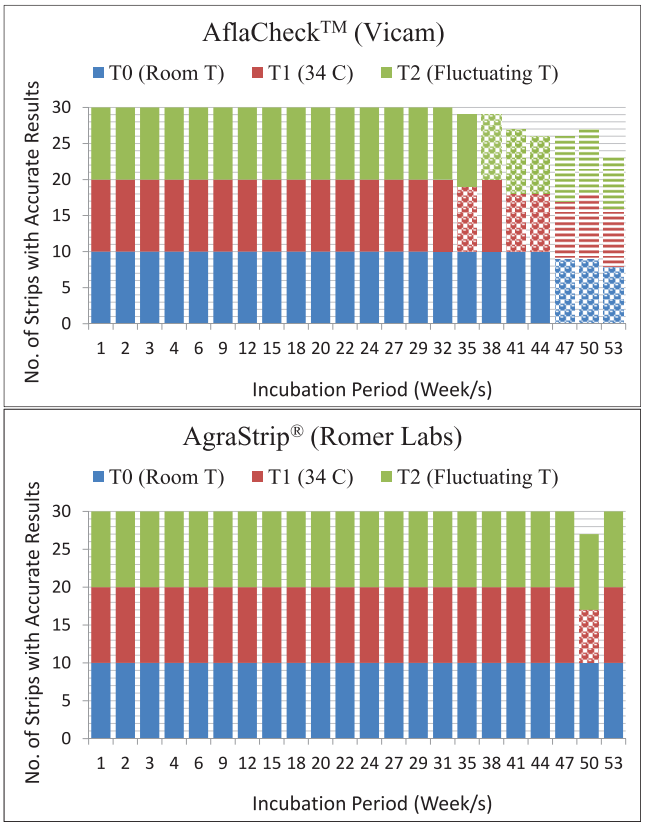

Fig. 3. Reaction of the immunochromatographic test strips as affected by high and fluctuating temperature in increasing incubation duration. A total of 30 test strips were tested per wk (10 for each temperature regime). Each treatment was tested on five solutions calibrated to contain aflatoxin level of 50 ppb, three solutions containing 10 ppb, and two solutions with no aflatoxin (distilled water). Specific problems observed are indicated by dotted (due to invalid result as no line was produced) and horizontal (positive result instead of a negative result and vice versa) lines.

strips to fluctuating temperatures mimicked two natural conditions in the field: fluctuating day and night storage temperatures; or the usage of the test strips in the field during the day then removed from the vehicle at night to be stored at room temperature when the test strips are not in use. Due to the ability of AflaCheck™ and AgraStrip® to remain stable for detecting aflatoxin levels under continuous high or fluctuating temperatures, the use of these test strips in the absence of a fluorometer or other technology for aflatoxin contamination in field locations that exhibit constant high or fluctuating temperatures is appropriate. Given that the test strips were incubated at temperatures beyond the manufacturer's recommendation, the accurate performance of the test strips is economically significant.

For future research, it would be beneficial to test the effect of sudden or short term temperature changes in the performance of the test strips, especially at temperatures that exceed 34 C. This could answer what would happen if the test strips are left in a hot car or under the sun during field testing. It is also suggested to test if additional factors such as humidity would have an effect in the storage of the test strips.

Conclusions

This study assessed the performance of immunochromatographic test strips to detect aflatoxin levels at a 20 ppb cut-off limit. Results revealed a significant association ($P<0.0001$) between the use of the test strips and the fluorometry method, showing that the test strips are highly accurate (97%), sensitive (94%) and specific (100%) in detecting aflatoxin. In addition, incubating the test strips at continuous high (34 C) and fluctuating (34 C for 8 hr, around 25 C for 16 hr daily) temperatures did not alter its efficiency in yielding accurate results for 32 and 47 wk (around 8 and 12 months) for AflaCheck™ and AgraStrip®, respectively. These test strips may, therefore, be used

Table 4. Number of AgraStrip® (Romer Labs) test strips that produced correct, incorrect and invalid results after incubation at room, high and fluctuating temperatures over 53 wk.

	Room temperature 25 C			High temperature 34 C			Fluctuating temperatures 34 C (8 hrs), 25 C (16 hrs)		
Week	Correct	Incorrect[a]	Invalid[b]	Correct	Incorrect[a]	Invalid[b]	Correct	Incorrect[a]	Invalid[b]
1-47	200	0	0	200	0	0	200	0	0
50	10	0	0	7	0	3	10	0	0
53	10	0	0	10	0	0	10	0	0
Total	220	0	0	217	0	3	220	0	0
%	100.00	0.00	0.00	98.64	0	1.36	100	0	0

[a]Incorrect indicates a result that is opposite of the expected negative or positive reaction as tested on aflatoxin standards calibrated at ≥20, 10 or 0 (distilled water) ppb.

[b]Invalid indicates no production of any visible line.

Table 5. Analysis of the number of immunochromatographic test strips that yielded correct detection reactions after incubation at three temperature regimes over 53 wk.

Immunochromatographic test strips	Mean[a] ± SD
AflaCheck™ (Vicam)	28.95 ± 0.64 b[b]
AgraStrip® (Romer Labs)	29.86 ± 1.91 a

[a]Mean was calculated from 22 sampling dates ranging from 1-53 wks of incubation at three temperature regimes. Thirty immunochromatographic test strips were tested per sampling date, 10 for each temperature regime. In each temperature regime, five test strips were tested on aflatoxin solutions calibrated at 50 ppb, three on 10 ppb, and two on distilled water (control, 0 ppb).

Means followed by different letters are significantly different ($P<0.05$) according to Fisher LSD test.

for the qualitative detection of aflatoxin at a 20 ppb cut-off limit in peanut production areas or clinical laboratories that lack specialized equipment like the fluorometer or in tropical locations where refrigeration is not a part of normal storage practice.

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