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Original Research Article

Development and Optimization of Advanced Fluorometric and ELISA Based Protocols for Comprehensive Aflatoxin Profiling in Areca (Betel) Nut

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Abstract

Areca nut, commonly known as betel nut, holds significant cultural importance in Asian societies. However, its aflatoxin associated losses are more common in Pakistan, Nepal, India and Bangladesh. This study aimed to optimize total aflatoxin determination in areca nut using cost effective, accurate and valid techniques. Various strategic modifications were applied during the method optimization. Blending an 80:20 methanol: water mixture with areca nut sample at high speed, followed by dilution with 15% Tween 20, was found to yield reliable and repeatable results. Validation results aligned with the EU Directive 2002/657/EC and AOAC standard validation guidelines, with % RSD < 20-21% and recovery rates between 60 – 110 %. Detection and quantification limits were low, indicating method sensitivity. Methods were found rugged but showed cross reactivity against tannin and phenolic compounds. Measurement uncertainty was ± 5.8µg/kg for fluorometry and ± 1.9 µg/kg for ELISA. Statistical comparison using ANOVA against the HPLC-FLD reference method revealed no significant differences ($p = 0.991$), confirming result consistency and homogeneity of variance (Levene's statistic = 0.009). In conclusion, optimized fluorometry and ELISA methods for aflatoxin analyses in areca nut are cost-effective, reproducible, sensitive, and statistically comparable to the reference method, making them ideal for routine analysis.

Keywords: Aflatoxin, Areca nut, Validation, Fluorometry, ELISA, AOAC.

1. INTRODUCTION

Areca nut (or betel nut), seed of oriental palm's fruit *Areca catechu,* is known to be grown mainly in the South and South East Asia, especially in India, China, Bangladesh, Indonesia, Thailand etc.[1]. It is typically used as a refreshment or antidepressant ingredient [2] particularly in Pakistan [3], where there is an alarming statistics of highest oral cavity cancer incidence in the world [4]. It is not only taken as a raw but in many forms such as supari, pan masala, gutka etc. [5].Several studies claimed its various medicinal benefits [6] like it's analgesic [7], hypolipidemic[8], antiallergic[9] and antibacterial [10] effects. On the other hand, some studies have also shown its harmful effects which includes aggravation of asthmatic condition [11], hypertension [12] and oral injuries leading to cancer in its long term effects [13].

Areca nut is known to be susceptible for aflatoxin infection [14] owing to high moisture content, bad procedures of processing or harvesting and sometimes because of drying their kernels in heaps [15]. Aflatoxin contamination in areca nut is reported by different countries notably its world's biggest producer India [16]. In Pakistan, where mostly it is imported from other South Asian countries i.e. Sri Lanka, India and Nepal, few studies have also been conducted to rule out its aflatoxin pollution [3, 14, 15, 17, 18].

Consumption of food high in aflatoxin is reported to have teratogenic, carcinogenic, or immunosuppressive, acute, sub-acute and chronic effects [19]. In some cases of animals and humans, high level of Aflatoxin contact reported to cause mild toxicity or at times turn out to be deadly [20]. Its major target organ is Liver [21]. Epidemiological and laboratory based studies claimed Aflatoxin as Group I cancer producing and Group 2 A probable human cancer producing toxin [22]. In betel nut users it is also reported to increase risk of

cirrhosis and hepatocellular cancer because of its targeting to liver [23].

The most effective way to protect the food consumers from deleterious effect of Aflatoxin is to abide standards set by different International or National bodies for their maximum levels in different food items. The extreme allowable level for AFB_1 is 1-20 ng/g and total aflatoxin (AFT) is 0-35 ng/g [24]. Food Drug and Administration (FDA) allowable limits for total aflatoxin is 4 to15 ng/g [25].

Validation study has usually been carried out to establish empirical data for determining fitness for use of newly developed analytical procedures which are laboratory established, with minor changes in standard methods or methods use outside of its scope [26].Commonly used tools of validation are Accuracy, Precision (repeatability, within laboratory reproducibility and inter laboratory reproducibility), trueness (recovery), limit of detection (LOD), limit of quantification (LOQ), specificity, robustness and measurement of uncertainty [27, 28].

Fluorometry and ELISA techniques are found to be rapid, efficient and economical for total aflatoxin determination in many food items but there is no any published method available for betel nut via these two techniques. However, recently one study was published about aflatoxin contamination in areca nut by HPLC-FLD technique [17]. This deficiency created a dire need of optimizing and validating method of aflatoxin determination in areca nut through easily available and widely used technique. With the help of which labs having insufficient resources could be able to analyze Aflatoxin easily and protect people in their community from its harmful effects.

Therefore, the main purpose of this research was to optimize and validate method of total aflatoxin determination by widely, inexpensive and easily available techniques i.e., fluorometry and ELISA. Moreover, the results obtained from optimized method have also been compared with HPLC reference method.

2. METHODOLOGY

2.1. Sample collection and preparation

Areca nut sample of about 3 Kg was purchased from Local market of Karachi. Sample was finely sorted into completely clean nuts, nuts with white small web and green fungus. Fungus free areca nuts were crushed into small pieces for sieving through 20-mesh and thoroughly homogenized after grinding. Prepared sample was tested for Total Aflatoxin concentration via HPLC-FLD reference method from accredited laboratory [17] and was utilized for the method optimization and the validation of the newly optimized test methods.

2.2. Extraction and Analysis

Areca nut sample of 25 g was blended with 5g salt NaCl in100mL mixture of methanol and water (80:20) at high speed for 1 minute. Extract was filtered. 5 mL of filtered extract was diluted with 20 mL of 15% Tween 20 solution and mixed thoroughly. Mixture was then again filtered through microfiber filter paper and 4mLof this was passed through IAC at a rate of 1-2 drops/seconds. After 10mL purified water washings (three times) at a rate of about 2 drops/seconds, elution from column with 1.0mL HPLC grade methanol at a rate of 1 -2 drop/second was done. For fluorometry method aflatoxin concentration was measured by adding 1mL developer (1:9) in 1mL elute on calibrated VICAM Fluorometer. For EISA method, *Celer* kit protocol was followed.

2.3. Validation

Validation was done as per international guide lines [28-31]. Parameters applied were Accuracy, Precision (Repeatability / Intra Laboratory Repeatability, Within Laboratory Reproducibility / Intermediate Precision and Inter Laboratory Reproducibility), Limit of detection (LOD)and Limit of quantification (LOQ), Ruggedness, Specificity and Measurement uncertainty [30-32].

2.3.1 Accuracy

Out of six ground areca nut samples half samples were processed without spiking and other half with spiking at10ug/kg. Average of Total Aflatoxin in non-spiked and spiked samples was represented as X_1 and X_2respectively. Recovery was calculated by the formula:

Recovery = $(X_2\text{-}X_1) \times 100 / X_{ADD}$.

2.3.2 Precision

For precision a series of the repeated analyses i.e., repeatability / Intra laboratory repeatability, within laboratory reproducibility and interlaboratory reproducibility were carried out.

2.3.2.1 Repeatability /Intra Laboratory repeatability and Within Laboratory Reproducibility/or Intermediate Precision

Areca nut samples of known concentration were spiked at three different concentrations i.e., 10ug/kg, 20ug/kg and 50 ug/kg. For repeatability data, triplicate analyses were carried out at each level by same analyst using same equipment at different occasions. For within laboratory reproducibility data, same set of analyses were carried out by two different analysts and in different environment. Overall Mean concentration, standard deviation, recovery and the coefficient of variation (CV) / Relative standard deviation (RSD) were calculated for analyses [33, 34].

For ELISA repeatability,10µg/kg and20µg/kg spiked areca nut samples were analyzed in duplicate by the same analyst on three days. For within Laboratory

reproducibility same set of analyses were carried out by two different analysts in different environment. Mean, recovery value, standard deviation, CV/RSD were calculated for both the repeatability and intermediate precision[31].

2.3.2.2 Interlaboratory Reproducibility

Reproducibility was carried out by analyzing the same sample from the different laboratories. The average of the aflatoxin concentration and standard deviation was provided by each laboratory which then later used for the calculation of the RSD.

2.3.3 Limit of Detection and Limit of Quantification (LOD and LOQ)

Standard curves for Fluorometry and ELISA methods were prepared using ground areca nut samples spiked at 0.5 µg/kg, 1 µg/kg, 2 µg/kg, 5µg/kg, 10 µg/kg, 20 µg/kg, 50 µg/kg. Regression analysis was done and LOD/LOQ were calculated using formula: LOQ=10×σ/S and LOD=3×σ/S respectively, where, σ is standard deviation of the Y-intercept and S is mean slope of calibration curve [35].

2.3.4 Ruggedness

For ruggedness, partial (three) factorial design was used (Table 1)[36]:

Table: 1

Experiment	Factor			Result
	A (Percentage of tween)	B (Ratio of methanol: water)	C	
1	15 %	80:20	Filter with one microfiber filter paper	Y_1
2	25 %	80:20	Filter with two microfiber filter paper	Y_2
3	15 %	60:40	Filter with one microfiber filter paper	Y_3
4	25 %	60:40	Filter with two microfiber filter paper	Y_4

Effect for factors was calculated by formula:

Effect A (absolute)= (Σ YA+) - (Σ Y A-) / 2

ΣY_{A+} = sum of results Y_i, where factor A has + sign (i.e., $Y_1 + Y_3$; n=2)

ΣY_{A-} = sum of results Y_i, where factor A has - sign (i.e., $Y_2 + Y_4$; n=2)

Effect is significant if it exceeds 2× the standard deviation of the procedure, i.e.,

Or

$$2 \times s_{cc} / \sqrt{2} \quad Effect > 1.4 \times s_{cc}$$

Where s_{cc} is the standard deviation of the within-laboratory reproducibility.

Effect B and C were calculated by same formula.

2.3.5 Specificity

Yogita and Singhal(2013) method was followed with slight modification for extracting maximum tannin and polyphenols from areca nut sample [37]. Extracted tannin and polyphenols were added during the extraction stage of Total Aflatoxin analyses via fluorometry and ELISA. Recovery and cross reactivity were then calculated to check the specificity[38].

2.3.6 Measurement Uncertainty

Different uncertainty sources were identified as per the EUROCAM guide [39]. For type A uncertainty repeatability and reproducibility of the known Aflatoxin areca nut sample was carried out whereas the external uncertainty sources like uncertainty from equipment calibration certificate, reference material, glassware and standards were used for the calculation of Type B uncertainty. All the type A and B uncertainties were combined to calculate combined uncertainty. Combined uncertainty was then multiplied by a factor 2 at 95 % confidence internal for expanded uncertainty[40].

2.3.7 Statistical Analysis

Statistical analyses were carried out by ANOVA using IBM STATISTICS SPSS software 2.

3. RESULTS AND DISCUSSION

The aim of this study was to optimize areca nut total aflatoxin determination method via fluorometry and ELISA technique. To the best of our knowledge there was no any published, validated method of Total Aflatoxin quantification in areca nut via affordable techniques such as ELISA and Fluorometry. Although recently, there is a report of HPLC-FLD method [17], but it is still comparatively an expensive technique [41, 42].

Areca nut usually gives false positive results in fluorometry and ELISA. For tackling such interferences, tween 20 is used with specific concentration in extraction methods. This strategy was found to decrease false positive results considerably in both techniques. Tween 20 is a detergent (surfactant) that is extensively used in immunohistochemistry buffers and reagents. Its goal in automated and manual techniques is to reduce background staining and improve reagent distribution. It can also be utilized on a staining system that is automated [43].

For optimization, different extraction methods (Table 2) were tried, method A was chosen as per their resulted accuracy in comparison to the standard reference HPLC method. Table3 showed the results obtained from all the methods applied.

Table 2: List of different extraction methods used during optimization

Methods	
A	15 % Tween 20 with 80:20 methanol: water and filter through one microfiber filter paper for the final dilution.
B	25 % Tween 20 with 80:20 methanol: water and filter through layer of two microfiber filter paper for the final dilution.
C	15 % Tween 20 with 60:40 methanol: water and filter through one microfiber filter paper for the final dilution.
D	25 % Tween 20 with 60:40 methanol: water and filter through two microfiber filter paper for the final dilution.
E	15 % Tween 80 with 80:20 methanol: water and filter through one microfiber filter paper for the final dilution.

Table 3: Comparison of various extraction methods:

Method	Total Aflatoxin concentration by Fluorometry µg/kg	Total Aflatoxin concentration by ELISA µg/kg
A	1.4	1.4
B	4.6	1.6
C	6.2	1.0
D	11	2.0
E	0	0.19

The optimized method was then validated as per different standards and published guidelines. Validation of both methods was done using the following performance parameters accuracy, precision (repeatability / intra laboratory repeatability, within laboratory reproducibility and inter laboratory reproducibility), limit of detection and limit of quantification (LOD and LOQ), ruggedness, specificity and measurement uncertainty [31-35, 44].

For accuracy / trueness certified reference material (CRM) is mandatory. CRM and blank sample of areca nut were not available. Preparation of completely blank sample was not possible because of improper transport and storage condition. Consequently, 0 µg/kg Total Aflatoxin areca nut was not possible for blank spiking recovery method. Best possible clean nuts were purchased. Sorting was carried out and four different types of areca nuts were separated i) clean areca nuts ii) Areca nuts with central white web, iii) areca nuts with central green web iv) areca nuts with central yellowish white soft region. For identification of the higher aflatoxigenic areca nuts, all samples were separately tested. Results obtained were as follows:0 µg/kg for cleaned areca nut, 0µg/kg for central white web, 2 µg/kg for yellowish white central portion areca nuts and higher aflatoxin in areca nut with green central web [17]Figure 1.

Figure: 1 a.yellow centered; b. central green webbed; c. cleaned;d. with central white web

3.1 Validation of Total Aflatoxin Determination Method in Areca nut by Fluorometry and ELISA

3.1.1 Accuracy

Accuracy of the methods was determined using areca nut sample of known (low level) aflatoxin concentration, confirmed by HPLC reference method [17]. The results obtained from fluorometry and ELISA showed the accuracy of 102 and 99.11 % respectively at 10 µg/kg spiking level. Acceptance criteria for aflatoxin recovery as per EC Regulation is 70-110%, 50-120% and 80 – 110 % in the concentrations range of 1-10 µg/kg, less than 1 µg/kg and more than 10 µg/kg respectively [45].AOAC Standard manual [32]also specified the acceptance criteria for the percentage of recoveries at different concentration levels as mentioned in Table 4.

Table 4: AOAC standard acceptance criteria for recoveries at different concentration levels

Active content	Analytical relationship	Unit	Mid recovery (%)
0.001	10^{-5}	10 ppm	80-110 %
0.0001	10^{-6}	1 ppm	80-110 %
0.00001	10^{-7}	100 µg/kg	80-110 %
0.000001	10^{-8}	10 µg/kg	60-115 %
0.0000001	10^{-9}	1 µg/kg	40 -120 %

This showed that results were in agreement with both European commission and AOAC standard validation guidelines specified limits[32, 45].

3.1.2 Precision (Repeatability / Intra laboratory repeatability, Within Laboratory Reproducibility and Inter laboratory Reproducibility):

For computing precision various repeatability / intra laboratory repeatability, within laboratory reproducibility and interlaboratory reproducibility experiments were carried out. Acceptance criteria for precision's coefficient of variance (CV) / Relative Standard Deviation (RSD) stated by the European Union (EU) directives is smaller than 20 % [33] and as per AOAC Standard manual [32]at different concentration levels should be as in Table 5.

Table 5: AOAC standard acceptance criteria for the precision at different concentration levels

Active content	Analytical Relationship	Unit	CV (RSD %)
0.001	10^{-5}	10 ppm	7.3
0.0001	10^{-6}	1 ppm	11
0.00001	10^{-7}	100 µg/kg	15
0.000001	10^{-8}	10 µg/kg	21
0.0000001	10^{-9}	1 µg/kg	30

*Coefficient of variance (Relative standard deviation)

In case of Fluorometry method, percent RSD and recovery attained for repeatability at 10, 20 and 50 µg/kg were 18.42 and 99.1 %, 14.3and 113 %,10.6 and 102.8 % respectively. Average intra laboratory repeatability was found 14.4 %. Percent RSD and recovery for within laboratory reproducibility at 10, 20 and 50 µg/kg were found17.5 and 99.11 %, 9.7and 99.7 %, 8.5 and 103.2 % respectively. Average intra or within laboratory reproducibility was found 10.6 %.

In case of ELISA method, percent RSD and recovery attained for repeatability at 10and 20 µg/kg were 18.07 and 102.02 %, 14.9 and 102.15 % respectively. Average intra laboratory repeatability was found 16.4 %. Within laboratory reproducibility percent RSD and recovery at 10and 20 µg/kg were observed 19.7 and 109.8 %, 18.3 and 111.1 % respectively. Average within laboratory reproducibility / intermediate precision for ELISA method was found 18.85%. For both methods interlaboratory reproducibility as averaged percent RSD was found 4.12 %.

Therefore, it was observed that all the values of RSD and recoveries for both methods were found in good compliance with the EU directives i.e. RSDs were found less than20 %[46]and recoveries were found between 70-110% (at 1-10 µg/kg Aflatoxin concentration) and 80 – 110 % (for more than 10 µg/kg concentration)[45].These results were also found in agreement with AOAC standard validation guideline specified limits i.e. RSD was found less than 21 % and recoveries at 10and≥ 20µg/kg concentration were between 60 -115 %[32].

3.1.3 LOD /LOQ of fluorometry and ELISA method:

Standard curve method ascertained the adherence to the Beer's law for increasing total aflatoxin spiked concentration with the observed concentration by regression coefficient (R^2) of 0.9978 and 0.9993 for Fluorometry and ELISA, respectively (Figure2and3). The LOD and LOQ for Fluorometry and ELISA methods were found 3.8and 11.5µg/kg (Table 6) and 3.3and 10.12µg/kg (Table 7) respectively. Low values of LOD and LOQ showed the adequate sensitivity of the methods. LOD/LOQ of total aflatoxin determination

method in areca nut were found in agreement with the values of method published in study for total aflatoxin in other food stuff by the same technique[47]. In case of ELISA results were found in agreement with ELISA Kit LOD / LOQ [32].

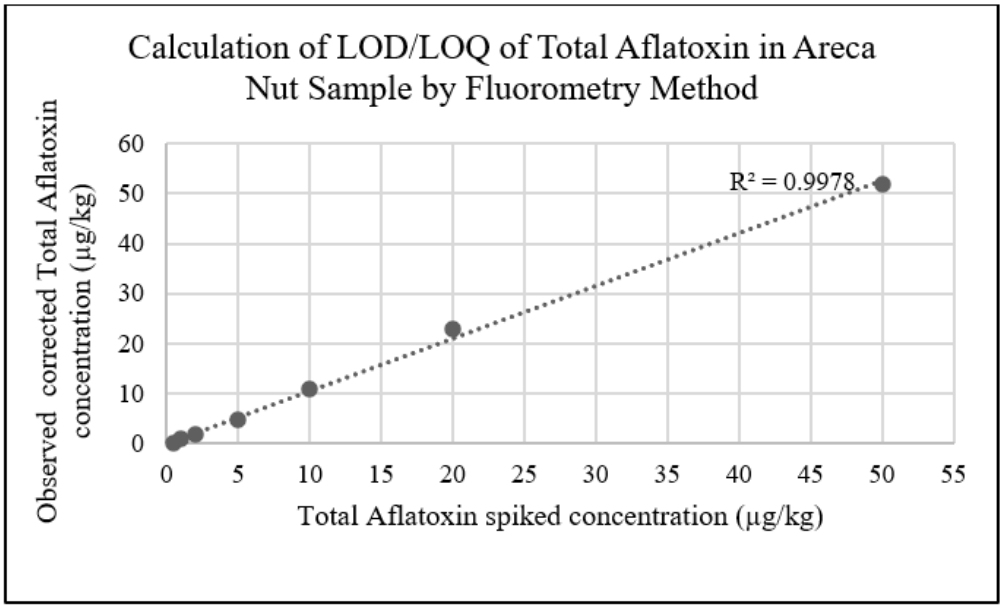

Figure: 2 Standard curve for LOQ/LOD of Fluorometry method

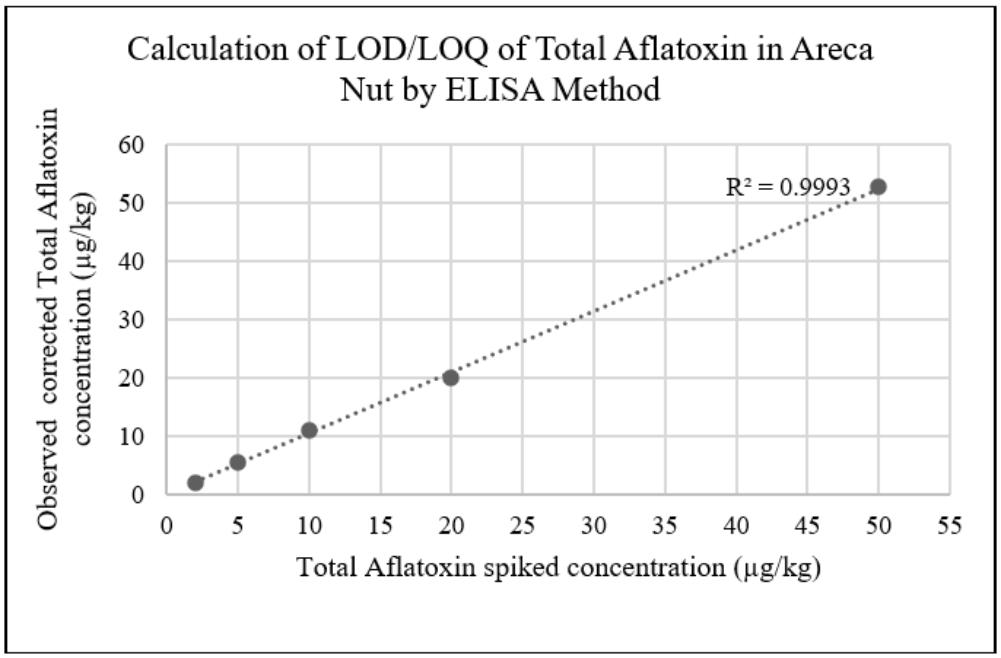

Figure: 3 Standard curve for LOQ/LOD of ELISA method method

Table 6: Calculation Table of Fluorometry method

S. No	Parameters	Formulas	Values from excel
1.	SE OF INTERCEPT	Excel Function (Data analysis regression third table	0.4581
2.	SD OF INTERCEPT	SE of Intercept* √N	1.2120
3.	√7	√N	2.646
4.	SLOPE	A	1.0520
5.	LOD	3.3*(SD OF INTERCEPT/SLOPE)	3.80 ppb
6.	LOQ	10*(SD OF INTERCEPT/SLOPE)	11.52 ppb

Table 7: Excel Calculation Table of ELISA method

S. No	Parameters	Formulas	Values from excel
1.	SE OF INTERCEPT	Excel Function (Data analysis regression third table	0.4045
2.	SD OF INTERCEPT	SE of Intercept* √N	1.0702
3.	√7	√N	2.65
4.	SLOPE	A	1.0572
5.	LOD	3.3*(SD OF INTERCEPT/SLOPE)	3.3
6.	LOQ	10*(SD OF INTERCEPT/SLOPE)	10.12

3.1.4 Ruggedness of fluorometry and ELISA optimized method

Data was obtained (Table8) illustrating the robustness of the two optimized methods. Partial factorial design was followed by the analyses of areca nut sample of known concentration under four conditions and utilizing three distinct types of factors as mentioned in Table: 1.

Table 8: Ruggedness data

Experiment	Fluorometry	ELISA
1	Y_1 = 1.4 µg/kg	Y_1= 1.4µg/kg
2	Y_2= 4.6 µg/kg	Y_2 = 1.6µg/kg
3	Y_3 = 6.2 µg/kg	Y_3 =1.0µg/kg
4	Y_4 = 11 µg/kg	Y_4 =2.035µg/kg

Alteration of tween 20 concentration from 15 to 25 %, ratio of the extraction solvent into water from 80:20 to 60:40 and numbers of microfiber filter paper used during filtration appeared to have non- significant effect on the total aflatoxin determination via fluorometry and ELISA method as their calculated effect values were found less than within laboratory reproducibility of the methods.

Therefore, the two methods were found rugged against the changes.

3.1.5 Specificity

Effect of intervening compounds on the determination of Total Aflatoxin in both Fluorometry and ELISA (Table 9) methods were quantified. Tannin and phenolic compounds were reported to effect aflatoxin determination by exhibiting false high positive result through co-extraction[48]. For checking the effect of intervening substances i.e. tannin and phenolic compounds, 80 % acetone extract of areca nut was added that was reported to contain1.73 mg of tannin and 407.47 mg of phenolic compounds per gram of areca nut[37]. Percent recovery and cross reactivity were found 30 % and 70 % respectively in case of Fluorometry, whereas 4.64 % and 95 %in case of ELISA. Recoveries were found significantly outlier as per both European Commission Regulations and AOAC standard validation guidelines specified limits because according to these guidelines' recoveries should be 70-110 % and 60 -114 % respectively at 10 µg/kg spiking level. Therefore, it was established that addition of tannin and phenolic compounds has a significant effect on the total aflatoxin determination via both optimized methods.

Table 9: Data for Specificity test of optimized methods

Number of Test	Fluorometry	ELISA
	(Spiked Total Aflatoxin (10 µg/kg) + added 1 g 80 % acetone extract of areca nut) µg/kg	Spiked Total Aflatoxin (10 µg/kg) + added 1 g 80 % acetone extract of areca nut) µg/kg
1	4.3	2.282
2	4.4	2.36
3	4.5	2.33
Mean concentration (µg/kg)	4.4	2.324
Recovery (%)	30	4.64
Cross Reactivity (%)	70	95.4

3.1.6 Measurement uncertainty of fluorometry and ELISA optimized method of total aflatoxin in areca nut

Measurement uncertainty for total aflatoxin via both methods was calculated as per the measurement uncertainty guidelines (Eurochem). Measurement Uncertainty for fluorometry and ELISA method was found ± 1.9 and ±5.8µg/kg respectively.

Over all higher uncertainty of both the methods showed the homogeneity problem in the preparation of the areca sample[49].

As per the validation parameters results of total aflatoxin determination in areca nut via fluorometry and ELISA techniques, (Table 10) it was found that both optimized methods have been validated as per the

European Commission and AOAC standard validation guidelines and they are fit for use for the intended purpose with a good repeatability, reproducibility, recovery and LOD/LOQ.

Despite a lot of disputes over banning of areca nut in the world, it is still use at different place and is also the part of trading i.e. import and export in different countries. As a lot of people

benefits are associated with its use. Not waiting for the complete ban of its use overall the world we should take prevention of using aflatoxin contaminated kernels which may be the biggest reason of its cancer producing ability along with the harmful synergetic effects of areca nut alkaloids [50]. Preventing consumption of contaminated kernel will only be possible by testing areca nut efficiently and cost eff effective way. Therefore, the validated methods of areca nut will be of great role in ruling out import of highly aflatoxin contaminated areca nut in Pakistan.

Table 10: Summary of Fluorometry and ELISA methods validation results

Validation parameter	Results	
	Fluorometry	**ELISA**
Accuracy	102 %	99.11 %
Intra laboratory repeatability (%)	14.4 %	16.4 %
Within Laboratory reproducibility / Intermediate precision (%)	10.6 %	18.85 %
Interlaboratory Reproducibility (%)	4.122 %	
LOD	3.80 µg/kg	3.3µg/kg
LOQ	11.52µg/kg	10.12 µg/kg
Ruggedness	Rugged	Rugged
Specificity (Cross Reactivity %)	70.5 %	95.4 %
Measurement Uncertainty	±5.85 µg/kg	± 1.906 µg/kg

3.2 Comparison of Total Aflatoxin in areca nut obtained by three different Techniques:

For further confirmation of the validation results, few areca nut samples (n=4) were taken and analyses was performed by both methods and their results were compared with HPLC-FLD reference method in Table 11 [17]. Statistical calculation for the comparison of these results was done by carrying out ANOVA using IBM STATISTICS SPSS software 21 (Table 12**)**. As per the homogeneity of variance table 12 statistics i.e., 0.009 was not found close to its significance i.e., 0.991. Hence, the methods were found non-significantly different or they have equal or homogenous variance. As per the F test f (2, 9) =0.004, p value i.e., 0.996 >0.05 thus it was also confirmed that there is a non-significant difference between the three methods results. Multiple comparison table also showed that p values > 0.05 for every possible comparison between them hence further confirmed that three methods were giving non-significantly different results i.e., they were giving homogenous results for aflatoxin. As per the homogenous subset table all the three methods were found in the same group i.e., they were all giving result homogenously. Therefore, the statistical analysis established that the two newly optimized methods for total aflatoxin determination in areca nut via Fluorometry and ELSIA are statistically similar to HPLC reference method [17].

Table 11: Total Aflatoxin determination in Areca nut via three different techniques

Sample ID	Fluorometry method (µg/kg)	ELISA Method (µg/kg)	HPLC method (µg/kg)
Sample 1	7.8	8.87	8.49
Sample 2	1.4	1.86	1.3
Sample 3	69	73	68.42
Sample 4	21	22	21.2

Table 12:

Oneway

Test of Homogeneity of Variances			
TotalAflatoxin			
Levene Statistic	df1	df_2	Sig.
.009	2	9	.991

ANOVA					
TotalAflatoxin					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.886	2	3.443	.004	.996
Within Groups	8638.988	9	959.888		
Total	8645.873	11			

Post Hoc Tests

Multiple Comparisons						
Dependent Variable: Total Aflatoxin						
Tukey HSD						
(I) coding	(J) coding	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Method A	Method B	-1.63250	21.90762	.997	-62.7987	59.5337
	Method C	-.05250	21.90762	1.000	-61.2187	61.1137
Method B	Method A	1.63250	21.90762	.997	-59.5337	62.7987
	Method C	1.58000	21.90762	.997	-59.5862	62.7462
Method C	Method A	.05250	21.90762	1.000	-61.1137	61.2187
	Method B	-1.58000	21.90762	.997	-62.7462	59.5862

Homogeneous Subsets

Total Aflatoxin		
Tukey HSD[a]		
coding	N	Subset for alpha = 0.05
		1
Method A	4	24.8000
Method C	4	24.8525
Method B	4	26.4325
Sig.		.997

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

The findings of this study confirm that both fluorometric and ELISA-based methods are viable, accurate, and reproducible for detecting total aflatoxins in areca nut. The validation results—recovery rates of 102% (fluorometry) and 99.1% (ELISA), and RSD values within acceptable limits—demonstrate compliance with internationally recognized guidelines [32; 46]. Additionally, the low LOD and LOQ values (3.8/11.5 µg/kg for fluorometry and 3.3/10.1 µg/kg for ELISA) indicate strong sensitivity, making both methods suitable for routine surveillance against maximum permissible aflatoxin levels [25]. These results are particularly relevant in South Asian countries like Pakistan, where areca nut consumption is high and aflatoxin contamination has been reported frequently [3;17]. While HPLC-FLD remains the reference method for aflatoxin quantification, its cost and technical demands limit its accessibility in many resource-constrained settings [41]. Hence, the validated methods presented in this study provide practical, low-cost alternatives with performance characteristics comparable to the reference technique.

However, specificity analyses revealed significant matrix interference due to tannins and polyphenolic compounds naturally present in areca nut. These compounds led to false positive or suppressed readings, particularly in ELISA, which showed 95.4% cross-reactivity and significantly reduced recovery rates. Such interference has also been reported in previous analytical studies and remains a known limitation of immunochemical methods when applied to complex food matrices [37; 48]. Despite this limitation, the results obtained using fluorometry and ELISA were statistically comparable to HPLC-FLD, as shown through ANOVA, indicating no significant differences ($p > 0.05$). Therefore, with further refinement in sample clean-up or matrix-matched calibration, these methods can be confidently applied for aflatoxin monitoring in areca nut products, supporting safer consumption practices and compliance with food safety regulations in importing countries.

4. CONCLUSION

As this study was carried out to optimize methods for the determination of Total Aflatoxin in areca nut via Fluorometry and ELISA technique, therefore it contained a detailed validation experiments by using different performance tools, resulted in the confirmation of reliability of the methods for testing Aflatoxin contamination, as per the European Union and AOAC validation guidelines. An importance of these optimized and validated methods is reflected in its cost effectiveness, validity of results, and its utilization of commonly used techniques, its user friendliness and simplicity. These methods will provide a validated procedure for aflatoxin testing in areca nut in a low budget laboratory where it is difficult to buy expensive and sophisticated equipment for analysis and hence abide by the regulations.

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