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Rapid Non-invasive NIR Method ofQuantification of Aflatoxins in Sifted Maize Flour

Joseph B Skudi, Hudson Nyambaka, Mildred Nawiri, Ruth Wanjau and Daniel Oyoo

Abstract
The study presents a rapid quantification method of aflatoxin in sifted maize flour using diffused reflectance NIR spectroscopy. Quantitative model protocolfor levels of aflatoxin contamination below, within and above the Legal Tolerance Limits of 20ppb were done on partitioned samples sets of calibration, validation and prediction.Confirmatory HPLC applied on each set before subjecting the sample on diffuse NIR measurements. Integrated peak areas of chromatograms representing various concentrations were recorded for each level.Multiplicative Signal Correction, Standard Normale Variate Detrending and Savitsky Golay Filtration were all applied in enhancementof thesignals at the preprocessing stage. On the Calibration set Partial Least Square Regression (PLSR)was done on the spectral data to develop best fit model on contaminated and neat spectral data set. To ensure the data fitted well in the model it was checked by Root Mean Square Error of calibration (RMSEC). The true performance of the model was checked using Prediction sample set and optimization done by application of Root Mean Square Error of Prediction (RMSEP). Overall performance of Quantitative model was evaluated by Root Mean Square Error of Cross Validation (RMSECV). The values of RMSEC, RMSEP and RMSECV were all stable and verylow with their values close to each other for all levels of aflatoxin in sifted maize flour. These results were quite promising andindicating robustness and accuracy of model prediction of aflatoxin concentration in sifted flour. This demonstrated the potential of NIR to be used in the rapid determination of aflatoxin contaminant insifted maize flour

Key words:*Aflatoxins, Chemometric method, Noninvasive, Calibration technique, Validation method, Prediction, Partial Least Square Regression, NIR Spectroscopy, Optimization techniques, Quantitative determination*

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I. Introduction

The paper presents a rapid and non-invasive method of detection and quantification of aflatoxin in sifted maize flour under high production premise. It's based on NIR diffused reflectance spectroscopy, directly applied and unfettered by the long analytical procedures of quantification. This method leveraged on the absorbing chromophoresof perfectly conjugated system of aflatoxin molecules which readily absorbed and scattered the diffused NIR (Naes *et al.,* 2004). The resultant huge spectral data generated comprised of both physical and chemical attributes modeled using various selected chemometric tests to link only relevant aflatoxin absorption signals with the corresponding concentrations from reference confirmatory HPLC (Zhao *et al.,*2016).

The increasing demand for sifted maize flour in Kenya has resulted in over production, thus out pacing current detection and quantification methods of aflatoxin as prime quality determinantfor suitability of flour. As a result of this,there has been tremendous decline in the quality of flour (khamila *et al.,* 2019). Thedegenerate production spreehas often triggered off alarms from various regulatory bodies (KEBS, 2012). The delay in the reaction time due to lack of rapid detection and quantification techniques by the authoritycould hardly save the consumers from such contamination (KNBS, 2017).Even though, current detection and quantification methodssuch as HPLC, TLC, HPTLC and ELISA are sensitive their long analytical procedures of detection and quantification cannot remotely keep up pace with the huge production (Kamruzzaman *et al.,* 2015). Furthermore, these methods are unsustainable due to heavy deployment of toxic and carsinogenic solvents. Nevertheless, these methods have proven to increase the chemical load into the environment thus being in violation of Kyoto protocols of 1997.To hasten detection and quantification under massive production premise,the long analytical procedure delaying detection and quantificationwas linked to NIR as afast means of quantification and detection giving the entire process a high impetusmatching production rates (Li and Zhang, 2016).

Development of Rapid, Accurate and Robust Quantitative Model protocol

The building of accurate and robust quantitative model involved partitioning of flour Samples and subjection on both NIR and confirmatory HPLC (Elzey *et al.,* 2016).The design of calibration model was developed on test sets using Partial Least Square Regression (PLS-R) method as per Wold, 2011. The Optimization and validation process conductedby application of the Root Means Square Error (RMSE) on Calibration, Cross validation and Prediction sets (Naes *et al.,*2002). Overall robust, accurate and reliable model built on the stable,lowest and closevalues from RMSEC, RMSECV and RMSEP (ASTM, 2013).

Signal Enhancement Techniques

To minimize signal interferences due to various effects of scattered light from diffused reflectance, variationsin moisture content in particles, differences in the path length amongst other interferences in the flour were corrected by application of preprocessing methods (Abookasis and Workman, 2012). They ranged from Multiplicative Signal Correction (MSC), Savitzk Golay filtration, Derivative methods and Standard Normale Variate Detrending (SNVD) (Barnes *et al.,* 1989).

Rapid Classification Methods

Generally both supervised and unsupervised methods were integrated and employed in the rapid determination of different samples withvarying concentrations of aflatoxin in sifted maize flour. For unsupervised method, Principle Component Analysis (PCA) was used to establish the structure of the calibration population sample to be assessed (Kamal and Karoui, 2015). This was done by plotting the principle component scores in two and 3 dimensions to give an overview of the general shape of calibration and how the spectra related to each other(Martens and Naes, 1996).

Fig (1.0) and (2.0) shows the PCA of the NIR calibrated data for sifted flour contaminated with aflatoxin and neat sifted flour respectively.

The supervised methods used were Partial Least Square Discriminant Analysis (PLS-DA) and Least Square- Support Vector Machine (LS-SVM). The efficiency and effectiveness of classification accuracy and validation of the two methods were determined from the preprocessed data comprising of both contaminated and uncontaminated flour (Bouabidi *et al.,* 2010).

Optimization Processes

The modeling of spectra on calibration set was done by application of Partial Least Square Regression(PLSR) method and captured all variability likely to be encounter as per work done byWold 2011. The ability of the model to fit the data set well was checked by application of the Root mean Square Error of Calibration (RMSEC) (Liu *et al.,* 2009). As expressed in the equation (1.0)

$$RMSEC = \sqrt{\sum_{i=1}^{n}\left(\left(\left(y_{ipred} - y_i\right)^2\right)/n\right)} \ldots\ldots \quad (1.0)$$

Where y_{ipred} is the predicted concentration of the sample that is included in the model formation, $y_i$represents the sample i concentration of aflatoxin from reference HPLC method and n is the total number of samples in the data set (Mantanus *et al.,* 2009).

The quantitative performance of the model was evaluated by the application of Root Mean Square Error of Cross Validation (RMSECV)as a fastest way to estimate the ability of model to predict samples which were not included in the calibration set (Jerome and Workman, 2018). This was done as in the equation (2.0)

$$RMSECV = \sqrt{\left(\sum_{i=1}^{n}\left(\left(y_{ipred} - y_i\right)^2\right)/n\right)} \ldots\ldots \quad (2.0)$$

Where y_{ipred} is the estimated concentration of the sample by cross validation, where the value of each sample predicted using a model that did not include sample *i* and the rest of the values were same as in the RMSEC.

After fully optimization of the model, it was validated with external validation set, and checked by the Root Mean Square Error of Prediction (RMSEP) as in equation (3.0)

$$RMSEP = \sqrt{\sum_{i=1}^{n}\left(\left(y_{ipred} - y_i\right)^2\right)/n} \ldots\ldots\ldots \quad (3.0)$$

y_{ipred} are samples which were not included in the calibration. This gave a true performance of the model since it was determined by samples which were not in the calibration set (Bouabidi *et al.,* 2010).

Reduced Wave Number Predictive Model

Thereduced absorption wave lengths or wave numbers were developed to predict the concentration as described by equation (4.0)

It was expressed in terms of percentage concentration (Naes *et al.*, 2002).
The multivariate regression equation for calibration was constructed to incooperated the Beer's law in its regression coefficient both Molar absorptivity and path length were encapsulated in the B term as per the equation (4.0) below

$$Y = B_O + B_i(-logR_i)_N + E \quad \ldots\ldots (4.0)$$

Where Y= percentage concentration of absorber, B_O = intercept from regression, B_i =regression coefficient, i=index of the wavelength used and its corresponding reflectance, N =total number of wavelength used in regression and E =random error.

Experimental
Materials and Reagents
The Aflatoxin Standards (AFB_1, AFB_2, AFG_1 and AFG_2) were purchased from ABRAXIS, Laboratories, Domingo Spain under batch number 17-001 and CRM 7220-81-7.
The certified concentration were based on results obtained from the gravimetric preparations of solution and quantity determined by H-qNMR Varian 500MHZ equipment
Concentrations of AFB_1, AFB_2, AFG_1 and AFG_2 were given at 95% confidence interval.
AFB_1 were set at (9.33±0.43)µmol/kg and (2.91±0.13)µg/g, AFB_2 were set at (8.72±0.50)µmol/kg and (2.74±0.15)µg/g, AFG_1 were set at (8.60±0.41)µmol/kg and (2.82±0.13) µg/g. for the AFG_2 were set at (8.150±0.54)µmol/kg and (2.69±0.18)µg/g
These standards were in ampoules form, stored under -20^0C and kept upright in the laboratory inside the original container.
The standards were used for calibrations and validations purposes.

Flour samples
Sample size
The sample size was designed as per Cochran's formula in equation 8.0 below
$n_o = \frac{z^2pq}{e^2}$ …… (8.0)
Where n_o is the number of batches, e is the desired level of precision (margin error), p is the estimated proportion of the population which has the attribute in question and q is 1- p
Z value was obtained from the z table.
From the previous study in regard to aflatoxin contamination in the flour, it was established that 60% of the flour sold around the country had levels of aflatoxin above the legal contaminant limits (KEBS,2021). Therefore, estimated proportion with the attribute in question was 60 % such that the p=0.6 at 95% confidence limits with a precision of $\pm$ 5% and e =0.05 with z value at 1.96
The total number of batches was computed as below
$(total\ number\ of\ batches) n_o = \frac{(0.4)(0.6)(1.966)^2}{(0.05)^2}$= 368.79
Therefore, 368 batches were sufficient for this study. 400 to 450 samples from 360 batches were used in this study. The samples were purchased from licensed formal super markets and convenient stores with dealerships from registered millers within the premises of Nairobi, Kenya.

Sample preparation methods
Extraction of aflatoxin from the flour samples
25g of maize flour samples were Weighed to the nearest 0.1g and poured into the blender. 5g of sodium chloride and 125ml of extraction solvent were added. The extraction solvent constituted of 7 parts per volume of methanol with 3 parts per volume of ISO grade 1water (3696-1987) were added then homogenized with the mixer for 2min at high speed. The blending time and speed were carefully controlled to minimize negative influence on the extraction efficiency. The mixture were filtered through fluted filter paper and recorded as v_1.
15ml were pipetted from v_1 and labeled as (v_2) then poured into a conical flask of 250ml with glass stopper. 30ml of water was added in v_2, then flask stoppered tightly and mixture shaken gently to mix well. Before starting HPLC the mixture was filtered then diluted, extracted through a glass microfiber paper (v_3). The filtrate (v_3) was clear and ready for qualitative determinations on HPLC. This was done to establish the initial concentration of aflatoxin in the flour prior to contaminating it to different concentrations.

Sample contamination matrices
The flour samples with nil concentration of B_1 or neat samples were mixed with flour sample with high concentration of B_2. The same procedures and processes were replicated for flour sample with nil concentration

of B_2 with high concentration of B_1.The mixing and blending to attain uniform distribution was done as per Nestle and Nalubola procedures.
These procedures and processes were adopted for flour samples with G_1 and G_2 contaminations with neat or nil concentrations in an alternatively manner.
Finally the samples mixtures were split into two equal portions. One of each portions subjected on HPLC for quantification and validation of the contaminant. The remaining portions were then subjected on NIR diffuse reflectance for spectra generation.

NIR Method of spectral acquisition.
5g of each neat and contaminated samples of sifted maize flour were weighed accurately on multi-purpose analyzer spectrometer equipped with an integrated sphere and InGaAs detector (Bruker-optics, Germany)
Spectra were obtained in the range of 12500 cm^{-1} and 4000cm^{-1}. An average of 32 scans was made with a spectra resolution of 4cm^{-1} and the repetition of 3 times. The average spectra were then recorded.

Calibration samples
150 samples constituting of both neat and contaminated flour were selected randomly and used as a calibration set. The calibration technique involved PLSR and Principle Component Analysis (PCA).
Cross Validation samples
100 samples were picked randomly from both contaminated flour and neat. They were used in the evaluation of quantitative performance of calibration model.
Prediction samples
150 samples were selected randomly from both neat and contaminated;they were used in the evaluationof robustness and accuracy of the prediction model as per Mantanus *et al.,* 2009 procedure.

II. Resultsand Discussion.

HPLC Performance on Quantification

The quantitative determination was performed by the external standard method involvingintegration of peak area. This was then related to the four types of aflatoxins and the precision of the concentration deduced as in table 1.0 below. The precision data was described in terms of the Relative Standard Deviation (RSD), Repeatability Standard Deviation, Repeatability Coefficient of Variation, Repeatability Limits, Reproducibility Standard Deviation, Reproducibility Coefficient of Variation and Reproducibility limits

Table 1.0
Precision data for contaminated sifted maize flour

Parameter	Aflatoxin			
	B_1	B_2	G_1	G_2
Number of accepted results	20	20	18	18
Mean value (µg/kg)	14.88	17.23	22.36	24.49
Repeatability standard deviation	0.68	0.35	0.68	0.20
Repeatability coefficient of variation%	5.80	20.00	9.50	19.00
Repeatability limits(µg/kg)	2.40	0.90	1.90	0.56
Reproducibility standard deviation(µg/kg)	1.56	0.41	0.68	0.53
Reproducibility coefficient of variation %	10	25	10	49
Reproducibility limits(µg/kg)	4.20	1.15	1.50	1.48

Multivariate Data Analysis
Calibration and Signal Preprocessing Methods

The Partial Least Square Regression, First Derivative (order 2, window 15 points corresponding 57.5cm-1) Standard Normal Variate (SNV) and Multiplicative signal correction were carried out with PLS tool box 5.0 Matlab. The predictive model was performed by PLS on calibration model as seen in the tables below.

Random subset cross validation was performed to validate the model, the number of data splits were selected against N (total number of samples and r the number of iteratives). The different test sets were determined through random selection of N/S objects in the data set. This was repeated r-times. The model ability to predict aflatoxins was further tested with external validation set

The external validation of new batches from manufacturers who were not included in the studywas used in this process. In order to demonstrate that four aflatoxin B_1, B_2, G_1 and G_2 concentration levels were sufficient to build a robust calibration and fully validated the model, all four toxin of concentration above and

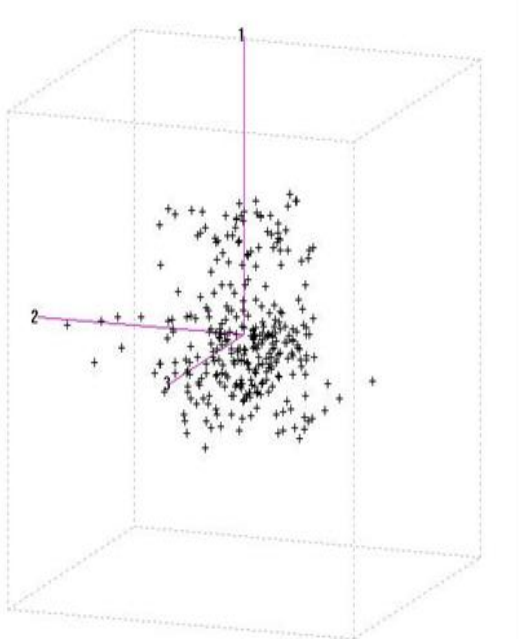
below legal limits were integrated in the external validation. Validation results were recorded as in the tables below for all the toxins.

Principle component Analysis

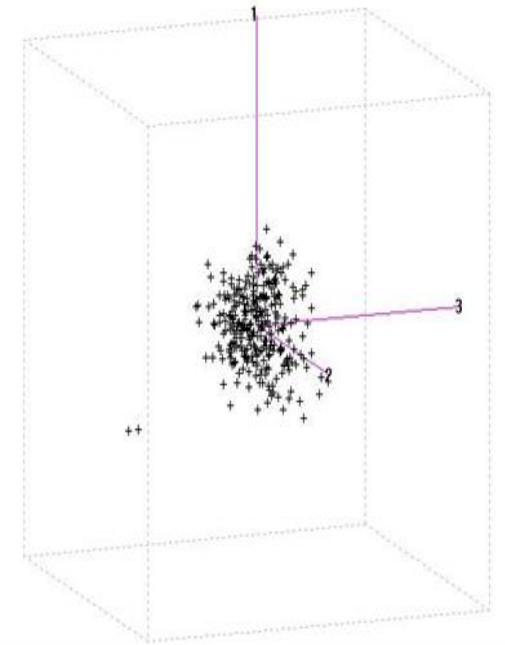
The selection of the number of PCA after centering and removal of all off set from the data sets of both contaminated and neat flour gave a steady value. The entire process of determination of optimal PCA number relied on the PRESS, Predicted Residues Sum of Squares through Cross Validation method. The values were tabulated for various concentrations from table 2.0 to 4.0 tables below.

Fig (1.0) and (2.0) shows the PCA of the NIR calibrated data for sifted flour contaminated with aflatoxin and neat sifted flour respectively.

Fig (1.0) sifted flour contaminated with aflatoxin



(2.0) Neat sifted flour



Performance on NIR calibration developed using PLS on sifted aflatoxin contaminated flour of concentration between 20ppb and 50ppb

Table 2.0

Sample	Quality Parameters	N	PCS	Preprocessing Technique	R^2	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB_1**	400	8.0	SNVD,MSC,1st and 2nd Derivatve	> 0.99	0.005	0.001	0.0005
Sifted maize flour	**AFB2**	400	8.0	SNVD,MSC,1st and 2nd derivatives	> 0.99	0.0041	0.021	0.0077
sifted maize flour	**AFG_1**	400	6.0	1st , 2nd Derivative, MSC	> 0.99	0.001	0.011	0.0058
Sifted maize flour	**AFG_2**	400	6.0	1st ,2nd Derivative, MSC	> 0.99	0.0019	0.001	0.0006

From table 2.0, the overall lowervalues in the RMSECV forall aflatoxin in sifted flour sample indicated a robust and accurate calibration model. The RMSEP was low and close to RMSECV, demonstrating excellent performance and reliability of the model in the future predictions of levels above the Legal Tolerance Limits.

Table 3.0:Performance on NIR calibration developed using PLS on sifted aflatoxin contaminated flour of concentration between 50ppb to 100ppb

Sample	Quality Parameter	N	PCS	Preprocessing Technique	R^2	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB_1**	400	8.0	SNVD,MSC,1st and 2nd Derivatve	> 0.99	0.005	0.001	0.0020
Sifted maize flour	**AFB2**	400	8.0	SNVD,MSC,1st and 2nd derivatives	> 0.99	0.0041	0.021	0.0400
sifted maize flour	**AFG_1**	400	6.0	1st , 2nd Derivative, MSC	> 0.99	0.001	0.011	0.0210
Sifted maize flour	**AFG_2**	400	6.0	1st ,2nd Derivative, MSC	> 0.99	0.0019	0.001	0.0022

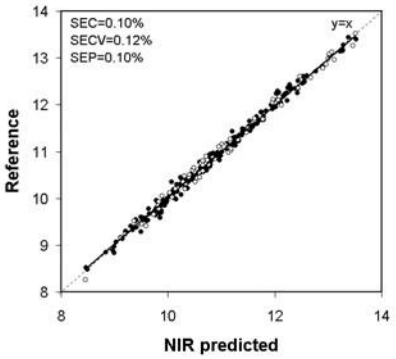
Fromtable 3.0, the acute contamination levels of aflatoxin in sifted maize flour with Principle component of 8 and 6 with a perfect correlation coefficient of above 0.99 almost 1 and very low values of RMSECV which were real close to RMSEC and RMSEP, demonstrated a perfect calibrated model with excellent model performance. The model was robust and accurate and could be used on prediction of acute levels of contamination for many different types of sifted flour.

Table 4.0: Performance on NIR calibration developed using PLS on sifted aflatoxin contaminated flour of concentration between 100ppb to 200ppb

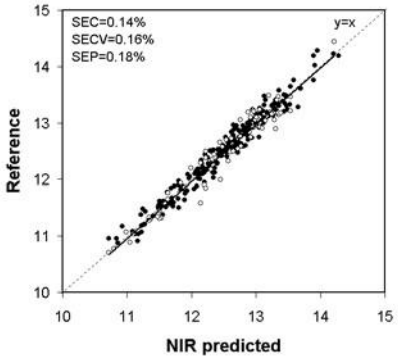
Sample	Quality Parameter	N	PCS	Preprocessing Technique	R^2	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB_1**	400	8.0	SNVD,2MSC,1st and 2nd Derivatve	> 0.99	0.005	0.001	0.0021
Sifted maize flour	**AFB2**	400	8.0	SNVD,2MSC,1st and 2nd derivatives	> 0.99	0.0041	0.021	0.0110
sifted maize flour	**AFG_1**	400	6.0	1st , 2nd Derivative, 2MSC	> 0.99	0.001	0.011	0.0320
Sifted maize flour	**AFG_2**	400	6.0	1st ,2nd Derivative, 2MSC	> 0.99	0.0019	0.001	0.0021

From table 4.0 the extreme levels of contamination way above the legal tolerance limit,with MultiplicativeSignal Correction (MSC) done twice and low values of RMSEP and close to the RMSEC suggested a robust and accurate model ability to predict high levels beyond the tolerance limit accurately.

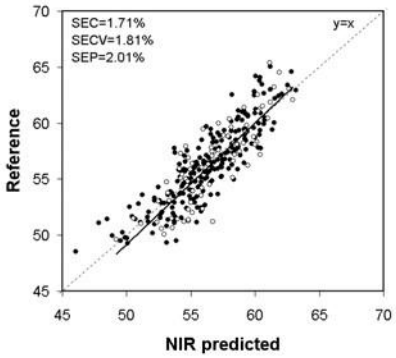
Calibration Performance of Aflatoxin Level in Sifted Maize Flour below Legal Tolerance Limits



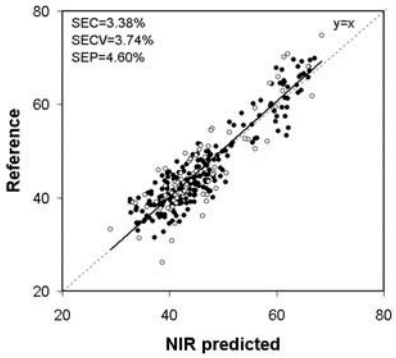
Sample contaminated with B_1



Samples contaminated with B_2

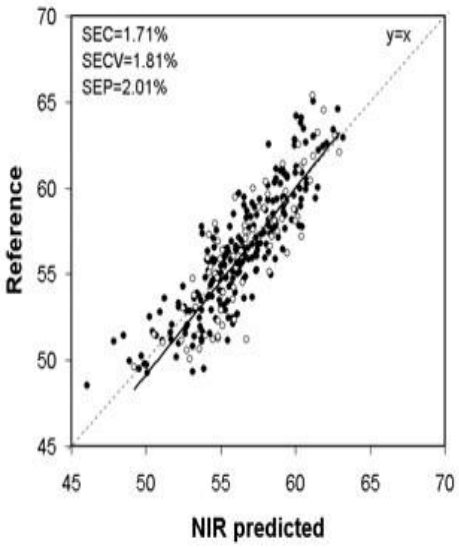


Samples contaminated with G_1

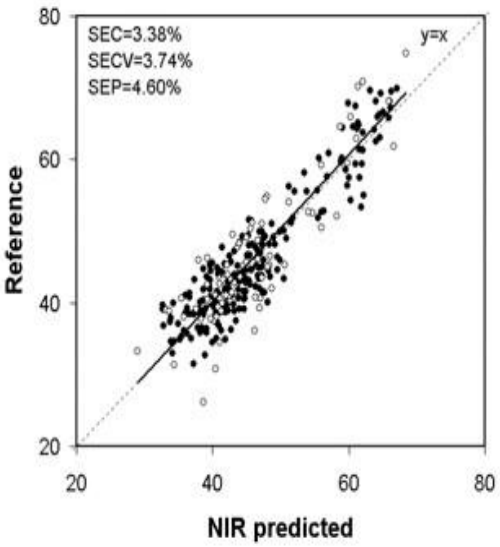


Samples contaminated with G_2

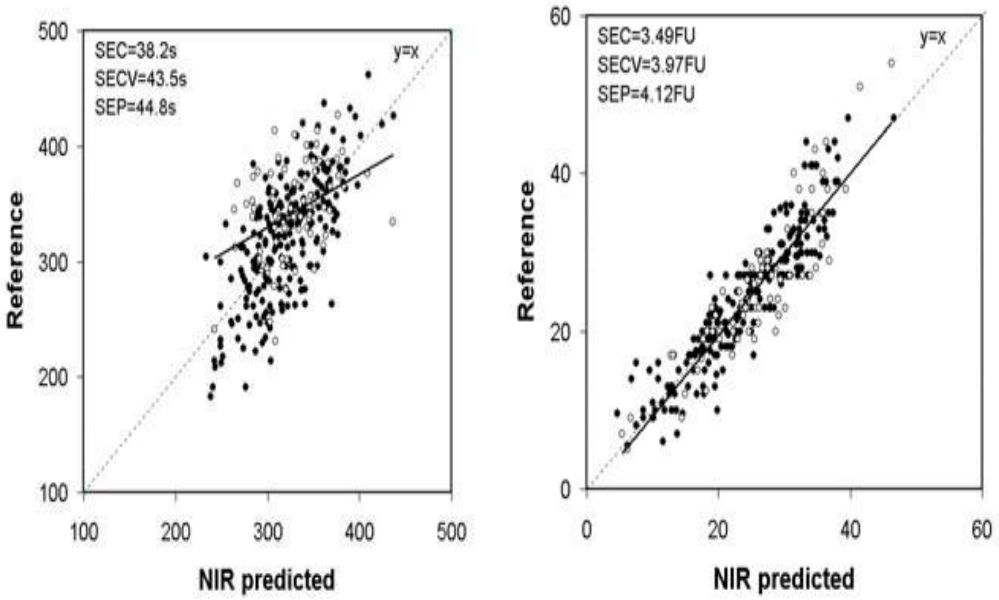
Calibration Performance for Level of Aflatoxin in Sifted Maize Flour above the Legal Tolerance Limits



Samples contaminated with B_1



Samples contaminated with B_2



Sample contaminated with G_1 **Sample contaminated with G_2**

III. Conclusion

A robust and accurate NIR model was able to quantify levels of aflatoxin in sifted flour below, within and above the legal tolerance limits. The model was successfully validated for aflatoxin content ranging from 20ppb to above 100ppb using external validation sets. The RMSEP values of model suggested the overall model accuracy.

Last but not least, the developed method may be used to monitor the levels of aflatoxin through the milling process of sifted maize flour. This may eventually reduce milling flour with aflatoxin above the legal tolerance limits.

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