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Real Time NIR Applications in Qualitative Discrimination and Authentication of Aflatoxin $B_1$and B_2, G_1 and G_2 in Sifted Maize Flour

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Abstract
The study presents a real time method based on non-invasive technique of NIR diffuse reflectance spectroscopy in qualitative and authentications of aflatoxins in sifted maize flour, amid claims of high levels of aflatoxin contamination in maize flour in Kenya. The differentiations of AFB_1 with AFB_2 and AFG_1 with AFG_2 were done on generated spectra using chemometric techniques. Aflatoxins were extracted from each flour sample and authenticated using HPLC on the basis of retention time (R_t). The remaining part of flour analyzed on NIR spectrometer equipped with InGaAs detector. Samples were partitioned randomly into calibration, validation and prediction. Calibration data was partitioned as per Kennard –Stone algorithm. An average of 32 scans were made on each sample and recorded. Multiplicative Scatter Correction, Savitzky –Golay, Standard Normale Variate Detrending and the derivatives methods were used for signal preprocessing. Spectral variable selection for calibration and validation was done using Successive Projection Algorithm coupled on Partial Least Square Discriminant Analysis. Least Square-Support Vector Machine and Partial Least Square - Discriminant Analysis were used for Classification, Validation and prediction. The results showed classification ability with an accuracy of 92.00% for the validation and 95.00% for the prediction of aflatoxins in flour in real time by LS-SVM were superior to P L S- DA. These results were quite promising and demonstrated the potential of NIR spectroscopy for qualitative discrimination and authentication of Aflatoxins in sifted maize flour in real time.

Key words*:*
Aflatoxins, NIR spectroscopy, Least Square- Support Vector Machine, Partial Least Square -Discriminant Analysis, Chemometrics, Qualitative Discrimination

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I. Introduction

The aflatoxins contaminant in sifted maize flour and other cereals is a perennial problem in Kenya and other developing countries. The analysis of such contaminant has always been reported in terms of total aflatoxin concentration and furthermore, not in real time. This kind of report has always elicited some concerns amongst millers and consumers considering some toxins being carsinogenic. Nonetheless, such reports are devoid of qualitative aspects which could be of great value on evaluation of toxicity levels impacting the most on consumer's health. In consideration of variations in toxicity, it is imperative to report each single individual toxin and its level in the matrix in real time. The need for real time, affordable, non-invasive and discriminating techniques for analysis is a pressing matter deserving National priority since maize flour is a staple food for many communities living in Kenya (Kang'ethe, 2011). This study therefore, presents real time post milling methods which could be used for discriminating each toxin in the matrix such that, each toxin could be reported as per its presence in real time using their absorption bands within the NIR region (Ge *et al.,* 2016).

The qualitative analysis of NIR spectra involved rapid pattern recognition of significant overtones and combination bands of aflatoxins using appropriate chemometric tools to describe true identity of each toxin in a sample (Cheng *et al.,* 2016).

Other methods like HPLC and ELISA have been used before in analysis of aflatoxin but they have long analytical procedures which escalates analytical cost. Such methods with prohibitive analytical costs are easily compromised with no real time results.

Real Time Discrimination Processes

Optimization techniques were applied on series of analytical signals of aflatoxins and generated spectral library (Campos *et al.,* 2 014). The process ensured all sources of variables were included (Wang *et al.,*

2016). Prediction accuracy and robustness were evaluated using Standard Error of Prediction (SEP) and Standard Error of Calibration. These were done on the basis of mathematical and statistical distance measurement such as Mahalanobis spaces, Euclidean distances and Manhattan for similarities tests (Burns and Ciurczak, 2002).

The applications of co-relation co-efficiencies were equally used in an integrated manner to test the viability and applicability of NIR method in determination of the parameters for similarities as per equation 1.0 (Hubert *et al.,* 2007). The equation (1.0) was used in determination of co-relation coefficients using absorbance spectra for various sample of sifted maize flour.

$$\boldsymbol{r = \frac{\sum_{i=1}^{N}(y_i-\bar{y})(X_i-\bar{x})}{\sqrt{\sum_{i=1}^{N}(y_i-\bar{y})^2 \sum_{i=1}^{N}(X_i-\bar{x})^2}}} \textbf{..... (1.0)}$$

Either **r** or **R** is applicable, for simple linear regression **r** is applicable but for multi linear regression **R** is applicable.

$\boldsymbol{x_{i=}}$ Values predicted by NIR for sample i

$\boldsymbol{y_i}$= Reference value for the sample i

x=mean of the predicted values

$\bar{\mathbf{y}}$= mean of the reference value

N= number of samples

Signal enhancement processes

The pretreatments techniques used in this study included derivatives methods of 1st, 2nd and 3rd, Standard Normale Variates Detrending (SNVD) and Multiplicative Scatter Correction (MSC). They were used in minimization of particle size effect due to variation in path length and the scattering effects respectively (Hubert *et al.,* 2007).

Once the entire spectral data was fully processed and all interferences removed, calibration models were developed.

The calibration equation (2.0) was used to develop the models for various aflatoxin contaminants in the flour (Wu *et al.,* 2016). The calibration equation related NIR response of instrument to sample composition.

$$\boldsymbol{y = a + bx} \textbf{..... (2.0)}$$

Where **y** are predicted values

a is the intercept

b is multiplicative scatter constant and **x** is the response used for prediction.

Due to the nature of the flour matrix,

Multiple Linear Regression methods were widely used for calibration as per the equation (3.0) below

$$\boldsymbol{y = a + b_1x_1 + b_2x_2 \ldots\ldots b_nx_n} \textbf{..... (3.0)}$$

Where **y** is the predicted value, **a** is the intercept constant and $\boldsymbol{b_1}$ *to* $\boldsymbol{b_n}$ are the multiplicative constants,$\boldsymbol{x_1}$ *to* $\boldsymbol{x_n}$ are the response used for prediction.

A regression coefficient **R** was used to determine the relationship of the references method and NIR methods.

Rapid Classification Methods

Generally both supervised and unsupervised methods were integrated and employed in the rapid determination of different classes and categories 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 as seen in figure 1.0 and 2.0 (Martens and Naes, 1996).

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.

Experimentals

Materials and Reagents

Standards

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 concentration were as (9.33±0.43)µmol/kg and (2.91±0.13)µg/g, AFB_2 concentrations were (8.72±0.50)µmol/kg and (2.74±0.15)µg/g, AFG_1 concentration were (8.60±0.41)µmol/kg and (2.82±0.13) µg/g. for the AFG_2 were (8.150±0.54)µmol/kg and (2.69±0.18)µg/g
These standards were in ampoules form, stored under -20 ^{0}C and kept upright in the laboratory inside the original container.
The standards were used for calibrations and validations purposes.

Reagents and Apparatus
Water, (grade 1 of ISO 3696)
Sodium chloride,
Iodine or Pyridinium hydrobromide Perbromide (PBPB)
Aflatoxins, (crystals and film ampoule)
Acetonitrile, HPLC grade
Methanol, analytical grade
Methanol, HPLC-grade
Toluene, analytical grade
Sulphuric acid
Immunoaffinity (IA) column
Flute paper filter, 25 cm diameter
Glass microfiber paper 11cm diameter
Volumetric flask, class A grade of capacity 2ml
Spectrometer for measuring wavelength between 200nm and 300nm
Quartz glass cells, of optical path length 1 cm and no significant absorption between wavelength of 300nm and 370nm
Membrane filter for aqueous solution, made of polytetraflourethylene (PTFE), with a diameter of 4mm and pore size of 0.45µm
HPLC pump with ability of producing a flow rate at 1ml/min, injection system with a syringe-loading injection valve with 50µL loop.
Separating column, (C_{18} − Analytical reverse phase) with ability of resolving baseline resolution of aflatoxin B_1 B_2G_1 $andG_2$ from all other peaks with the following characteristics
Length of 250mm
Internal diameter 4.6mm
Spherical particle size 5µm
Post column derivatization system
Fluorescence detector: with excitation at wavelength of 365nm and emission at wavelength of 435nm. Ability to detect 0.05ng of B_1 per injection volume of atleast 50µl

Sampling method and sample size
The design of the sample size from the batches of flour was formulated using Cochran's formula as per equation 4.0 below

$$\square_o = \frac{z^2pq}{e^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4.0)$$

Where $\square_o$ 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. 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

$$(total\ number\ of\ batches)\square_o = \frac{(0.4)(0.6)(1.966)^2}{(0.05)^2} = 368.79$$

Therefore, samples for this study were obtained from a selection constituting of 369 batches, about 450 -500 samples were used in this study. The samples were purchased from licensed 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 very clear and ready for qualitative determinations on HPLC.

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 authentication and validation of the contaminant. The remaining portions were then subjected on NIR diffuse reflectance for spectra generation.

The generated spectral data from the NIR reflectance of samples were treated using various chemometric techniques. These techniques employed various algorithms aimed at developing rapid and sensitive classification models for real time qualitative discrimination of aflatoxins as discussed in the subsequent sections.

NIR Method of spectral acquisition for aflatoxins from sifted maize flour.

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.

Data Partition

The 450 samples of neat and contaminated sifted maize flour were randomly divided into calibration, validation and prediction sets. This gave identical content ranges containing 150, 150 and 150 samples respectively. The partition of calibration data and validation were done according to Kennard-Stone algorithm. These were done in different days, calibration and validation data were collected in one day while prediction data on the next day. The reference data and the NIR calibrated data were compared using Multivariate regression method as in the equation (1.0) to establish their relationship.

Signal Preprocessing Technique

Multiplicative Scatter Correction (MSC)

The multiplicative scatter correction was employed to estimate the relation of the scatter of each sample with respect to the sample reference. The average spectrum was selected as reference. Then each sample spectrum regressed against the reference.

The corrected data was reconstructed by the original ones subtracted the intercept and divided by the multiplicative constant as per Wold *et al*...1998. Equation (4.0) below was used in reconstruction of correct data.

$x_{corr}=(X-A)/B$…….. (4.0)

Where x_{corr} is the corrected spectra, X is the original spectra and B is the multiplicative constant.

Standard Normale Variate and Detrending (SNVD)

This technique was applied to minimize or rather to reduce multi collinearity, baseline shifts and curvature. These attributes were due to variable interaction of moisture and particles effects across the NIR spectral range. This was done according to the work of Naes *et al.,* 2002.

Successive Projection Algorithm (SPA)

The successive projection algorithm was used to find a small representative set of variables with the emphasis on minimization of collinearity as per the work of Galvo *et al.*2001.

Calibration matrix was treated using SPA in iterative projection such that an ordered chain of variable was obtained. Multiple Linear Regression was coupled on SPA to estimate the necessary number of variables to be included in the calibration. The necessary number of variables was obtained using the average Risk G of misclassification by linear discrimination analysis as a target function. This was done according to Pontes *et al.,* 2005.

II. Results and Discussion

HPLC Performances

The total aflatoxins content in the portions of sifted maize flour comprised of AFB_1, AFB_2, AFG_1 and AFG_2.

These toxins were spiked at10µg/kg, 20µg/kg, 30µg/kg and 40µg/kg together with the references and their retention time (R_t) compared for identification and authentication.

The precision was described as Relative Standard Deviation (RSD) through determination of 16 samples containing 10µg/kg, 20µg/kg, 30µg/kg and 40µg/kg of aflatoxins. The recovery was 81% and LOD 0.1ng/kg with inter -day and intra- day precision set at 4.7 % and 5%.

The retention time of reference standards confirmed the identities of aflatoxins in the entire portion for all samples. The elution time confirmed presence of all G_2 contamination at 6mins, G_1 at 8mins, B_2 at 9mins and B_1 at 11mins.

Table 1.0
Performance of NIR Calibrations Developed using Datasets Generated from Aflatoxin Contaminated sifted Maize Flour in the range of 0.00ppb to20.00ppb

Measured Parameter	n	Concentration range	No of PCs	R^2	SEC	SECV
AFB_1	450	0.00-20.00ppb	8	>0.99	0.0030	0.0010
AFB_2	450	0.00-20.00ppb	8	>0.99	0.0041	0.021
AFG_1	450	0.00-20.00ppb	8	>0.99	0.001	0.011
AFG_2	450	0.00-20.00 ppb	8	>0.99	0.0019	0.001

As seen in table 1.0 from the 450 samples with Principle Component averaging at 8 examined by NIR, R value was greater than 0.99, implying that NIR could be used in the discrimination of the measured parameters in real time. The standard error of calibration (SEC) which described the behavior of the spectra along the regression line clearly indicated a good calibration performance. The values of SEC and SECV were low and quite close to each other indicating accuracy and robustness of the model. The values of standard Error of Calibration (SEC) and Standard Error of Cross Validation (SECV) for the four Aflatoxins were all low with a difference of less than 0.002 not quite significant, this implied the model was accurate and robust. It could be used in real time discrimination of aflatoxin within the Legal Tolerance Limits in sifted maize flour.

Table 2.0
Performance of NIR Calibrations Developed using datasets Generated from Aflatoxin Contaminated sifted Maize Flour in the range of 20ppb to100ppb

Measured Parameter	n	Concentration range	Number of PCs	R^2	SEC	SECV
AFB_1	450	20.00-100.00ppb	8	>0.99	0.0010	0.0010
AFB_2	450	20.00-100.00ppb	8	>0.99	0.0020	0.0021
AFG_1	450	20.00.00-100.00ppb	8	>0.99	0.0012	0.011
AFG_2	450	20.00-100.00ppb	8	>0.99	0.0019	0.0020

The NIR calibration performance as seen in table 2.0 for levels of aflatoxin above the legal tolerance limit was accurate and robust. The standard error of calibration and standard error of cross validation were all very low and real close to each other. This indicated a perfect, accurate and robust model performance which could be used for routine discrimination of aflatoxin in real time from sifted maize flour

NIR Spectra characteristics of neat, contaminated and unknown contaminated sifted flour

The generated overtones and combination bands were derived from, neat, known contaminated and unknown contaminated sifted flour samples in the absorbance ranges of the following wave numbers 4000cm^{-1}-5500cm^{-1}, 5600cm^{-1} to 6800cm^{-1}, 6800cm^{-1} to 8500cm^{-1} and 8900cm^{-1} to 12500cm^{-1}.

These absorbance spectra constituted various messages of all flour matrices including physical properties. The chemical properties of interest were in absorbance associated with CH-CH, CH_3, NH, OC, CHO. H_2O, Ar-OH and Ar- O. They were from the overall molecular formula of C_{17} $H_{14}O_6$ .They generated various overtones and combination bands. The region between 4000 -5000cm^{-1}, the absorbance was very strong due to combination band effects compared to other regions. The flour usually contain13-14% moisture, the overtone due to absorbance of water is quite unstable and was excluded in the analysis. It was between5000-5300cm^{-1} and 6300-7000cm^{-1}. The first overtone of CH which trickled down to2nd overtone messages of -CH,-NH, and OH,

the absorbance are very weak and therefore excluded in both qualitative and quantitative analysis (Ozaki *et al.,* 2007).

In order to identify a representative spectrum from unknown samples, for the purpose of classification of unknown, the unknown spectra were averaged. The average of the unknown spectra with largest similarity to the known helped in selection from the known category the type of aflatoxin category. Then the unknown spectra with largest similarity to the unknown average spectra in the unknown samples were selected for classification by application of PLS-DA and LS-SVM. The huge nature of spectra data with all information was treated to eliminate irrelevant information. To narrow it to the relevant information under investigation both, signal pretreatment and variables selection were applied. To enhance the signals SNVD, Derivative (1^{st},2^{nd} and 3^{rd}), Savitzky-Golay methods and variable selection techniques were applied. Principle Component Analysis (PCA) was used to establish the structure of the calibration population sample to be assessed as seen in the diagram 1.0 and 2.0 below

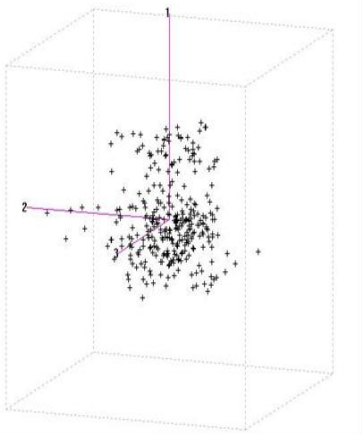

Fig (1.0) sifted flour contaminated with aflatoxin

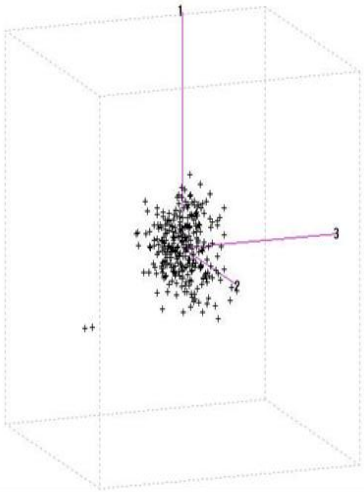

Fig (2.0) Neat sifted flour

Variable selection

The Successive Projection Algorithm (SPA) was used in the extraction of the spectra with relevant and useful information for analysis and differentiations. This excluded areas with irrelevant information in regard to the classification of toxins. The necessary number for computation was determined by validation cost as a target function of the chained. The 150 variables were selected by the SPA. This gave a minimum point of cost function at 106^{th} wavelength.

Selected Classification Methods

The Partial least Square Discriminant Analysis (PLS-DA) and Least Square Support Vector Machine (LS-SVM) were used in this study. These methods were compared and evaluated on their effectiveness in classification of Aflatoxins in real time.

Partial Least Squares –Discriminant Analysis (PLS-DA)

The PLS-DA was used to set up a model similar to the PLS Regression. The concentration matrix was a dummy matrix in which each element was either -1 or +1 (-1 for sample without AFB_1 and +1 for sample with AFB_1)

The number of significant PLS factors which were the least were selected, after optimization according to Root Mean Square Error of Cross Validation (RMSECV) and Root Mean Square Error of Calibration (RMSEC). This was finally set at 6 and threshold for screening AFB_1 set at 0.

Least Square-Support Vector Machine (LS-SVM)

The first two inputs of principle component were obtained from the PLS-DA. Then the output vectors were assumed to be -1 or +1.

-1 meant absences of AFB_1 and +1 meant presence of AFB_1. This was followed by selection of proper kernel function and the parameter in the calibration data. Secondly, application of the parameter for similar operation in the prediction set. The kernel function used in this modeling was Radial Based function Kernel (RBF). This was done in computation of the total prediction error rate. The two crucial model parameters were regularization parameter (γ) and the kernel function parameter (δ^2).The regularization parameter was used in the trade off determination between the fitting error minimization and smoothness of the estimated function. They were set in the range of 0.005232-147.3241.

Finally the optimization of the range gave the value of regularization at 10.1063 and the kernel value at 0.115063.

Results Comparison for PLS –DA and LS-SVM

Both PLS-DA and LS-SVM had some similarities in identifying AFB_1 and AFB_2 above 20ppb. The LS-SVM was more superior in classifying all the toxins in real time. The LS-SVM enjoyed a superior classification with an accuracy of 92.00% with a prediction of 94.67% for all the toxins. For samples contaminated with AFG_1 and AFG_2 above 50ppb, again LS-SVM gave a superior classification with an accuracy of 92.00% and validation of 95.00%

The PLS-DA also showed some good results in reducing the matrix effects in analysis. Nonetheless, both methods failed to completely eliminate the effects of Matrix in the analysis.

III. Conclusions

Though NIR was not as sensitive as HPLC, the model could be used for real time differentiation of toxin in sifted flour. The accuracy and robustness of the models were sufficient to sustain rapid discrimination of aflatoxin from sifted flour in real time.

This model could be applied in its designed space for routine checkup to enhance flour quality.

Post milling analysis of aflatoxins could be avoided by application of these models through the milling process. This will significantly reduce the cost of production hence, lowering the price of the commodity. Though the Regulatory requirements prescribe post milling determination of the levels of aflatoxin in the flour, but they are easily compromised and have proven to increases the cost of production.

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