METHOD DEVELOPMENT FOR A NON INVASIVE DEDICATED NIR CALIBRATION MODEL OF AFLATOXIN B1 FOR REAL TIME ANALYSIS OF SIFTED MAIZE FLOUR

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Abstract

The high demand for sifted flour and association of Aflatoxin B1 with cancer raises the need for extensive mitigation measures .Therefore; herein a method was developed exclusively for real time quantification and detection of aflatoxin B1 in all ranges of sifted flour. This method presents a fast reliable dedicated model for noninvasive real time detection and quantification of the dreaded aflatoxin B1 in all ranges of sifted maize flour. The NIR diffused reflectance Spectra of AFB1 at different levels of concentration were generated from branded and non-branded sifted maize flour with all variability captured in the model development. Aflatoxin standards of B1 was used in contamination at different levels and parallel confirmed by HPLC methods as reference primary method. Signals generated from diffused reflectance NIR were preprocessed by selected applications of signal enhancement techniques. Multiplicative signal correction, 1st and 2nd derivative and standard Normale Variate Detrending (SNVD) were all applied to minimize interferences on the signals. Quantitative model building involved multiple linear Regression (MLR) methods built on Beer's law and qualitative method for authentication of B1 involved Partial Least Square-Discriminant Analysis (PLS-DA) with Successive Projection Algorithm (SPA) used in the spectral selection. The resultant model was tested for all ranges of sifted flour which were not included in the calibration set and a good percentage of accuracy of 97% in prediction and 98.5% in validation of the model obtained. This showed the potential of the model to be used selectively in the determinations of B1   on-line, in-line and at-line in sifted flour

Key words: Aflatoxin B1,Dedicated Calibration, NIR spectroscopy, Multiple Linear Regression, Successive Projection Algorithm, Validation, Prediction, Optimization Techniques, Noninvasive, Quantitative Determination,

Introduction

This paper demonstrates the potency of a dedicated calibration model as a rapid noninvasive procedure for online, in line and at line identification and quantification of Aflatoxin B1 in all branded sifted flour. The up surging cancer index which could be associated to acute contamination of B1 could be checked down significantly or mitigated through real-time model of measurement at line in line and on line (Tan *et al.,* 2021). These measurements could detect Out of Specification (OOS) batches thus preventing it from under going further processing or consumption.

The Spectra of contaminated flour at different levels were all generated by diffused reflectance NIR method. This involved two fundamental steps, namely: recording NIR spectra for the constituents of interest also called spectral library and validating them on validation set (Wang *et al.,* 2017). Majorly the calibration model developed was meant to relate the concentration of analyte found in the sample to the spectral data collected from the sample. The development of accurate calibration model relied on the variance of the sample set used in the calibration equation (Pezzei *et al.,* 2017). Both conventional chemical analysis and inclusion of enough source of variability through the identification processes were performed as a key role in the development of accurate calibration model (Chang *et al.,* 2016).

The two main sources of spectral variability from batches through manufacturing process and sample recorded by different operators in different days were included in the calibration (Hertrampf *et al.,* 2015). The variability in the sample was captured in the account of sample from 5 to 10 different batches (Kirchler *et al.,* 2017).

Calibration Model Development

A simple and convenient procedure was developed for a feasible calibration; it involved collection of approximately 30 to 50 samples from different batches to cover the entire anticipated concentration ranges of aflatoxin B1 in sifted flour samples (Silva *et al.,* 2012). In addition to that, the conventional chemical analysis on the sample was done accurately (Feyziyev *et al.,* 2016). The accuracy of the value generated from the reference HPLC analysis is presented in the table 2.0. (Fikrat *et al.,* 2016)

The calibration set was narrowed with small variance in sample to ensure an increase in the accuracy of analyzing sample within the prescribed range (Kirchler *et al.,* 2017).

$$y_i = b_o + \sum_{i=1}^{k} b_i x_i + e_{ij} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (1)$$

Where b_i is the computed coefficient.

x_i is the absorbance at each considered wavelength and e_{ij} is the error.

The above calibration equation could also be expressed in terms of percentage concentration (Naes *et al.,* 2002).

These absorptions under specific wavelengths obey Beer's law (Naes *et al.,* 2004). The Beer's law is expressed as per equation (2.).

$$A = Mcd \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (2)$$

Where A= absorbance (optical density)

    M= molar absorptivity

    c= concentration, d= sample path length

The multivariate regression equation used for calibration 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 (3)

$$Y = B_O + B_i(-logR_i)_N + E \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (3)$$

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.

The above equation was rearranged to demonstrate sensitivities relationship between concentration and optical density of Aflatoxin B1 in terms of regression coefficient as per equation (4)

$$Concentration\ (Con) = \frac{change\ in\ concentration}{change\ in\ absorbance} \times Absorbance\ + Some\ Error..\ (4)$$

$$Con = K \times Absorbance + some\ Errors \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (5)$$

Where K=regression coefficient.

Using the above equation, a large change in concentration of calibration set with a relatively large change in the absorbance implied a small regression coefficient with high sensitivity

Optimization of Calibration Model

From the calibration set the model was built based on Partial Least Square regression. The ability of the model to fit the data was checked with statistical calculation of the Root Mean Square Error of Calibration (RMSEC) as per the equation (6)

$$RMSEC = \sqrt{\frac{\left(\sum_{i=1}^{n}(y_{i\,pred}-\bar{y}_i)^2\right)}{n}} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots (6)$$

Where $y_{i\,pred}$ is the predicted concentration of the sample i that is included in the model formation, while $\bar{y}$ represent the sample i concentration of the property of interest determined by the selected reference method, n is the total number of objects in the data set

Model validation

After model optimization the next step is to validate the model with external validation set, external validation comprised of samples that were not included in the calibration sets (Moffat *et al.*, 2000). For determination of the model efficacy, comparison is made on values generated by RMSEC and RMSEP (Wang *et al.*, 2017). If both values are low and close to each other, it implies the model is accurate (Kirchler *et al.*, 2017). Otherwise, if the RMSEP is too high, further optimization is necessary and if after optimization the values are still high, investigation of sufficient inclusion of outliers and other variability at the calibration set should be checked (Fikrat *et al.*, 2016). The RMSEP is computed as per equation (7)

$$RMSEP = \sqrt{\frac{\left(\sum_{i=1}^{n}(y_{ipre}-\bar{y}_i)^2\right)}{n}} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots. (7)$$

Where, y_{ipre} refers to sample that were never included in the calibration set, the value of i is predicted using a model that was built using a set of sample that was not included in the calibration set.

The RMSEP is a true performance indicator of the model as it is calculated with samples that were never included in the calibration set (Colliez, Dufrenois and Hamid, 2006). The approach in this technique gives a global indication of the overall performance of the model. Nonetheless, there could be some bias generated through systematic or random errors in the process of data acquisition leading to low rating of the model (Ge *et al.,* 2016).

Materials and Method

1) Apparatus
Multi-purpose Analyzer Spectrometer (Bruker Optics, Germany)

Immunoaffinity (IA) column (PG Instrument Ltd, England)

Sieve of 600 to 700 micron

Flute paper filter with the following specification;

-25 cm diameter

Glass microfiber paper with the following specification:

 -11cm diameter

Volumetric flask with the following specification:

-class A grade

 -Capacity 2mL

Spectrometer (PG Instrument Ltd, England)

-measuring wavelength between 300nm and 370nm

Quartz glass cells (Orion, Boston MA 02129 USA)

With the following specifications:

-optical path length 1 cm and

-no significant absorption between wavelength of 300nm and 370nm

Membrane filters with the following:

 -Made of PolyTetraFlourEthylene (PTFE) for aqueous solution,

-Diameter of 4mm

 -Pore size of 0.45μm

HPLC pump with the following specifications:

 -Ability of producing a flow rate at 1mL/min

- Injection system with a syringe-loading

-Injection valve with 50μL loop.

Separating column with the following specifications

-(C_{18} – Analytical reverse phase)

-Ability of resolving baseline resolution of aflatoxin B_1 $B_2 G_1$ $and G_2$ from all other peaks

-Length of 250mm

-Internal diameter 4.6mm

-Spherical particle size 5µm

-Post column derivatization system

Fluorescence detector with the following specification:

- Excitation at wavelength of 365nm

- Emission at wavelength of 435nm.

-Detection 0.05ng of B_1 per injection volume of atleast 50µL

2) Softwares
LS-SVM Lab 1.7 Tool box

PLS Tool box version 3.5

PLS Tool box version 5.0

Method

NIR Spectra Acquisition for Analysis of Aflatoxin
A 5g amount of contaminated and neat flour was separately measured on a multipurpose analyzer spectrometer from Bruker Optics Germany. Spectrometer equipped with an integrated sphere and an InGaAs detector. Samples were sieved through 700 micron super sieve from Staphorst, Netherlands and weighed accurately. An average of 32 scan were done on each sample to obtain the spectra between 12500cm^{-1} and 4000cm^{-1}. The scanner velocity was set at 10 KHz and a background of 32scans with frequency test of five times on each sample recorded, and then the average spectrum was obtained from the five frequencies

Data partition

The data partition was done by random selection of calibration, validation and prediction samples from total samples. The samples were randomly selected as follows: 150 samples for the generation of calibration data with concentrations spanning from LTL to acute level, 75samples for validation and 175 samples for the prediction.

The calibration and validation sample were selected on the same day while the prediction samples selected on the next day. The partition of calibration data was done using Kennard-stone algorithm as per Daszykowski procedure due to the uniformity in dispersion.

Details of samples of the Calibration, Validation and Prediction

Table 1.0

Subset of the Data	Calibration	Validation	Prediction
Number of samples	150	75	175
Range of aflatoxin contamination in (ppb)	AFB1>5.00ppb, AFB1<5.00ppb	AFB1 0.00-20.00ppb AFB1 0.00-5.00ppb	AFB1 0.00-20ppb

Results

Statistical analysis for Confirmatory HPLC describing precision and accuracy of the primary reference method is shown in table 1.0

Table 2.0

Parameter	Aflatoxin B$_1$
Number of accepted results in samples	30
Mean value (μg/kg)	10.86
Repeatability Standard Deviation (RSD)	0.66
Repeatability Coefficient of Variation% (RCV)	5.60
Repeatability Limit (μg/kg)	2.4
Reproducibility standard deviation (μg/kg)	1.56
Reproducibility Limits ((μg/kg)	4.2

The quantitative assaying by HPLC (Primary reference method) for different levels of aflatoxin B1 concentration in contaminated sifted flour indicated consistency and accurate determinations of levels. Therefore, allowed for a potent NIR calibration of B1 for the levels. Since calibration model relied on the accuracy of primary method

Table 3.0 Co-relation coefficients between NIR measurements and Primary Confirmatory HPLC method at different concentration levels

Quality Parameter	AFB$_1$	AFB$_1$	AFB$_1$	AFB$_1$	AFB$_1$
Concentration in ppb	5-10	10-20	20-50	50-100	100-200ppb
Correlation (r)	0.97	0.92	0.95	0.92	0.98
Coefficient (R)	97.00%	92%	95%	92%	98%

From table 3.0 The results of variables measured over wide spread concentration ranges and compared for the two methods using Pearson correlation coefficient showed a very strong linear correlation. The two methods could give similar results for aflatoxin B1 measurements in sifted flour samples under similar conditions (Schober *et al.,* 2018).

Therefore a Dedicated NIR Calibration method was developed further for both quantitative and qualitative measurements of aflatoxin in sifted flour (Damien *et al.,* 2022).

Performance on NIR Calibration Developed using PLS on Sifted Aflatoxin B1 Contaminated Flour of Concentration Between 0.00ppb to 5.00ppb

Table 4.0

Sample	Quality Parameter	N	PCS	Spectral range (cm⁻¹)	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB₁**	400	8.0	12000-9200 9200-6500	0.9805	0.9510	0.9921

From table 4.0 the values of the RMSEC, RMSECV and RMSEP were all low and close. This demonstrated good accuracy of the model in quantification of B1 in sifted flour.

Performance of NIR Calibration Developed using PLS on Sifted Aflatoxin Contaminated Flour of Concentration Between 50ppb to 100ppb.

Table 6.0

Sample	Quality Parameter	N	PCS	Spectral Range (cm⁻¹)	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB₁**	400	8.0	12000-9200 9200-6500	0.005	0.001	0.0020

The optimization parameters were all quite low at 50ppb to 100ppb , indicating high sensitive of the model in quantification of B1 in sifted flour

Performance on NIR Calibration Developed using PLS on Sifted Aflatoxin Contaminated Flour of Concentration Between 100ppb to 200ppb.

Table 5.0

Sample	Quality Parameter	N	PCS	Spectral range (cm^{-1})	RMSEC	RMSECV	RMSEP
Sifted maize flour	**AFB$_1$**	400	8.0	12000-9200 9200-6500	0.005	0.001	0.0021

The optimization parameter gave quite low values and real close to each other, demonstrating high sensitivity of the mode at extremely high concentration.

GRAPHICAL PRESENTATION OF QUANTITAIVE PREDICTIVE MODELS FOR DEDICATED CALIBRATION OF AFLATOXIN B1 IN SIFTED FLOUR

Aflatoxin B1 in sifted flour in the range of 10.00ppb to 50.00ppb

Graph 1.0
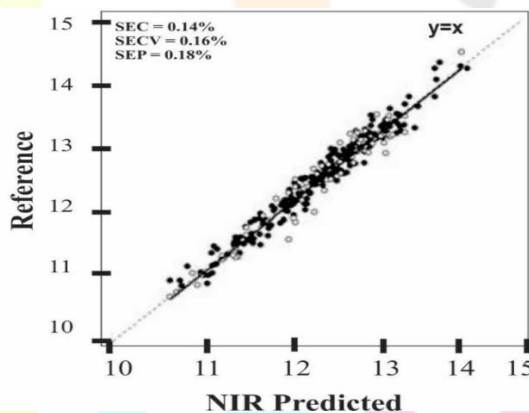
The NIR predictive model of aflatoxin level in the range of 10.00ppb to 50.00ppb had a linear response with the HPLC method. This demonstrate the whole potency and sensitivity of a dedicated model of B1 in the determinations of B1 in sifted flour.

Conclusions

The dedicated calibration model for B1 demonstrated an outstanding selectivity in the prediction of B1 only in all ranges of sifted maize flour. As the concentration of B1 in sifted flour increased the sensitivity of the model also increased linearly. Therefore the model could be used in on-line, in-line and at-line inspection of levels of B1 in sifted flour. This could help to minimize the incidence rate of chronic and acute exposure to B1, leading to significant reduction of hepatocellar casinoma indices.

Acknowledgement

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