



Prediction of Retinol in Fortified Maize Flour using Fourier Transform - Near Infrared Spectroscopy

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Abstract

Food fortification is one strategy for addressing micronutrient deficiencies among the population groups at risk. Non-compliance with fortification standards hinders the success of fortification programs. This is due to a lack of techniques to rapidly check the amounts of the added fortificants. Fourier transform - near-infrared (FT-NIR) spectroscopy is a fast and reliable technique that would be used to ensure adherence to requirements. This study aimed to investigate the potential of using FT-NIR spectroscopy to predict the amount of retinol in fortified maize flour. 150 fortified maize flour samples were used in this study. Partial least squares regression (PLS-R) was used to build calibration models based on the retinol reference values obtained by high-performance liquid chromatography (HPLC), and fortified maize flour NIR spectra acquired from the FT-NIR spectrophotometer. Two calibration models were developed to predict retinol above and below 1.0 mg/kg. The performance metrics of model one developed to predict retinol < 1.0 mg/kg were: $R^2_c = 0.81$, RMSEE = 0.08, RPD = 2.29 and $R^2_v = 0.82$, RMSEP = 0.09, RPD = 2.07 for the calibration and validation, respectively. The second model developed to predict retinol ≥ 1.0 mg/kg had the following performance metrics: $R^2_c = 0.93$, RMSEE = 0.16, RPD = 3.58 and $R^2_v = 0.81$, RMSEP = 0.22, RPD = 2.43 for the calibration and validation, respectively. Overall, the findings demonstrated that FT-NIR spectroscopy can be utilised to reliably predict retinol levels in fortified maize flour samples. FT-NIR spectroscopy, by replacing time-consuming and laborious wet chemistry laboratory procedures, has the potential to be used for rapid regulatory monitoring of fortification compliance for a large number of samples.



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Introduction

Micronutrient deficiency is a major global health concern affecting more than two billion people across the world.^{1,2} People in developing countries frequently lack a variety of micronutrients.¹ One of the major micronutrient deficiencies in Kenya is vitamin A deficiency (VAD).^{3,4} Approximately 24% of Kenyans are vitamin A deficient, with preschoolers being the most affected (53%).⁵

A regular intake of essential micronutrients can prevent or eliminate micronutrient deficiencies.⁶ Increasing the availability of micronutrients in the diet through fortification is a cost-effective strategy to reduce the prevalence of micronutrient deficiencies.^{5,7} A suitable fortification vehicle should be extensively consumed and easily accessible to consumers.^{5,8} Maize (*Zea mays* L.) is widely considered a staple food to a large population in Kenya.^{9,10} Maize flour is used as a vehicle to deliver retinol, thiamin, riboflavin, niacin, pyridoxine, folic acid, iron, and zinc in the diet.¹¹

Vitamin A is an essential micronutrient that must be supplied in the diet. Retinol is one of the naturally occurring active forms of vitamin A.¹² Adequate provision of vitamin A results in reduced risk of vitamin A deficiency (VAD) hence decreased susceptibility to infections and impaired immune responses. The set tolerable upper intake level of retinol is at 3000 mg/day for adults and lower for children.¹³ Vitamin A fortificant in the form of retinyl palmitate is usually added to flour after milling.^{14,15} According to East African Community,¹⁵ the minimum and maximum regulatory levels of retinol for maize flour fortification are 0.5 mg/kg and 1.4 mg/kg respectively.

Despite various interventions including fortification initiatives in many nations, vitamin A deficiency is still a common public health concern.⁵ The effectiveness of fortification as an intervention to curb micronutrient deficiencies is hindered by non-compliance with fortification standards, which is not easily determined due to a lack of techniques for quickly determining the quantity of fortificants added. Laboratory analysis is essential in monitoring successful fortification by checking compliance with fortification standards.¹⁶ High-Performance Liquid Chromatography (HPLC) is a traditional technique that is used to quantify retinol in fortified maize flour as a way of monitoring

compliance with fortification standards. However, due to the lengthy extraction operations, HPLC as a monitoring approach is time-consuming and tedious especially when a large number of samples are involved.¹⁷ Additionally, the use of HPLC for quantification of retinol in samples is highly prone to oxidation and many interfering compounds which is a source of likely errors in the method.¹⁸ There is a need, therefore, to develop a non-destructive, more accurate, and rapid technique for vitamin A determination such as the Fourier Transform Near-Infrared (FT-NIR) spectroscopy.

FT-NIR spectroscopy is a rapid analytical tool which allows the development of calibration models that can be used to predict various parameters in various food matrices.¹⁹ Calibration models are based on the association between the NIR spectrum and the chemical constituents of the food product.²⁰ Successful NIR spectroscopy models have been developed for predicting starch,²¹ moisture,²² protein, oil, carbohydrates²³ and biochemical components such as amylose, lysine, tryptophan, and phytic acid in maize/corn flour samples.²⁴

There are limited studies on the application of NIR spectroscopy to predict micronutrients such as vitamins. Most industries and research centres still rely on wet chemistry methods to obtain accurate estimates of vitamins since little research has been conducted on the application of NIR spectroscopy. This study therefore aimed to investigate the prospects of applying near-infrared spectroscopy to predict retinol amounts fortified maize flour.

Materials and Methods

Sample Collection and Preparation

This study included 150 samples of randomly selected packaged fortified maize flours. The samples were collected from retail outlets, wholesale shops and supermarkets in ten counties in Kenya. These counties included: Nairobi (n-21), Kiambu (n-18), Uasin Gishu (n-16), Nakuru (n-17), Elgeyo-Marakwet (n-12), Kwale (n-11), Kilifi (n-10), Kisumu (n-15), Busia (n-13) and Mombasa (n-17) (Table 2). This was done to adequately represent millers from the Central, Eastern, Western and Coastal regions of Kenya. To avoid duplication, fortified maize flours from the same region under the same commercial brand names with similar batch numbers (identification numbers assigned to

products made in the same manufacturing run) were pooled together. Thus, the maize flours included in the study were those belonging to different commercial brand names and those with different batch numbers within a brand. This resulted in a total of 150 samples. The different samples were mixed thoroughly using a commercial blender (Omniblend 1-TM-767) to ensure homogeneity. The homogenized fortified maize flour samples were then put in airtight zip-lock bags and kept in carton boxes at room temperature until the day of analysis. Storing samples in airtight zip-lock bags and carton boxes was done to reduce the adverse effects of environmental factors such as oxygen and light respectively.

Retinol Quantification using HPLC

Extraction and quantification of retinol in the form of retinyl palmitate was carried out according to the method described by Chepkoech, Sila and Orina.²⁵ Two grams of flour was weighed into a centrifuge tube. This was followed by adding 15 ml of ethanol containing 0.1% (wt/vol) ascorbic acid and then 2 ml of 50% (wt/vol) potassium hydroxide. The centrifuge tubes were capped, shaken well, and put in a water bath (Memmert WNB AC 230 V-50/60 HZ, Germany) at 80 °C for 20 minutes. The tubes were shaken intermittently throughout this period. Using running water, the tubes were cooled before 15 ml of hexane containing 0.01% butylated hydroxytoluene (BHT) (wt/vol) was added. The contents of the tubes were thoroughly mixed on a vortex mixer for one minute, and after standing for two minutes, the contents of the tubes were again mixed for another minute. Two ml of cold water (1°C) was added to each centrifuge tube and then the tubes were inverted 10 times. The samples were centrifuged at 1000 rpm for 10 min. Afterwards, the upper-organic layer was pipetted into a round-bottomed flask and the solvent was evaporated under vacuum at 40 °C using a rotary vacuum evaporator (Hahnshin HS-2005S, water bath HS-3001, Korea). The residue was dissolved in 1 ml of methanol, and put in vials, ready for HPLC analysis. Twenty (20) µl of the sample was injected into reverse-phase HPLC (Shimadzu RF-20A, Japan) fitted with column C-18 ODS size 250 mm × 4.6 mm × 0.5 µm. The mobile phase was methanol and water in a ratio of 98:2 and the flow rate was 0.8 ml/min. A UV-visible diode-array detector (SPD - M20A) was used for the identification of retinol at 324 nm. Concentrations of retinol were calculated

using peak areas of the samples and the standard curves of the retinol standards.

NIR Spectral Data Acquisition

Acquisition of NIR spectral data was done following the procedure outlined by Wafula *et al.*²⁶ Samples were thoroughly mixed before each scan. Three (3) sub-samples were drawn from each sample then transferred to a plastic cup measuring 10 cm in diameter, and put to about 3 cm deep. The scan range for the sub-samples was 12000 cm⁻¹ to 4000 cm⁻¹, and this was done using Bruker MPA (Multi-Purpose Analyzer) FT-NIR spectrometer fitted with a semi-conductor lead sulphide detector (RT-PbS). The integrating sphere was used to scan in reflectance mode at 30 °C with a resolution of 16 cm⁻¹. For each sample, three independent spectra (as determined during preliminary research) were recorded, similar to the collection of reference values using HPLC. The calibration models were then constructed using these data in triplicate. The average number of scans was 64 for each spectrum obtained. The wavelength range was narrowed to 9000 cm⁻¹ to 4000 cm⁻¹ by visual assessment of the spectra, in order to remove noisy regions.

NIR Spectral Pre-processing

Spectral pre-processing was done using OPUS software 7.8, according to the method adapted from Bag, Srivastav and Mishra.²⁷ This was done to reduce the prediction errors associated with spectral noise and the influences of temperature changes, particle size differences, light diffusion and baseline shifts on the NIR spectra while increasing signal from chemical information.^{28,29} Before calibration, the raw spectra were treated with a combination of multiple pre-processing techniques, including first derivative, second derivative, and standard normal variate (SNV). First derivative pre-processing technique was done to correct spectral baseline shifts while second derivative was used to improve spectral resolution. Standard normal variate (SNV) pre-processing technique was also employed to correct scatter in the spectra caused by path length variation occurring during scanning due to particle size differences between the samples. The most optimal pre-processing technique combination was first derivative + SNV. Additionally, before calibration, all spectral data were mean-centred. Mean centering (MC) was the basic pre-treatment used that reduced bias noise from all NIR spectra.

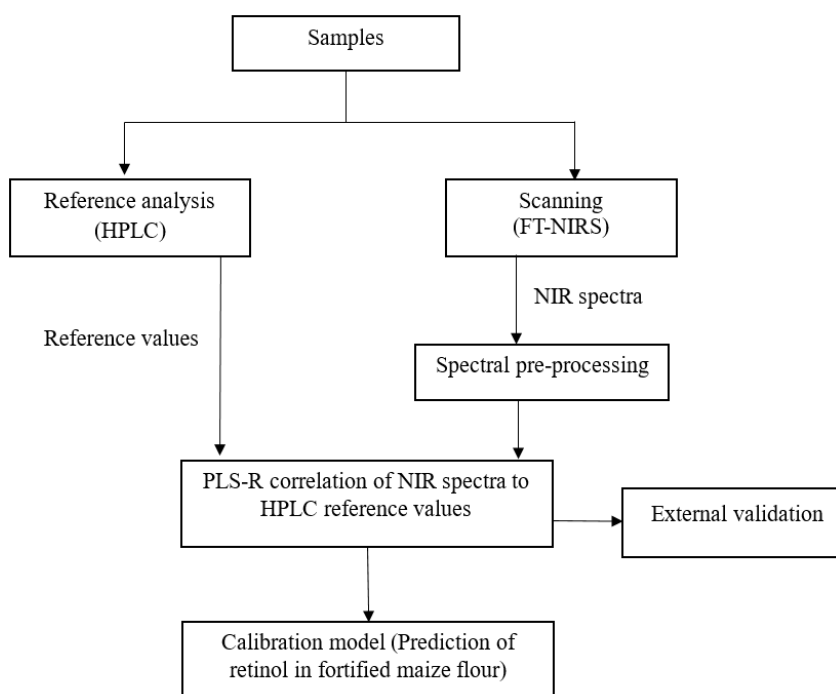
Calibration and Validation Set Sample Selection

The most ideal outcome of this study would have been the successful development of a single model capable of predicting retinol levels in fortified maize flour samples over a wide range of concentrations. However, a single calibration model for predicting retinol in fortified maize flour had a poor predictive capability hence the decision to develop two separate models. The fortified maize samples were grouped into two, retinol < 1.0 mg/kg and retinol \geq 1.0 mg/kg (retinol values from HPLC). This resulted in two data sets used in developing two (2) separate models. For

the two groups of data, samples used for calibration and validation were selected. The calibration and validation data sets were selected randomly in a way that they covered the whole range of the reference data. Calibration for model I was developed using 63 samples in the range of concentrations of 0 to 0.62 mg/kg retinol, whereas calibration for model II was developed using 37 samples in the range of concentrations of 1.0 to 3.32 mg/kg retinol (Table 1). A validation set of 31 samples and 19 samples were used for external validation of calibration for model I and model II respectively (Table 1).

Table 1: Mean, range, and standard deviation of calibration and validation data sets

	Calibration set				Validation set			
	n	Range (mg/kg)	Mean (mg/kg)	SD	n	Range (mg/kg)	Mean (mg/kg)	SD
< 1.0 mg/kg	63	0-0.62	0.34	0.18	31	0-0.6	0.28	0.18
\geq 1.0 mg/kg	37	1.0-3.32	1.47	0.53	19	1.0-2.89	1.34	0.43

**Fig. 1: Process flowchart for calibration model development and validation****Model Development and Validation**

Model development and model validation using was done according to the method adapted from

Wafula *et al.*²⁶, as shown in Figure 1. Calibration models I and II were generated using OPUS software 7.8. The calibrations were derived by

performing partial least squares regression (PLS-R) which correlated retinol reference values (y) with the pre-processed NIR spectra (x), meaning that retinol (y) was predicted from NIR spectra (x) using PLS-R. The default optimisation command of the OPUS software program, which combined the number of PLS factors, wavenumber ranges, and pre-treatment techniques, was used to choose the optimal model. In this study, only 5% of outlier samples with high errors were excluded from the calibration set. External validation was also done using the OPUS software to test the reliability of the models. For model validation, the samples used were independent of the calibration set.

Statistical Evaluation

Microsoft Office Excel 2019 was used to calculate the means and standard deviations of the retinol values obtained from HPLC (reference values). The prediction potential of the developed FT-NIR calibration models was evaluated by statistical terms of coefficient of determination for calibration (R^2c), and the root mean square error of estimation (RMSEE). The reliability of the generated models was assessed according to the coefficient of determination of validation (R^2v), root mean square error of prediction (RMSEP), ratio of performance to deviation (RPD), and bias values.

Table 2: HPLC retinol data used for FT-NIR spectroscopy modelling

County	Sample size (n)	Range (mg/kg)	Mean (mg/kg)	SD
Nairobi	21	0.00 - 4.03	0.96	0.94
Kiambu	18	0.06 - 1.92	0.65	0.42
Uasin Gishu	16	0.02 - 1.40	0.46	0.33
Nakuru	17	0.00 - 1.67	0.45	0.53
Elgeyo-Marakwet	12	0.00 - 0.75	0.19	0.25
Kwale	11	0.00 - 0.76	0.27	0.28
Kilifi	10	0.00 - 0.72	0.17	0.22
Kisumu	15	0.01 - 0.64	0.23	0.22
Busia	13	0.02 - 1.17	0.30	0.36
Mombasa	17	0.00 - 0.72	0.30	0.24

Results and Discussion

Retinol Content of the Fortified Maize Flours

The retinol content of fortified maize flours measured using the HPLC method are shown in Table 2. The total retinol content ranged between 0 mg/kg \pm 0.00 (not detectable) to 4.03 \pm 0.82 mg/kg. Only 26.9 % of the analysed samples complied with the set standards for retinol (0.5-1.4 mg/kg), meaning that quite a number of samples did not meet the fortification requirements. This is somewhat comparable to the report of a study conducted by Khamila, Sila and Makokha³⁰ which showed that of the total samples evaluated, only 33.3% met the acceptable requirements for retinol, with levels in fortified maize flour samples ranging from below detectable levels to 1.2 mg/kg.

Samples with retinol concentrations lower than 0.499 mg/kg were considered under-fortified. Whereas samples with retinol levels above 1.4 mg/

kg were termed over-fortified. Both of these sets of samples did not meet the regulatory requirements for compliance. Retinol is one of the micronutrients that is added to maize flour as a fortificant in the form of retinyl palmitate.^{14,15,31} Generally, the precision with which the chemical composition of the samples is determined using reliable and accepted reference procedures has a significant impact on the performance of the calibrations developed.³²

NIR Spectra of Fortified Maize Flour

The characteristic raw/unprocessed spectra of the fortified maize flour samples are shown in Figure 2. From this visual representation it can be seen that although all spectra had very similar shapes, there was variation in absorbance. NIR spectroscopy studies on various parameters of maize flour samples reported by Plumier²¹; Chen, Delaney and Johnson²² and Egesel and Kahrman²³ generated spectral data with a comparable shape to this study's

samples. The fortified maize flour spectra generally resembled those of other agricultural products such as wheat²⁸ and common beans.²⁶

Absorption bands were linked to functional groups and compounds present in fortified maize flour samples. The dominant absorption bands included: 9000 cm^{-1} - 8000 cm^{-1} (second overtone C-H stretching), 6800 cm^{-1} - 6600 cm^{-1} (first overtone N-H stretching; first overtone O-H stretching), 5300 cm^{-1} - 5200 cm^{-1} (combination C-H stretching), 5000 cm^{-1} - 4700 cm^{-1} (combination N-H stretching; combination O-H stretching), and 4545 cm^{-1} - 4065

cm^{-1} (combination C-H stretching).³³ The peaks in the spectra before pre-processing could be explained by quite high amounts of carbohydrates, proteins, and an substantial levels of moisture and vitamins in maize flour.^{34,35} The absorption bands in 5000 cm^{-1} - 4700 cm^{-1} are associated with protein and moisture content in the samples while absorption at 5300 - 5200 cm^{-1} is associated with polysaccharides such as amylose and amylopectin. Retinol, a minor parameter, mainly consists of C-H-O molecular bonds.³⁶ The absorption bands ranging from 4762 cm^{-1} - 4386 cm^{-1} are believed to be related to C-H-O structures such as retinol.³⁷

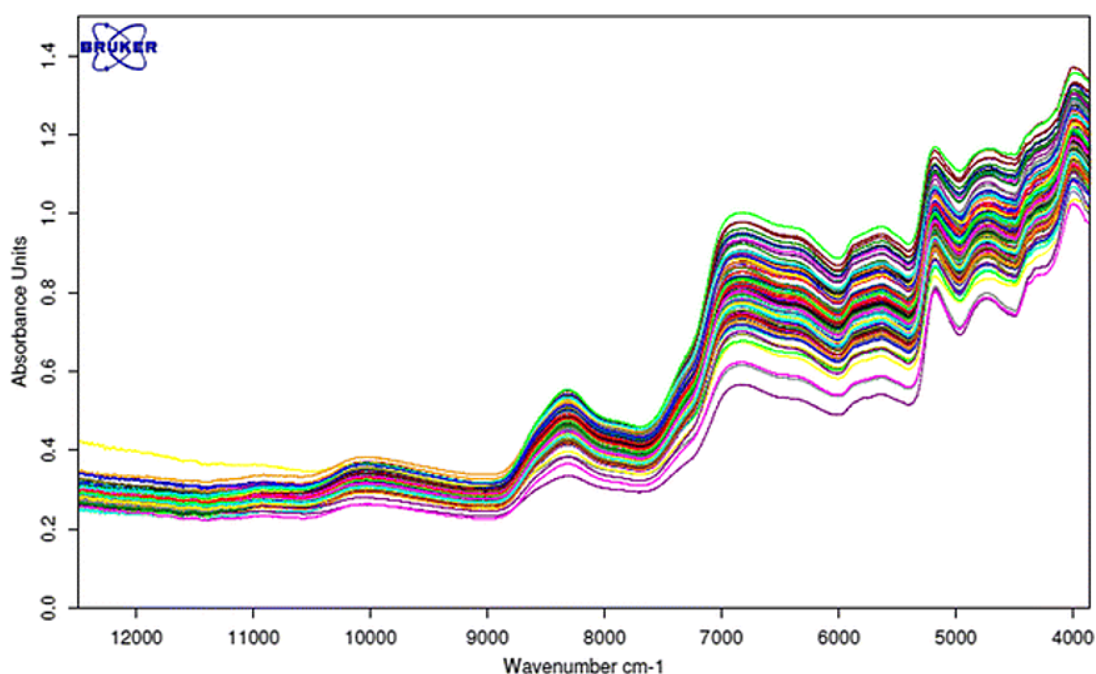


Fig. 2: Raw NIR spectra of fortified maize flour

PLS-R Models Predicting Retinol in Fortified Maize Flours

Successful building of a single model that has a capacity to predict retinol levels in fortified maize flour samples over a wide range of concentrations would have been the most ideal outcome of this study. However, as illustrated in Figure 3 a single calibration model to predict retinol in fortified maize flour had a poor predictive capability as demonstrated by low R^2 ($R_c^2 = 0.20$; $R_v^2 = 0.18$), low RPD (1.11, 0.92) and high RMSE (RMSEE = 0.70; RMSEP = 0.81) values. An acceptable model should have a high R^2 , a low RMSE and an

RPD higher than 2.5.³⁸ There are several actual calibration scenarios where it is difficult to develop a single universal calibration equation for the full population of interest, resulting in a lack of a suitable model for all components.³⁹ Various approaches to address non-linearity such as the use of new pre-treatment methods, the elimination of wavelengths, the addition of principal components/latent variables to the model or splitting the data into subsets or groups can be used.³ Based on this observation and knowledge, two separate models were developed to improve the model performance.

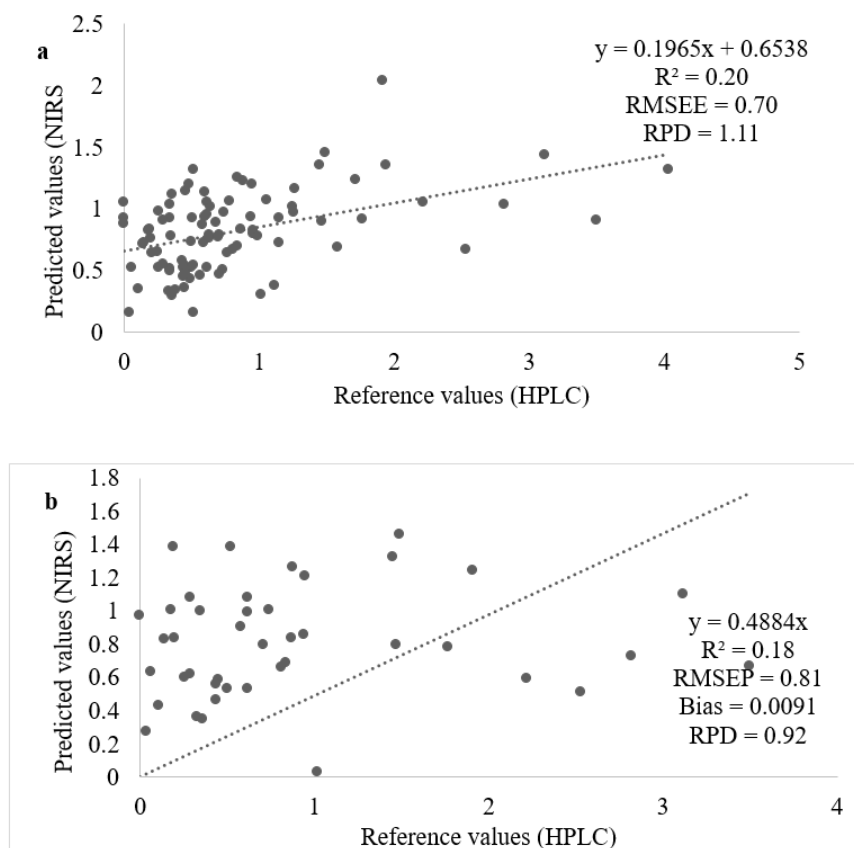


Fig. 3: Calibration (a) and validation (b) of the correlation between reference values and FT-NIR predicted values of retinol in fortified maize flour (single model). R²: coefficient of determination; RMSEE: root mean square error of estimation; RMSEP: root mean square error of prediction; RPD: ratio of performance to deviation

Table 3: Calibration and external validation statistics for prediction of retinol in fortified maize flour according to the models developed

Statistics	Single model		Model I Retinol (< 1.0 mg/kg)		Model II Retinol (≥ 1.0 mg/kg)	
	Calibration	Validation	Calibration	Validation	Calibration	Validation
R ²	0.20	0.18	0.81	0.82	0.93	0.81
RMSEE	0.70	-	0.08	-	0.16	-
RMSEP	-	0.81	-	0.09	-	0.22
Bias	-	0.0091	-	-0.0008	-	-0.0046
RPD	1.11	0.92	2.29	2.07	3.58	2.43
Rank	8	8	7	7	8	8

R²: coefficient of determination; RMSEE: root mean square error of estimation; RMSEP: root mean square error of prediction; RPD: ratio of performance to deviation.

It was observed that splitting the data into two groups, retinol < 1.0 mg/kg and retinol \geq 1.0 mg/kg, resulted in models I and II, respectively that showed better prediction performance (Figures 3 and 4). The performance metrics for the single calibration model, models I and II are summarized in Table 3. This table shows that, in comparison to the single calibration model, the RMSEE and RMSEP values of models I and II were lower and the R^2 values were higher.

The R^2_c , RMSEE, and RPD before data splitting were 0.20, 0.70, and 1.11 respectively. Model I on the other hand achieved R^2_c , RMSEE, and RPD of 0.81, 0.08, and 2.29 respectively while model

II showed equally good results with R^2_c , RMSEE, and RPD of 0.93, 0.16 and 3.58 respectively (Table 2 and Figures 4 & 5). When the models were applied to predict the retinol contents of the validation sets, the prediction results for model I were: $R^2_v = 0.82$, RMSEP= 0.09, bias = -0.00088, and RPD = 2.07 (Table 2 and Figure 4) while the prediction results for model II were: $R^2_v = 0.81$, RMSEP= 0.22, bias = -0.0046 and RPD = 2.43 (Table 2 and Figure 5). The reliability of the generated models was assessed according to R^2_v , RMSEP, RPD, and bias values. The models generally depicted high R^2 values, low RMSEP values, and low bias values hence reliable for application.^{19,23}

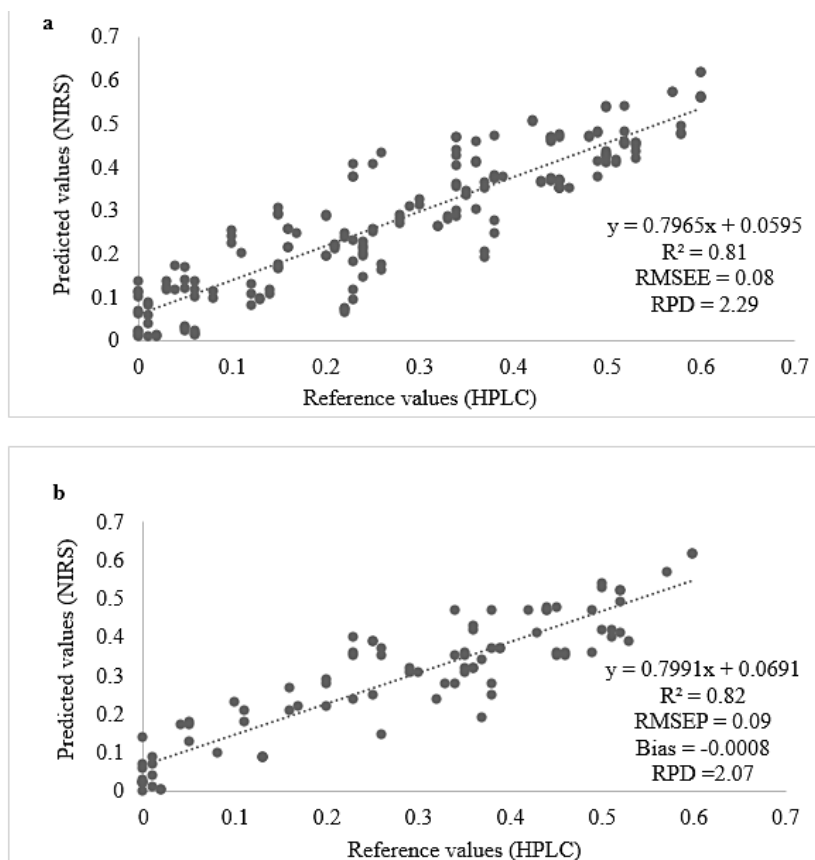


Fig. 4: Calibration (a) and validation (b) of the correlation between reference values and FT-NIR predicted values of retinol (< 1.0 mg/kg) in fortified maize flour

Comparing the two models, external validation indicated that the prediction errors (RMSEP) for model II were slightly higher (0.22) than those of model I (0.09). Model II had a fewer number of samples and this is probably why it had slightly

higher errors for the RMSEP. Further, retinol estimates were negatively biased (bias = -0.00088 and bias = -0.0046) for models I and II respectively (Figures 4 & 5). The bias value for an accurate calibration model should be close to 0.⁴⁰ The average

difference between the measured or reference values (x) and the NIRS estimate (y) is known as bias.⁴¹ A positive score indicates that the model is generally overestimating the composition by that amount, whereas a negative value indicates that the model is underestimating the composition.^{41,42} Although the models created would underestimate retinol amounts in fortified maize flour, the values were close to 0, indicating that the models developed were quite accurate and adequate, and hence can be adopted in food analysis.

Models I and II had R^{2v} of above 0.8 which implies that these models were acceptable and usable for screening and some approximate calibrations according to de Girolamo, Cervellieri and Visconti.²⁸ The RPD values, 2.07 (model I) and 2.43 (model

II), according to Xia, Li, Zhao and Chang⁴³ indicate that model I had slightly lower robustness. When the prediction accuracy of calibration models is relatively insensitive to unknown changes in external factors, the model is said to be robust.⁴⁴ Temperature fluctuations, shifts in wavelength, and changes in detector stability over time are some of the factors that could have impacted model performance. The RPD tests the robustness of a model by assessing how well the developed model predicts the retinol in the validation set, and the higher its value, the better the model's prediction capacity.^{28,43,45} Regression models with RPD values ≥ 6.5 are excellent and suitable for process control or any application.⁴³ Chang, Laird and Mausbach⁴⁶ however contrast by mentioning that calibration models with $RPD > 2$ are considered satisfactory.

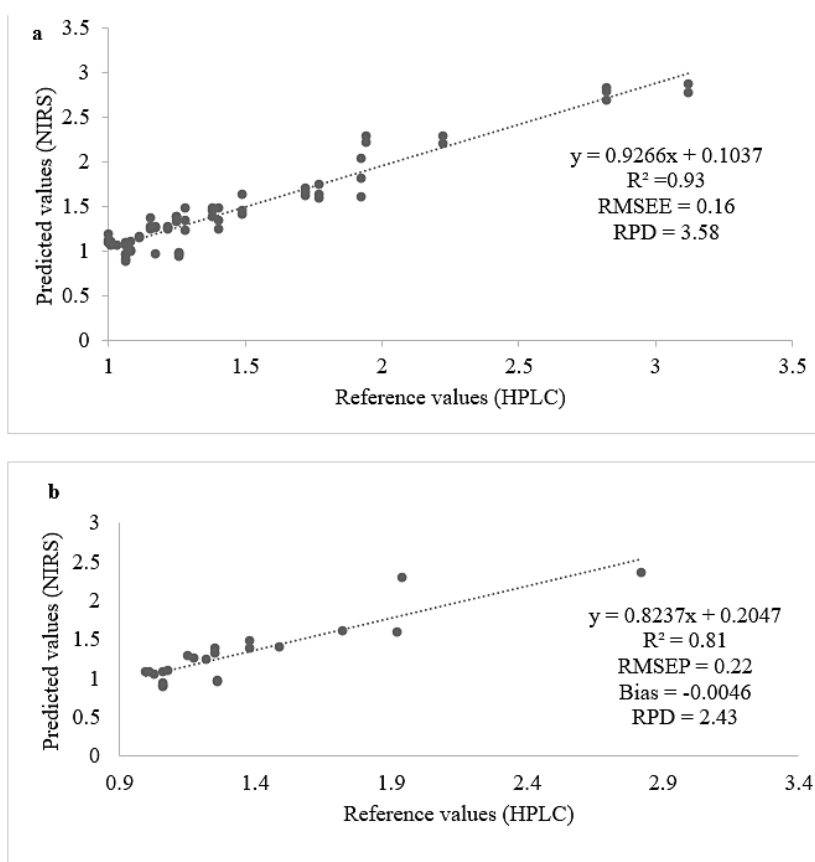


Fig. 5: Calibration (a) and validation (b) of the correlation between reference values and FT-NIR predicted values of retinol (≥ 1.0 mg/kg) in fortified maize flour

Limited studies have been done on applying NIR spectroscopy to predict retinol in maize flour. For

this reason, the discussion of this study's findings was mostly limited to NIR studies on the prediction

of retinol in various food matrices and the prediction of other parameters in maize flour. The work of Kahrıman, Onac, Oner and Egesel⁴⁷ obtained the following prediction performance of fat-soluble secondary metabolites in maize flour, carotenoids ($R^2_v = 0.721$; RMSECV = 3.069) and tocopherol ($R^2_v = 0.515$; RMSECV = 2.0943). The models presented in the current study showed higher R^2_v values and lower prediction errors than those of Kahrıman, Onac, Oner and Egesel⁴⁷ which applied NIR spectroscopy to predict carotenoids and tocopherol in maize flour. Furthermore, Soulat, Andueza, Graulet and Ferlay⁴⁸ reported a lower coefficient of determination ($R^2_v = 0.34$) for the prediction of retinol from cow's milk. The prediction errors reported by Soulat, Andueza, Graulet and Ferlay⁴⁸ were slightly higher (RMSEP = 0.15) than those of model I (RMSEP = 0.09), and slightly lower than those of model II (RMSEP = 0.22). Altogether, the results of this study and those of the studies discussed here demonstrate that there is potential to successfully use NIR spectroscopy to rapidly assess retinol in different food matrices.

Conclusions

The range of retinol values used as reference values in this study (0 mg/kg \pm 0.00 (not detectable) to 4.03 \pm 0.82 mg/kg) comprised the retinol levels that may be routinely encountered in fortified maize flour in the market. Approximately 26.9% of the analysed samples met the retinol fortification standards (0.5-1.4 mg/kg), indicating that a significant proportion of samples did not meet the fortification requirements. Initially, a single calibration model showed poor predictive performance. The model predictive performance was improved by splitting the dataset, hence developing two separate models. The model developed for predicting retinol \geq 1.0 mg/kg illustrated slightly better prediction performance

than the model for predicting retinol < 1.0 mg/kg. The models developed in this study had high R^2 values and low errors. FT-NIR spectroscopy can thus be utilised to adequately estimate retinol in fortified maize flour. NIRS, by replacing time-consuming and laborious wet chemistry laboratory procedures, has the potential to be used for rapid regulatory monitoring of fortification compliance for a large number of samples.

Recommendations

The robustness of the models can be improved in the future by the addition of more calibration data. Furthermore, linear discriminant analysis (LDA) studies should be done to generate an algorithm that will be used to differentiate the two prediction models developed (model I used to predict retinol < 1.0 mg/kg and model II used to predict retinol \geq 1.0 mg/kg). This will help determine which of the two models is to be employed for predicting the amount of retinol in a specific unknown sample. FT-NIRS-based models for predicting B-vitamins in fortified maize flour should also be developed to allow quick analysis.

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Conflict of Interest

The authors declare no conflict of interest.

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