Magna Scientia Advanced Research and Reviews

eISSN: 2582-9394

Cross Ref DOI: 10.30574/msarr

Journal homepage: https://magnascientiapub.com/journals/msarr/

(Research Article)

Validation of an ELISA test kit for the quantitative analyses of total Aflatoxins in spices marketed in Cameroon

Evelyne Nguegwouo [1, 2, *], Azoumboi Abassi Nouhou [2], Abel Wade [3], Gabriel Nama Medoua [1] and Elie Fokou [2]

[1] Center of Research in Food, Food Security and Nutrition, Institute of Medical Research and Studies of Medicinal Plants, Ministry of Research and Innovation, Yaoundé, Cameroon.
[2] Laboratory of food science and metabolism, Department of Biochemistry, Faculty of Science, University of Yaounde 1, Yaoundé, Cameroon.
[3] National Veterinary Laboratory, Ministry of Fisheries and Animal Industry, Yaoundé, Cameroon.

Magna Scientia Advanced Research and Reviews, 2023, 07(01), 086–097

Publication history: Received on 01 December 2022; revised on 21 February 2023; accepted on 23 February 2023

Article DOI: https://doi.org/10.30574/msarr.2023.7.1.0080

Abstract

Aflatoxins (mold-synthetized secondary metabolites) contamination associated with food such as spices continue to be a global problem in the world. The aim of this study was to measure the standard parameters of validation (Linearity, occuracy, repeatability, intermediate precision, reproducibility, limit of detection, limit of quantification and robustness) of the total Aflatoxins (AFt) low-matrix Enzyme-Linked ImmunoSorbent Assay (ELISA) Kit (Lot No. 062218, RENEKABIO, USA) and used this method to evaluate the contamination degree of AFt (AFB_1, AFB_2, AFG_1 and AFG_2) in 4 different spice matrices [white pepper (*Piper nigrum*), black pepper (*Piper nigrum*), *njansang* (*Ricinodendron heudelottii*) and *super épice mbongo* (Mix of *Aframomum citratum, Monodora myristica and Afrostyrax lepidophyllus*] marketed in Cameroon and commonly used as ingredients in Cameroonian sauces. The test was performed as a solid phase direct competitive ELISA using a horse radish peroxidase conjugate as the competing, measurable entity.

Data obtained from validation showed linearity in the range between 0 µg kg^{-1} and 20 µg kg^{-1}; an accuracy marked by a recovery rate ranging from 95.09 % to 110.29 %; a coefficient of variation (CV) of repeatability of 3.14 %, a coefficient of variation (CV) of intermediate precision of 6.82 % and a coefficient of variation (CV) of reproducibility of 13.96 %. Detection and quantification limits of 0.15 µg kg^{-1} and 0.50 µg kg^{-1} were determined, respectively. The robustness test revealed a critical point of the method as an incubation for 30 minutes at room temperature. The application of the method to our spices analyzed revealed 100 % contamination of 2.34 ± 0.25 µg kg^{-1} (white pepper), 2.37 ± 0.38 µg kg^{-1} (black pepper), 2.58 ± 0.87 µg kg^{-1} (*Njansang*) and 6.60 ± 5.21 µg kg^{-1} (*Super épice mbongo*).

The RENEKABIO low matrix ELISA kit used for this research enables an accurate, reliable and faithful screening and quantification of AFt in spice matrices. This will be an asset in quality control of spices marketed in Cameroon.

Keywords: Spices; Total Aflatoxins; ELISA method; Validation

1. Introduction

Spices are non-caloric substances used for diets and to bring a lot of flavor to food [1, 2] The Cameroonian cuisine uses a plethora of spices, some of which are imported and others are locally produced such as white pepper, black pepper, *njansan*, ginger, cinnamon, anise, clove etc. [3]. After harvest, they are stored in shops, storehouses, warehouses and households. However, the storage conditions of these spices are favorable for the development of molds, which produce mycotoxins that contaminate spices [4-6]. Ingestion of these toxins leads to diseases known as "mycotoxicoses",

* Corresponding author: Evelyne Nguegwouo

Copyright © 2023 Author(s) retain the copyright of this article. This article is published under the terms of the Creative Commons Attribution Liscense 4.0.

manifested by certain disorders (vomiting, abdominal pain, convulsions, coma ...) sometimes having a fatal outcome [7-8]. There are more than 400 types of mycotoxins [8-9] those mainly found in Africa are Aflatoxins, Ochratoxin A and Fumonisins. Of all these toxins, Aflatoxins are the most studied and the most feared, as some such as Aflatoxin B_1, the most toxic, is a class 1 carcinogen [10] naturally produced by a fungi of the family *Aspergillus*, especially *Aspergillus flavus*. This form of Aflatoxin is directly related to adverse health effects such as liver cancer and cirrhosis depending on the degree of exposure [11].

Conventional chemical methods for the analysis of Aflatoxins such as thin layer chromatography, column chromatography, high performance liquid chromatography, mass spectrometry are laborious, time-consuming, and expensive and require intensive sample cleaning [12]. New efforts have been made to develop and use the ELISA or Enzyme-Linked Immunosorbent Assay method for the determination of Aflatoxins in feeds and foodstuffs. ELISA methods although in some instances give acceptable performances have not been validated at sufficiently low levels and are limited in the range of matrices examined [13]. Therefore, an extensive study on the accuracy and precision of ELISA method over a wide range of commodities is needed and a full validation for an ELISA method is essential and critical [13]. Several research studies have been done on the level of mycotoxin contamination of spices and the results reveal a high level of contamination that sometimes exceeds recommended standards [14]. In order to prove that the protocol of an ELISA RENEKABIO test kit is sufficiently accurate and reliable to have confidence in the results provided for decision-making, this study is conducted to implement a quantitative ELISA test and to validate it, for the determination of total Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) in spices marketed in our country.

2. Material and methods

2.1. Sampling

We carried out a "snow ball" sampling nearby major spice [White pepper (*Piper nigrum),* Black pepper (*Piper nigrum),* Njansang (*Ricinodendron heudelottii*) and *Super épice mbongo* **(***Aframomum citratum, Monodora myristica, Afrostyrax lepidophyllus*)] distributors of some markets in *Yaoundé,* Cameroon) (*Mokolo, Mfoundi, Mvog-bi, marché huitième* and *Essos market*). The sampling was done during May 2019 according to the European Commission Regulation [15], which stipulates that the collection of elementary samples for mycotoxicological analyzes must be done at various points distributed over the entire batch or sublot. Then the overall sample is obtained by uniting the elementary samples. In each market, we took 4 global samples of 500g (one global sample per spice) and a total of 16 global samples. They were then tagged and sent to the Laboratory of Food Study and Quality Control in the Centre of Research in Food, Food Security and Nutrition of the Institute of Medical Research and Medicinal plants Studies in Cameroon where they followed various treatments.

2.2. Treatment of samples

The samples were dried in a "MEMMERT" brand ventilation oven at 50 ° C for 3 days, in order to facilitate grinding and to obtain a fairly fine powder having a particle size of at least 0.05. These samples were then ground in an electronic machine brand "Sanford SF5664CG" until the finest possible particle size. This done, the powder was introduced into hermetically sealed plastic containers and kept in the freezer for various analyzes.

2.3. Extraction of total Aflatoxins in spice samples

The extraction of total Aflatoxins in our samples was done with 80 % methanol as described by the kit "Total Aflatoxin Low Matrix ELISA Lot No: 062218". Firstly, we prepared the extraction solvent by adding 800 mL of Methanol (with a degree of purity of 100 %) to 200 mL of distilled water in an Erlenmeyer flask and homogenized with a magnetic stirrer for 5 minutes. Then we took 5 grams of our ground sample to which we added 25 mL of the extraction solvent, after which the mixture was vortexed for 5 minutes, centrifuged at 3500 rpm for 5 minutes and finally, the supernatant was extracted using a micropipette tip in a hemolysis tube. 5 mL of the extract was filtered using a N°1 Wattman paper and 1mL of the filtrate removed and diluted by adding 9 mL of the washing buffer. The latter was covered with aluminum foil and used for various analyzes. The total dilution factor was 1:50.

2.4. Determination of total Aflatoxins by the ELISA method

The total Aflatoxins assay in our spice samples was done by the ELISA (Enzyme-Linked ImmunoSorbent Assay) method. The ELISA kit (Total Aflatoxin low-matrix ELISA Kit, Lot No. 062218, RENEKABIO, USA) used for the detection and quantification of total Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) is said to be by direct competition.

For precaution, all the manipulations were done under the hood in order to avoid poisoning. At First, all the reagents and samples were brought back to room temperature and the wash buffer was brought to 1L with distilled water. Then, the mixing wells were placed in a micro-well holder and an exact number of antibody coated microtiter well were also placed in another micro-well holder. In each mixing well, we simultaneously dispensed 200 µL of the sample diluent using a monoclonal micropipette and then 100 µL of each standard (concentration 0.05 µg mL^{-1}, 0.02 µg mL^{-1}, 0.05 ηg mL^{-1}, 0.1 ηg mL^{-1}, 0.2 ηg mL^{-1} and 0.4 ηg mL^{-1} respectively) and prepared samples, all mixed by priming the micropipette 3 times. Using a new tip, we transferred 100µL of the contents of each mixing well into the corresponding antibody-coated microtiter wells. The whole was covered with aluminum foil and incubated for 30 minutes at room temperature. After incubation, the contents of each well were decanted into a discard basin and washed with washing buffer 3 times (this consisted of introducing into each microtiter 300 µL of the wash buffer with a micropipette, followed by the emptying of these and the whole process done three times). The residual water from the wells was subsequently removed by tapping the microwells face down on a layer of absorbent towels. After that, 100 µL of conjugate (Aflatoxin HRP-Conjugate) was added to each well and the whole covered with aluminum foil and then incubated again at room temperature for 30 minutes. After the incubation time, we washed the microwells again with the washing buffer 3 times. Subsequently, we added 100 µL of the substrate reagent (tetramethylbenzidine) to each well, covered with aluminum foil and incubated at room temperature for 10 minutes. After the incubation, 100 µL of stop solution (acid solution) was added per well and the optical densities read at 450 nm using the Bexim ELISA microplate reader (LEDETECT 96). The total incubation time of the kit was 1 hour 10 minutes.

2.5. Determination of the standard validation parameters of the ELISA test

2.5.1. Choice of the area of validity of the method (Linearity)

Validation interval: the validation range was from 0.00 ηg mL^{-1} to 0.4 ηg mL^{-1} (Table 1).

Table 1 Validation domain

Level	Concentration (ηg mL^{-1})
1	0.0
2	0.02
3	0.05
4	0.1
5	0.2
6	0.4

2.5.2. Experimental design of the other parameters of validation of the ELISA kit

The validation plan is formed on one side of I = 2 series (coded day 1 and day 2) for accuracy and intermediate fidelity with K = 4 concentration levels (X_1, X_2, X_3 and X_4) and K = 1 concentration level (X_5), J = 3 repetitions and J = 10 repetitions per day respectively. On the other hand, it consists of I = 1 series (coded day 1), with K = 1 level of concentration and J = 10 repetitions per day (for repeatability, reproducibility and limit of detection) and J = 3 repetitions per day (for robustness) (Table 2).

Table 2 Experimental design of other parameters of validation

Parameter	Level (K)	Concentration Level (X) (µg kg^{-1})	Days (I)	Number of repetitions (J)
Limit of detection/quantification	1	X_1	1	10
Accuracy	1	X_2	1	3
			2	3
	2	X_3	1	3
			2	3
	3	X_4	1	3
			2	3
	4	X_5	1	3
			2	3
Precision (repeatability)	1	X_6	1(morning)	5
			1(evening)	5
Precision (Intermediate precision)	1	X_6	1	10
			2	10
precision (reproducibility)	1	X_6	1	10
Robustness	1	X_7	1 (37°C)	3
			1 (15min)	3

2.5.3. Limit Of Detection (LOD)

The detection limit of our ELISA test was determined by a method described by the "Center of Expertise in Environmental Analysis of *Québec*" [16].

Exactly 10 replicas of the white pepper sample (from the samples analyzed above) with a concentration of X_1 = 2.28 µg kg^{-1} were analyzed following all the steps of the analytical method, after which we calculated the resulting concentrations and their averages. From the mean of the concentrations, we calculated the standard deviation and the coefficient of variation (CV) using the formulas below.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(m - x_i)^2}{n-1}}$$

$$CV(\%) = \frac{s}{m} \times 100$$

m: Arithmetic mean of a series of measurements;
xi: Individual measures;
n: Number of measurements;
s: Standard Deviation of a series of measurements
CV: Coefficient of Variation

The limit of detection was calculated from the following formula;

$$LOD = 3 \times s$$

2.5.4. LOD: Limit of detection

S : standard deviation of a series of measurements

The detection limit being obtained, we went forth by calculating the validity of the approach (compliance ratio (R)). In general, if the result of the calculation for a ratio R used to establish a limit of detection is not greater than 4, the procedure for establishing the limit of detection is applied once more with a sample that has a higher concentration. We calculated the compliance ratio with the formula below;

$$R = m \times LOD$$

R: Compliance ratio;
m: Arithmetic mean of the n replicas;
LOD: Limit of detection

2.5.5. Limit Of Quantification (LOQ)

The LOQ was determined by a method described by [16]. It was obtained from the standard deviation in the evaluation of the limit of detection. The following formula was used to calculate the limit of quantification;

$$LOQ = 10 \times s$$

LOQ: Limit Of Quantification
s: Standard Deviation

2.5.6. Accuracy (systematic error)

The accuracy of our ELISA test was determined by a method described by [16].

About 100 µL of each certified reference material (standard) with a concentration level of X_2 = 0.05 ηg mL^{-1}, X_3 = 0.1 ηg mL^{-1}, X_4 = 0.2 ηg mL^{-1} and X_5 = 0.4 ηg mL^{-1}, were analyzed by the ELISA method (whose procedure is described above) in 6 tests (3 on the first day and the other 3 on the second day), then the concentrations of each test, followed by their averages were calculated. The accuracy of this method has been determined and expressed in terms of bias and recovery rate by the formulas presented in Table 3 below.

Table 3 Validation Values for Accuracy

Parameters of accuracy	Expression	Equation
Absolute accuracy bias	B	$\bar{\bar{y}} - V$
Relative accuracy bias	B(%)	$(\bar{\bar{y}}$ - V) / V x 100
Recovery rate	TR(%)	$\bar{\bar{y}}$ /V x100

where

$\bar{\bar{y}}$ = arithmetic mean, *V* = given concentration

2.5.7. Precision (random error)

The precision of our ELISA test was determined by a method described by [16].

2.5.8. Repeatability

Ten replicas of the white pepper samples with a concentration of X_6 = 2.70 µg kg^{-1} were analyzed by the same analyst, the same apparatus and the same day at all the stages of the analytical method. Five replicas were analyzed in the morning and five more in the evening. Then we calculated the mean (y_1) and the standard deviation (δ_1) of the concentrations obtained, followed by their coefficient of variation (CV_r). The value of the repeatability was determined from the equation below.

$$CV_r = \delta_1/y_1 \times 100$$

CV_r = Coefficient of Variation of repeatability
$\boldsymbol{\delta_1}$ = Standard Deviation of a series of measurements referring to repeatability

y_1= Mean

2.5.9. Intermediate precision

Twenty replicas of the white pepper samples with a concentration of X_6 = 2.70 µg kg^{-1} were analyzed according to all the steps of the analysis by the same analyst, the same apparatus but on different days. Ten samples were analyzed the same day and the other 10 the next day. Then we calculated the mean (y_2), the standard deviation (δ_2) and the coefficient of variation (CV_i) of the concentrations obtained. The value of the intermediate precision was determined from the equation below.

$$CV_i = \delta_2 / y_2 \times 100$$

CV_i = Coefficient of Variation of intermediate precision
δ_2 = Standard Deviation of a series of measurements referring to intermediate precision
y_2= Mean

2.5.10. Reproducibility

Ten replicas of the white pepper samples with a concentration of X_6 = 2.70 µg kg^{-1} were analyzed according to all the steps of the analysis in another laboratory (the quality control laboratory of the Center for Research in Nutrition of the Institute of Medical Research and Studies of Medicinal Plants). The analysis were done by a different analyst, different devices and on a different day. Subsequently, we calculated the mean (y_3), standard deviation (δ_3) and coefficient of variation (CV_R) of the concentrations obtained. The reproducibility value was determined from the equation below.

$$CV_R = \delta_3 / y_3 \times 100$$

CV_R = Coefficient of Variation of reproducibility
δ_3 = Standard Deviation of a series of measurements referring to reproducibility
y_3= Mean

2.5.11. Robustness

For the determination of the robustness of our method, we chose the following parameters: incubation temperature (37 ° C) and incubation time (15 min) according to a method proposed by the Codex Alimentarius Commission [17]. Three sets of three samples of white pepper with a concentration of X_7 = 2.26 µg kg^{-1} were analyzed according to all the steps of the analysis method at an incubation temperature of 37 ° C, an incubation time of 15 minutes and at an initial volume of the measured extract of 50 µl respectively. Then, concentrations and their averages were calculated.

2.6. Determination of total Aflatoxins in prepared spice samples by the validated method.

After the validation of the ELISA test used, we applied it in the assay of the spice samples collected according to the protocol described above. A total of 16 global spice samples were assayed.

2.7. Statistical analysis

The results of the various analyzes were presented as mean ± standard deviation with a significance level of 5 % and in the form of coefficient of variation (CV). The statistical analysis was performed by the IBM / SPSS 20.0 for Windows software using the ANOVA test to compare averages and the Microsoft Office Excel 2013 software was used for graphical representations.

3. Results

3.1. Linearity

Figure 1 below is the calibration curve of 6 standards assayed by the ELISA method. This figure is the representation of y (percentage of binding, obtained by the division of the optical densities (OD) of each standard by the OD of the standard at the concentration 0 ηg mL^{-1}, and the whole multiplied by 100) against the concentrations of the standards (0 ηg mL^{-1}, 0.05 ηg mL^{-1}, 0.1 ηg mL^{-1}, 0.2 ηg mL^{-1} and 0.4 ηg mL^{-1} respectively) provided in the kit. It has an equation of the form y = ax + b with y =% of binding, x = concentration of AFt to be determined, and b constants generated when drawing the line in Excel. The cloud of points obtained enabled us to determine the equation of the curve y = -4.1366x + 98.036, with a correlation coefficient (R^2) of 0.9856.

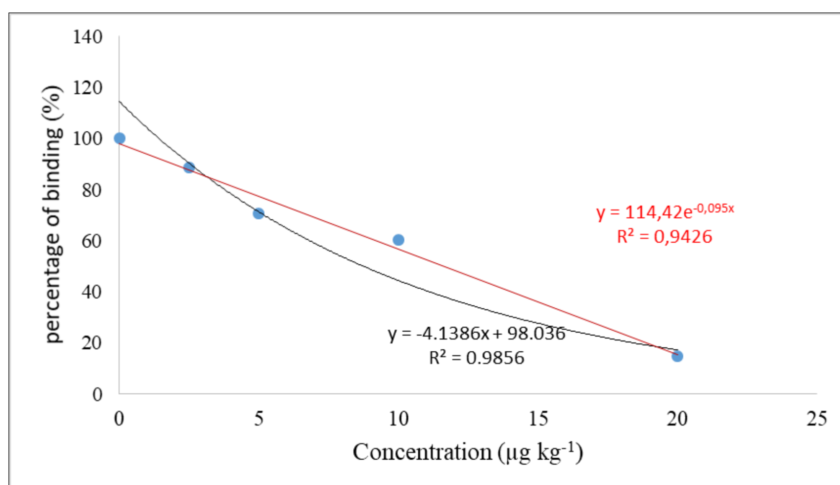

Figure 1 Calibration curve of standards assayed by the ELISA method

3.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The detection limit and the limit of quantification of our quantitative ELISA kit are shown in Table 5 below. According to this table, the smallest amount that can be detected by our ELISA kit is 0.14 µg kg^{-1}. This result was obtained from 10 tests having an average concentration of 2.35 µg kg^{-1}, a standard deviation of 0.05 µg kg^{-1} and a repeatability CV of 2.11 %. The calculation of the compliance ratio gave us a value of 15.77 indicating that the concentration used was good and fair. The limit of quantification found by our method is 0.5 µg kg^{-1}. The latter was determined through 10 tests having an average concentration of 2.35 µg kg^{-1}, a standard deviation of 0.05 µg kg^{-1} and a repeatability CV of 2.11 %.

Table 4 Limits of detection and quantification of the kit

Parameter	Mean (µg kg-^{1})	Standard deviation (µg kg^{-1})	Concentration (µg kg^{-1})	CV	compliance ratio (R)
LOD	2.35	0.05	0.15	2.11	15.77
LOQ	2.35	0.05	0.50	2.11	--------

3.3. Accuracy

The values of the parameters of accuracy are presented in Table 6 below. Based on these results, the absolute accuracy bias shows deviations ranging from -0.49 to 0.51 µg kg^{-1}. As for the relative accuracy bias or relative error, we obtained values ranging from -4.91 to 10.29 % and a recovery rate ranging from 95.09 to 110.29 %.

Table 5 Results of the parameters of accuracy

Concentration levels (ηg mL^{-1})	Parameters of accuracy			
	Absolute accuracy bias (µg kg^{-1})	Relative accuracy bias or relative error (%)	Recovery rate (%)	Standard deviation (µg kg $^{-1}$)
0.05	-0.08	-3.12	96.88	0.46
0.1	0.51	10.29	110.29	1.03
0.2	-0.49	-4.91	95.09	0.44
0.4	0.24	1.22	101.22	0.16

According to European Commission 2002/658 / EC [18], in the case of repeated analyzes of a certified reference material, the difference between the corrected average weight fraction of the experimentally determined recovery and the certified value shall be within the limits set out in Table 7 below. This table stipulates that for a certified reference

material (standard) with a concentration between 1 and 10 μg kg^{-1} and a concentration equal to or greater than 10 μg kg^{-1}, the recovery must be between 70 % - 110 % and 80 % -110 % respectively.

Table 6 Limits on the recovery rate at given concentrations

Concentration fractions (μg kg^{-1})	**Recovery rate interval (%)**
< 1	-50 to + 20
>1-10	-30 to + 10
≥10	-20 to +10
Weight fraction (ppb)	**Beach (%)**
< 1	-50 to+20
>1-10	-30 to + 10
≥10	-20 to +10

3.4. Repeatability, intermediate precision and reproducibility

As shown in Table 8 below, the coefficient of variation of repeatability that reflects intraday precision is 3.14 % with a standard deviation of 0.08 μg kg^{-1}. That of the intermediate precision which reflects the inter-day precision is 6.82 % with a standard deviation of 0.18 μg kg^{-1} and finally the CV of reproducibility is 13 % with a standard deviation of 0.32. This difference in the coefficients of variation could be due to errors of inter-day and intraday manipulation. Moreover, the difference between the devices and tools of the two laboratories (National Veterinary Laboratory and CRASAN's (Center for Research of Food, Food Security and Nutrition of the Institute of Medical Research and Studies of Medicinal Plants) Quality Control Laboratory), the handling conditions presented by the two laboratories and the laboratory technicians could be at the origin of these differences.

Table 7 Results of the precision's parameters [18]

Parameter of precision	**Mean (μg kg^{-1})**	**Standard deviation (μg kg^{-1})**	**Coefficient of variation (CV) /%**
Repeatability	2.68	0.08	3.14
Intermediate precision	2.61	0.18	6.82
Reproducibility	2.28	0.32	13.96

The requirements of FSA[19] on the coefficients of variation (CV) to be respected for the repeatability, intermediate precision and reproducibility of quantitative methods are presented in Table 9 below.

Table 8 FSA requirements for coefficients of variation (CV) of repeatability, intermediate precision and reproducibility **[19]**

Concentration (μg kg^{-1})	**CV of repeatability and intermediate precision (%)**	**CV de reproducibility (%)**
1	≤ 20	≤ 30
10	≤ 20	≤ 30
100	≤ 15	≤ 25
1000	≤ 12	≤ 20

3.5. Robustness

Table 10 below presents the robustness test data obtained by our ELISA method. Regarding the robustness parameter referring to incubation for 15 minutes at room temperature, we obtained an average of 3 tests amounting to 0.75 ± 0.13

μg kg^{-1}. Regarding the parameter of robustness having time of incubation 30 minutes at 37 ° C, we obtained an average of 1.41 ± 1.03 μg kg^{-1} for 3 tests.

Table 9 Robustness parameter results

Parameters of robustness	test 1 (μg kg^{-1})	test 2 (μg kg^{-1})	test 3 (μg kg^{-1})	mean (μg kg^{-1})	Concentration in normal conditions (μg kg^{-1})	Lower limite (-30%) (μg kg^{-1})	Upper limit (+30%) (μg kg^{-1})
Incubation for 15 min at room temperature	0.63	0.75	0.88	0.75 ± 0.13	2.26	1,58	2,94
Incubation at 37°C for 30 minutes	2.51	1.24	0.48	1.41 ± 1.03	2.26	1.58	2.94

3.6. Quantitative total Aflatoxins in spices samples

The calibration curve (Figure 1) allowed us to calculate the total Aflatoxins concentrations in our spice samples, the results of which are shown in figure 2. As this shows, the samples of white pepper, black pepper, *njansang* and *super épice mbongo* are 100 % contaminated, with the contamination of white pepper, black pepper and *njansang* significantly different from that of *super épice mbongo.* Total Aflatoxins concentration in white pepper had an average of 2.34 ± 0.25 μg kg^{-1}. In black pepper, we had average concentration of total Aflatoxins of 2.37 ± 0.38 μg kg^{-1}. *Njansang* had total aflatoxins concentrations average of 2.58 ± 0.87 μg kg^{-1}. The *super épice mbongo* contains average total Aflatoxins of 6.60 ± 5.21 μg kg^{-1}.

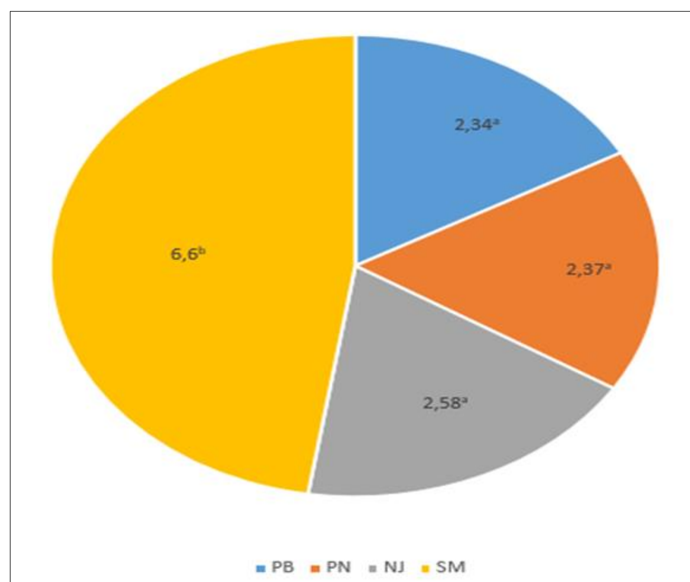

Figure 2 Mean of total Aflatoxins concentrations in spices samples

4. Discussion

The pattern of the calibration line (figure 1) shows that it is linear in the range between 0 μg kg^{-1} and 20 μg kg^{-1} with a correlation coefficient (R^2) of 0.99. This implies that the method in question can quantify total aflatoxins in a concentration range going from 0 μg kg^{-1} to 20 μg kg^{-1}. These values are similar to those obtained by Raquel *et al.* [20] who worked on the development and validation of an HPLC method for the detection and quantification of Ochratoxin A in green tea and obtained a linearity ranging from 3 μg kg^{-1} to 23 μg kg^{-1}.

The detection limit obtained (0.15 µg kg^{-1}) is lower than the maximum limit of Aft in spices set by the European Commission (10 µg kg^{-1}) and the Codex Alimentarius Commission (FAO / WHO) (20 µg kg^{-1} at 30 µg kg^{-1}) [21]. Thereby, the ELISA test can detect concentrations far below the limits set by the regulation. In addition to that, the limit of detection obtained is lower than that obtained by the HPLC method in the determination of AFt in wheat, carried out by Felipe *et al* [22] which obtained a detection limit of 0.6µg kg^{-1}.The limit of quantification determined is lower than the maximum limit of Aft in spices set by the European Commission (10 µg kg^{-1}), thus showing that the method is capable of quantifying AFt in spices at very low concentrations below the regulatory limit. Furthermore, this limit is lower than that obtained by Felipe *et al [*22], who noted a limit of quantification of 1.2 µg kg^{-1}, by the HPLC method.

Thus, the concentrations of our standards used being included in these ranges (2.5 – 20 µg kg^{-1}), we can qualify our method as being fair, because it gave us a recovery rate ranging from 95.09 to 110.29 %, which is within the range provided by the regulations.

According to the table 9, samples with concentrations ranging from 1 to 10 µg kg^{-1} must have a CV ≤ 20 % for repeatability and intermediate precision and a CV ≤ 30 % for reproducibility. Those with a concentration of 100 µg kg^{-1} and 1000 µg kg^{-1} must have CV ≤ 15 % and CV ≤ 12 % for repeatability and intermediate precision respectively and CV ≤ 25 % and ≤ 20 % for reproducibility respectively. The concentration of the white pepper sample analyzed for this parameter was 2.70 µg kg^{-1}. This concentration being found between 1 and 10 µg kg^{-1} must according to the regulations below have a CV ≤ 20 % for repeatability and intermediate precision and a CV ≤ 30 % for reproducibility. In Table 8 above, we obtained on one hand a CV of repeatability and intermediate fidelity of 3.14 % and 6.82 % respectively and on the other hand a CV of reproducibility of 13.96 %. These CV obtained are respectively < 20 % and < 30 %. Thus, based on the regulations in force above, we can qualify our method as being faithful.

According to standards established by the Codex Alimentarius Commission [17], on guidelines for performance criteria and validation of methods for the detection, identification and quantification of specific DNA sequences and specific proteins contained in food, the response of a robustness test in the presence of these small changes (incubation time, incubation temperature) should not deviate by ± 30 percent from the response obtained in the original conditions. The result obtained for the normal handling conditions (incubation at room temperature for 30 min) was 2.26 µg kg^{-1}. From the latter we established a lower bound of 1.58 µg kg^{-1} (-30%) and an upper limit of 2.94 µg kg^{-1} (+ 30 %). By observing the results obtained after a modification of the incubation time and the incubation temperature (Table 10), we can affirm that the kit is not robust neither when the incubation time is reduced to 15 minutes, nor even when the incubation temperature is increased to 37 ° C. This could be explained by the fact that the incubation time of 15 minutes is short and insufficient to allow AFt to bind to the fixed antibodies in the microtiter wells. And also by the fact that the incubation temperature of 37 ° C is not optimal for the binding between antibody and AFt.

The absoluteness of Aflatoxin contamination in spices (white pepper, black pepper, *njansang* and *super épice mbongo*) sold in the city of Yaoundé could be attributed in part to the hot and humid weather conditions, which could favor the proliferation of toxigenic fungi. This environment provides optimal conditions for mold growth and subsequent accumulation of mycotoxins in spices over time as spices are a natural medium for mold growth. Moreover, agricultural practices such as irrigation, harvesting and post-harvest handling of spices could also contribute to contamination by toxigenic fungi. This can be remedied by using good agricultural practices such as the use of genetically modified seeds to resist mold growth, crop rotation, cleaning of grain harvesting or transporting machinery and storage silos. These levels of total Aflatoxins found in our different spice samples are below the maximum limit set by the European Commission [23], which states that spices intended for human consumption must not exceed a rate greater than 10 µg kg^{-1}. In addition to that, the Codex Alimentarius Commission, following a study conducted in 2018, has set a maximum total Aflatoxins limit of 20 to 30 µg kg^{-1} in spices in general [20]. This would also mean that the deleterious nature of the spices which are the subject of our study is negligible. Water content, which is one of the fundamental parameters in mold development and AF production, may also explain lower concentrations than the observed regulation. These water contents obtained not being above the recommended limit (12 to 13 g per 100g of dry matter), explains the low production of AFt in our spice samples, due to a limited development of the molds producing them. Moreover, since the water content is directly proportional to the activity of water, as shown by the sorption isotherm curve, it normally indicates a small Aw which cannot reach the threshold value (0.99) of mold growth and production of Aflatoxins.

5. Conclusion

The goal of our study was to validate a quantitative ELISA for the determination of total aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) in spices, it appears that the ELISA test kit performed is specific, accurate and precise. Furthermore, it has a limit of detection and quantification at a relatively small concentration thus allowing screening at almost negligible doses. Moreover, the ELISA test performed has a critical point as being incubation at room temperature for 30 minutes. All the

samples that took part in the screening are contaminated at a rate lower than that prescribed by the European Union's current regulations. Based on these findings, the ELISA test kit produces reliable results and can be used for control analyzes. The spices (white pepper, black pepper, *njansang* and *super épice mbongo*) sold in the local markets of the city of *Yaoundé* are save in what aflatoxins are concerned, because they have concentrations below the maximum limit set by the European Union (<10 µg kg^{-1})

Compliance with ethical standards

Acknowledgments

The authors specially thank the staff of the markets involved in this study.

Disclosure of conflict of interest

The authors declare that they have no competing interests.

Funding

No funding was obtained for this study.

Availability of data and materials

All datasets on which the conclusions of the manuscript rely are presented in the paper.

References

[1] UNIDO and FAO. Herbes, spices and essential oils, post-harvest operations in developing countries, Austria, 2005. pp. 70.

[2] Uhl R.S. Hand book of spices, seasonings, and flavorings, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2006; ISBN 978-1-4200-0436-6.

[3] Pegie A., Spice mix for *mbongo*, 2015, available on http://www.alicepegie.com, consulted on the 07 january 2019.

[4] Abd El-Tawab AA, El-Diasty ME, Khater FD, Al-baaly MY. Mycological identification of some fungi isolated from meat products and spices with molecular identification of some Penicillium isolates, Adv. Anim. Vet. Sci. 2020; 8:124 – 129.

[5] Rajarajan PN, Rajasekaran KM, Devi N. K. A. Aflatoxin Contamination in Agricultural Commodities. IJPBR. 2013;1:148-151.

[6] Iha HM, Trucksess WM. Management of mycotoxins in spices, J. AOAC Int. 102 2019;102: 1732 – 1739.

[7] Essawet J. Aflatoxin contamination of spices sold collected from local market in tripoli. Int. J. Curr. Microbiol. App. Sci. 2017;6: 1468-1473.

[8] Haque AM, Wang Y, Shen Z, Li X, Saleemi KM, He C. Mycotoxin contamination and control strategy in human domestic animal and poultry : A review, Microb. Pathog. 2020;142: 104095.

[9] FAO, Worldwide Regulations for Mycotoxins. FAO, Food and Nutrition Papers, Rome, Italy, v 81, 2004, pp. 183.

[10] IARC, Monographs on the evaluation of carcinogenic risks to human. Some traditional herbal medicine, some mycotoxins, naphthalene and styrene, Lyon, France, No. 82, 2002.

[11] Ojuri TO, Ezekiel C. Sulyok M, Ezeokoli TO, Oyedele AO, Ayeni IK. Eskola KM, Sarkanj B, Hajslovà J, Adeleke AR. Assessing the mycotoxicological risk from consumption of complementary foods by infants and young children in Nigeria, Food Chem. Toxicol. 2018;121: 37 – 50.

[12] Gilbert J, Anklam E. Validation of analytical methods for determining mycotoxins in foodstuffs. Trac-Trend Anal. Chem. 2002 ;21: 468-486.

[13] National Health Security Agency (NHSA), validation guide for analytical methods. NHSA/PR3/07/01 version a, 2015, pp. 67.

[14] Erdogan A. The aflatoxin contamination of some pepper types sold in Turkey, Chemosphere. 56 2018 ;56: 321-25.

[15] European Commission (EC) No 401/2006, laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. OJ L, 70, 2006, pp. 12.

[16] Center of expertise in environmental analysis of *Québec* (CEEAQ), Protocol for the validation of a method of analysis in chemistry, DR-12-VMC, *Quebec.* Ministry of sustainable development, environment and the fight against climate change, 2015, pp. 29.

[17] CAC. Guidelines for performance criteria and validation of methods for the detection, identification and quantification of specific DNA sequences and specific proteins in foods, 2010, pp. 23.

[18] European commission (EC), Decision 2002/658/EC: methods of application of the directive 96/23/EC of the council with regard to the performance of the methods of analysis and the interpretation of the results, OJEC. L221/8, 2002.

[19] French standardization agency (FSA), Validation of alternative methods of analysis, application to the food industry, 2017, pp. 65.

[20] Raquel DCCB, Uekane TM, Cunha CP, Valnei SC, Janaína MR, Godoy R LO, Marcus HCC. Development and validation of a method for detection and quantification of ochratoxin A in green coffee using liquid chromatography coupled to mass spectrometry, Ciência Tecnol. Alime. 32 2012;32: 775-782.

[21] FAO/OMS, Proposed draft maximum levels for total aflatoxins and ochratoxin A in nutmeg, chili and paprika, ginger, pepper and turmeric and associated sampling plans: Joint FAO/WHO food standards programme codex committee on contaminants in foods, Twelfth Session Utrecht, The Netherlands, CX/CF 18/12/11, 2018, pp. 18.

[22] Felipe MT, Thaís BS, Direito GM, Fraga ME, Saldanha T. In-house validation of a method for determining aflatoxins B1, B2, G1 and G2 in wheat and wheat by-products. Pesqui. Agropecu. Trop. 2014 ;44: 255-262.

[23] European Commission (EC) No 2174/2003, Amending Regulation (EC) No 466/2001 as regards aflatoxins (Text with EEA relevance), 2003.