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# Analysis of aflatoxin B1 in Brazil nuts: method optimization and validation

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**Abstract:** A method for the determination of aflatoxin B1 was validated according to criteria by SANTÉ 11945/2015. The samples were extracted with acetonitrile and the cleaning step was not performed. Quantification was made by liquid chromatography with fluorescence detector and pre-column derivatization with trifluoroacetic acid. No matrix effect was observed and linearity was obtained in the range of 5 - 80 µg $L^{-1}$. The recoveries ranged from 73% to 85% with a coefficient of variation of less than 5%. The quantification limit was 6 µg $kg^{-1}$ and the measurement uncertainty was ± 0.18 µg $kg^{-1}$ for this level. This method was applied to 14 real samples purchased in the local market and contamination was found in one sample above the limit tolerated by the legislation (59 µg $kg^{-1}$). It is a low cost method with good sensitivity and easy to execute.

**Keywords:** validation, liquid chromatography, pre-column derivatization, aflatoxin B1, Brazil nuts

## INTRODUCTION

Mycotoxins are defined as low-molecular-weight natural products produced by fungi as secondary metabolites[1]. The great diversity in their chemical structure can be correlated with the variability on their toxicities and target organs[2]. Aflatoxin is most frequently produced by *Aspergillus flavus* and *A. parasiticus*[3-4], and in addition to liver cancer, it has been associated with acute poisoning, immune-system

dysfunction and stunted growth in children[5]. Approximately 18 aflatoxins analogues are known, being the most toxic analogues the B1, B2, G1 and G2, although all aflatoxins have carcinogenic, teratogenic, mutagenic and immunosuppressive effects[6]. The International Agency for Research on Cancer (IARC) classifies aflatoxin B1 (AFB1) as the highest carcinogenic natural compound with hepatotoxicity and cumulative effects[7].

According to Food and Agriculture Organization (FAO), more than 25 % of the world's agricultural production is contaminated with mycotoxins[8]. Thus, several actions have been initiated worldwide aiming control mycotoxin contamination, such as regulatory limits, sampling and analytical procedures, decontamination, and diversion to less risk uses for contaminated product[9-10].

Brazil nut is an important commodity in the north of Brazil, but the production still considered extractive and with low technological level associated to the inadequate conditions of nut management leads to aflatoxin contamination. Recently was reported that Brazil nuts are preferred substrate of Aspergillus[11]. So, regulations have been implanted to monitor the levels of contamination, mainly based on several analytical methods, were realised throughout the last decades for the identification and accurate quantification of single or chemically related mycotoxins in different matrices[12]. In Brazilian legislation, Brazil nuts in shell have a limit for aflatoxins contamination[13] of 20 μg $kg^{-1}$ .

Several approaches have been reported for the determination of aflatoxins such as capillary electrophoresis[14], thin layer chromatography[15], high performance liquid chromatography (HPLC)[16] and enzyme-linked immunosorbent assay[17].

Many of the pioneering studies on mycotoxins relied on thin-layer chromatography[18]. It was an extremely powerful, rapid, low-cost, and one of the most widely used separation techniques in aflatoxin analysis before HPLC techniques became popular[19]. Modern techniques as gas chromatography (GC) could be used for mycotoxin detection[20]. The insufficient volatility of analytes associated with lack of suitable derivatization agents or easier-to-use methods are the mainly limit for using GC in mycotoxins quantification[21].

Nowadays, HPLC technique is the most popular method used for mycotoxins analysis, and has been established as official AOAC methods[22]. HPLC with fluorescence detection (FD) becomes widely used due to available high-resolution columns and the sensitivity of fluorescence detectors allied with its potential for automation[23]. HPLC - FD is largely used in aflatoxins analysis.

Aflatoxin B1 has to be derivatized to enhance its fluorescence[24-25]. The most usual method is the derivatization of the hemiacetal form prior to chromatographic separation, using trifluoroacetic acid[26]. On the other hand, HPLC using mass spectrometry (MS) detection provides analysis of mycotoxins eliminating the need of sample derivatization for fluorescence activity enhancement, but the cost for equipment acquisition is still high[27].

The objective of this study is to present an in-house validation method based on AOAC 994.08[22] with modifications related to the cleaning step, eliminating the use of immunoaffinity columns (IAC), multifunction columns (MFC) or solid-phase extraction (SPE) and maintaining extraction efficiency, further reducing the cost of analysis, allowing the quantification of AFB1 in Brazil nuts.

## MATERIAL AND METHODS

**Chemicals, reagents and standard solutions:** Standard of AFB1 was purchased from Sigma-Aldrich, with 99% of purity. The individual stock standard solution was prepared by weighing 5 mg of AFB1 into 20 mL volumetric flask. The volume was completed with acetonitrile and the solution was stored at -20 ± 5 °C. The working standard solutions were prepared by diluting the stock solution in mobile phase in the concentration range of 5 – 80 µg $L^{-1}$. Acetonitrile and Methanol HPLC grade were purchased from JT Baker (USA). Trifluoroacetic acid (TFA) from Sigma-Aldrich (USA) and acetic acid from Dinamica (Brazil) were of analytical grade. Water was obtained from a Milli-Q water system with a resistivity of 18.2 MΩ $cm^{-1}$ (USA).

**Sample preparation:** Brazil nuts samples in the shell were peeled manually before milling. All samples were processed in blender and stored at -20 ± 5 ° C until analyzes were performed.

Aliquots of 30.0 g of sample were transferred to a 250 mL centrifuge tube, extracted with 50 mL of acetonitrile using a shaker for 1 h at high speed, and then centrifuged for 5 min at 4300 g in a refrigerated centrifuge (18 – 23°C). The supernatant was filtered in a glass funnel with wool glass. An aliquot of 2 mL purified extract was transferred into derivatization vial and it was evaporated under nitrogen flow at room temperature until dried. The dried extract was derived as recommended by AOAC 994.08[22] with 700 µl of acetic acid: water: TFA (1: 7: 2) and kept in a 65 ° C water bath for 17 minutes. After complete derivatization of aflatoxin, the flask was cooled to room temperature for 4 minutes; the volume was completed to 1 mL with mobile phase, filtered through a Millex HV filter (0.45 µm, Millipore) and proceeded with HPLC-FD.

**Instrumentation and conditions:** Analyses were performed on a HPLC Shimadzu LC 20A system equipped with a fluorescence detector RF10AxL (Tokyo, Japan). Chromatographic separation was accomplished using a C18 column (Zorbax SB-C18 150 x 4.6 mm, particle size 3.5 µm) at 30 °C. An isocratic flow rate of 1.0 mL $min^{-1}$ was employed with mobile phase composition in methanol: water: acetonitrile (1: 3:1 v/v/v). The fluorescent derivate was detected with excitation wavelength of 360 nm and emission wavelength of 440 nm. Data acquisition was performed by LC Solutions software.

**Method Validation:** The in house validation was performed according to the criteria and recommendations by SANTÉ 11945/2015[28] and on good laboratory practice (GLP), supported in good scientific practice[29-30]. The parameters considered were: linearity, LOQ, specificity, recovery and precision (repeatability and within-laboratory) and matrix effect. Besides that, the stability and measurement uncertainty was verified.

Linearity was determined with calibration curve injected three times with five levels ranging of 5 - 80 µg $L^{-1}$ for AFB1. The presence of outliers was evaluated by Hubber test and the homoscedasticity by Cochran test. The residual analysis with normal distribution of the calibration points were performed. Matrix effect was evaluated preparing the same five levels (5 - 80 µg $L^{-1}$) in blank matrix extract and compared with the external calibration curve. This comparison was performed employing Student´s and Fisher's test.

Specificity was checked by analysis control samples of Brazil nuts and comparison between signals at the retention time. The LOQ was determined as the level at which the analyte could be accurately detected and quantified.

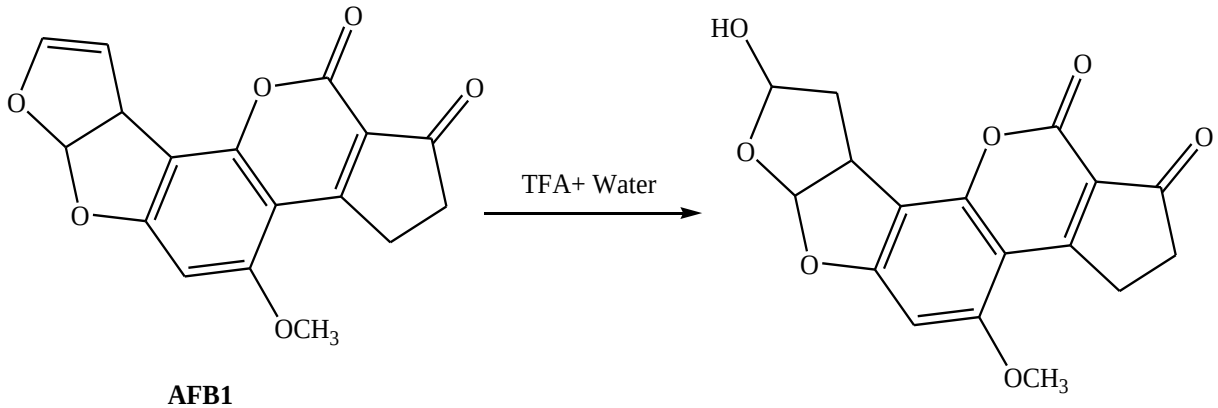
Recovery studies and precision experiments were performed by spiking samples in two different concentrations corresponding to 6 and 20 μg $kg^{-1}$ in five replicates each. Two different operators performed the analysis and all spiked samples were processed and analyzed according to the procedure describe in sample preparation section. The recovery data were calculated by comparison between the added amount and obtained concentration after the analysis and the results were expressed by percentage recovery. Precision results were expressed by coefficient of variation (CV %). Standard solutions stability was tested in triplicate during 24 days under refrigeration (5 ± 3 °C).

For the uncertainty measurement, the top-down approach has been used according to EURACHEM/CITAC Guide[31]. The $U_{95\%}$ is the expanded uncertainty which is twice the value of the combined uncertainty at 95% confidence level.

**Real samples application:** The validated method was applied for the analysis of 14 commercial samples of Brazil nuts in shell and shelled from local market in triplicate.

## RESULTS AND DISCUSSION

**Sample preparation:** Brazil nuts have a high content of lipids that can compromise extraction efficiency. Thus, the choice of solvents for extraction should be made based on this characteristic[11]. The choice of acetonitrile presented a great extraction power of AFB1 avoiding to remove much of the lipids and promoting a clarified extract. The use of this solvent also provides easy evaporation after filtration and obtaining sharp peaks as compared to extraction with methanol. Usually in the purification and enrichment step, SPE, IAC or MFC are used. The elimination of this cleaning step reduces the cost and sources of experimental errors reaching the levels required by the legislation as demonstrated in method validation item. In determining the AFB1 contamination in Brazil nuts, the derivatization procedure requires care and attention due to the stability of the formed species. In acidic solution, with TFA, the double bond in the dihydrofuran group is rapidly rehydrated leading to the production of the fluorescent specie B2a (**Figure 1**).




**AFB1** **AFB1 derivate**

**Figure 1:** Aflatoxin B1 and its fluorescent derivate.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According to Kok, W. Th. (1994)[32] the reaction rate increases with a factor of 1.8 at every 10 ° C of temperature increase, and 95% of AFB1 is converted to fluorescent derivative from 3 hours at 40 °C to 10 min at 100 °C. The official method indicates that the reaction should occur at 65 ° C and at a time greater than or equal to 8.5 min, then maintaining the bath temperature as mentioned the reaction time was studied in order to obtain the best conversion rate. So, the reaction was assayed at 15, 17 and 20 minutes using a standard 10 µg $L^{-1}$ of AFB1. The reaction time in 17 minutes was the one that showed the best response for the conversion.

The main advantage of the TFA reagent is its simplicity, and disadvantage is the stability of the TFA derivate[32]. The time stability of the derivate was observed and even with the addition of acetic acid during the reaction (to stabilize the derivative), it should be immediately injected into the chromatograph.

**Method validation:** The extraction method was evaluated through internal validation using the parameters recommended in the SANTE 11945/2015[28] guideline, among them: selectivity, linearity, recovery, precision, matrix effect and quantification limit in addition to uncertainty calculation. A blank sample with no contamination of the target compound was chosen for the validation procedures. The selectivity of the method was verified by the analysis of blank sample and no interference of the matrix was observed next to the retention time of the AFB1. Chromatograms representative of the blank and fortified samples are shown in **Figure 2 (A and B)**. Chromatographic conditions allowed a run time total of 7 minutes and the retention time of AFB1 was 5.1 minutes.

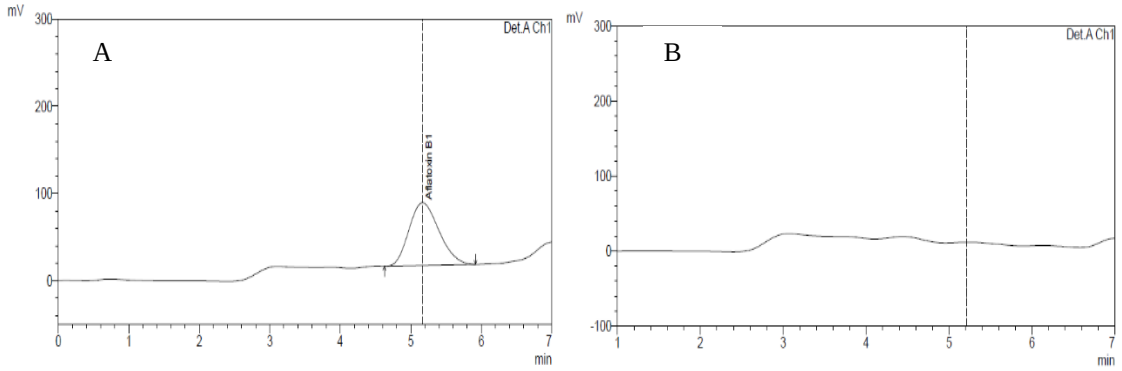


**Figure 2:** Chromatograms of (A) Brazil nut sample fortified in 6 µg $kg^{-1}$ and (B) Blank sample.

Linearity was assessed in solvent and matrix-based calibrations, which included five concentration levels in triplicate. The range analyzed in calibration curves in solvent was between 5 to 80 µg $L^{-1}$. The curve was submitted to ANOVA test and no deviation linearity ($p < 0.05$) was showed. The linear regression equation obtained for AFB1 was $y = 429406x - 182771$ with determination coefficient ($R^2$) higher than 0.999 and the residual distribution was randomly distributed and lower than ±10%. Matrix effects are known sources of error lead to overestimation or underestimation of result. So, it was studied with a matrix-based calibration curve in the same range evaluated in the solvent. F-test and Student test were used to evaluate the homogeneity of the variance and to compare the means between the results. No significant differences were observed between the values, as can be observed in **Table 1**.

**Table 1:** Matrix effect data using *F* and t tests.

| Concentration AFB1 (μg $L^{-1}$) | *F* | t | Matrix Effect |
|---|---|---|---|
| 5 | 1.84 | -1.55 | No |
| 10 | 0.45 | -2.19 | No |
| 20 | 0.32 | -2.89 | No |
| 40 | 0.21 | -2.19 | No |
| 80 | 0.23 | -2.20 | No |

The LOQ was established by successive chromatographic analysis of extracts with decreasing concentrations of the compound until reaching the lowest concentration with precision and accuracy. The level of 6 g $kg^{-1}$ attended this considerations, so we set this level as LOQ. The result is satisfactory with respect to criteria established at 10 and 20 μg $kg^{-1}$ maximum level tolerated (MLT) for aflatoxin in Brazil nuts shelled and in shell, respectively[13].

Recovery studies were conducted at two concentration levels, LOQ (6 μg $kg^{-1}$) and de MLT (20 μg $kg^{-1}$) in five independent replicates. As recommended by SANTÉ 11945/2015 the recovery studies must present values of recovery between 70%-120% and precision studies, evaluated by the coefficient of variation, should not exceed 20%. The results are shown in **Table 2** where media recoveries were 80% for the two fortification levels and the precision studied for different operators, in the same laboratory and instrument in agreement with the performance criteria defined in SANTÉ 11945/2015[28].

**Table 2:** Recoveries and precision for AFB1 in Brazil nuts.

| | Accuracy | | Precision | |
|---|---|---|---|---|
| AFB1 added (μg $kg^{-1}$) | Found ± S.D (μg $kg^{-1}$) | Recovery ± CV (%) | Operator 1 | Operator 2 |
| 6 | 4.50 ± 0.221 | 75 ± 5.0 | 77 ± 3.0 | 73 ± 4.0 |
| 20 | 17.0 ± 0.600 | 85 ± 4.0 | 85 ± 1.0 | 84 ± 0.5 |

**S.D = standard deviation CV= coefficient of variation**

AFB1 standard solutions remained stable for 24 days in refrigerator. During the test time the concentration of AFB1 declined only 3% of total.

The uncertainty estimation was calculated based on the data obtained from the validation of the method. Thus, four main sources of uncertainty were considered: accuracy, precision, linearity and analytical standard. The uncertainty expanded was calculated using a coverage factor of 2 corresponding a 95% confidence level. Uncertainty values at the contamination levels of 6 μg $kg^{-1}$ and 20 μg $kg^{-1}$ for AFB1 were ± 0.18 μg $kg^{-1}$ and ± 0.60 μg $kg^{-1}$, respectively.

The main contribution to $U_{95\%}$ in this study of residues comes from precision tests, corresponding to a 76% contribution, whereas the uncertainty associated to linearity did not reach 1% of contribution. Considering the characteristics of the matrix and the concentrations evaluated in the validation of the method, the result of the calculation of uncertainty estimation is consistent and it is according with Decision 401/2006/EC[33].

**Method application:** To evaluate the applicability of the method, 14 samples were purchased in the local market, 8 samples of Brazil nuts in shell and the remaining shelled. In each batch of analyzes, the fortification tests were performed as quality control; the results of these assays ranged from 80% -92%.Two samples showed levels of AFB1 above the LOQ, represents 14% of the samples. In one of them, the level of contamination reached 59 µg $kg^{-1}$ on average (**Figure 3**).

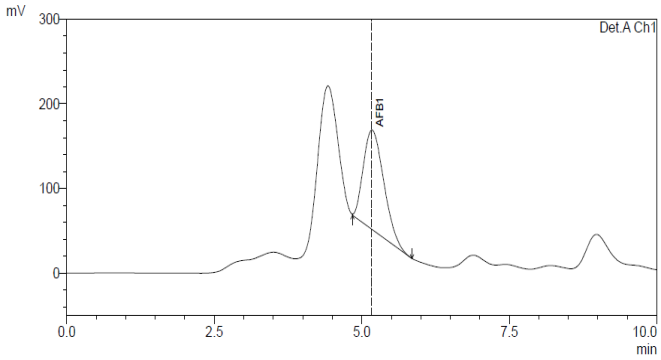


**Figure 3:** Chromatogram obtained from commercial sample which AFB1 contamination level was 59 µg $kg^{-1}$ on average.

This sample with shell has a high contamination content that exceeds the MLT for Brazil nuts in shell, which is 20 µg $kg^{-1}$ **(Table 3)**. Considering the quantifiable samples, the mean concentration of AFB1 contamination is 29 µg $kg^{-1}$.

**Table 3:** Real samples results.

| Sample ID | 1 | 2 | 3 | 7 | 9 | 10 | 11 | 14 | 4 | 5 | 6 | 8 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Average ± SD (µg $kg^{-1}$) | 8.79 ± 0.318 | < LOQ | ND | < LOQ | 59 ± 1.4 | ND | <LOQ | ND | ND | ND | ND | ND | ND | ND |
| Min-Max (µg $kg^{-1}$) | 8.42 – 8.98 | – | – | – | 58.1 – 60.0 | – | – | – | – | – | – | – | – | – |
| Type | In shell | | | | | | | | Shelled | | | | | |

**SD = standard deviation ND = no detected LOQ = limit of quantification**

In the Brazilian Amazon, local where the Brazil nuts are produced, the temperature varies between 24.3 and 27.2 ° C, besides that having a mean precipitation between 1400 and 2800 mm and a humidity between 79% and 86%[11]. These are ideal conditions for the growth of toxigenic fungi and contamination by AFB1. The presence of toxigenic fungi in Brazilian nuts is critical, since sanitary barriers are rigid and impose low limits for a presence of AFB1. Thus, Brazil has difficulty in exporting, especially Brazil nuts in shell, that do not go through processing stages and as seen are those with higher content of AFB1.

## CONCLUSION

This work described the optimization and validation of a method for detection and quantification of AFB1 contamination levels in Brazil nuts without the clean-up step. It is a low cost method, with good sensitivity and it uses a kind of detector accessible to any laboratory. Therefore, it can be recommended as an alternative to solid-phase extraction (SPE), immunoaffinity columns (IAC) or multifunction columns (MFC) for AFB1 determination on Brazil nuts. The validation also showed that the method is robust and precise. The method applied to 14 natural samples, showing that it is suitable for its purpose.

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