Comparison of the Predictive Ability of Multiway Calibration Methods in the Determination of Aflatoxin Af-B2 in the Presence of Af-G2 in Peanut Samples using Spectrofluorimetry

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Abstract

This paper compares different multi-way calibration methods for the determination of aflatoxin Af-B2 content in the presence of aflatoxin Af-G2 in peanuts using excitation–emission matrix (EEM) fluorescence spectra. The multi-way methods assayed were parallel factor analysis (PARAFAC), unfolded partial least-squares (U-PLS), and multidimensional partial least-squares (N-PLS), the last two combined with the residual bilinearization procedure (RBL). Fluorescence spectra were obtained in the range from 300 to 370 nm (step of 2 nm) for excitation and 427 to 550 nm (step of 1 nm) for emission. The accuracy of each model was evaluated through elliptical joint confidence region (EJCR) tests. All methods gave excellent results, but the PARAFAC model performed slightly better than the others, respectively.

Keywords: Second-order calibration methods; Spectrofluorimetry; Peanuts; PARAFAC; N-PLS/RBL; U-PLS/RBL

1. Introduction

The primary producers/exporters of peanuts are the United States, Argentina, Sudan, Senegal, and Brazil. One of the major problems in peanut production worldwide is the contamination with Aspergillus section Flavi and aflatoxins, being these mycotoxins of great concern due to their toxicological effects to human and animals (Torres et al., 2014).

Aflatoxins are secondary metabolites produced by fungi of the genus Aspergillus (A. flavus, A. parasiticus, and A. nomius) present in foods such as peanuts, corn, and wheat (Fig. 1). Because of
their carcinogenic, mutagenic, teratogenic and immunosuppressive effects (Cole & Cox, 2004), they generate serious food safety problems for producers and consumers (Abbas et al., 2004).

**Fig. 1.** Chemical structure of Aflatoxins B2 and G2, respectively

Aflatoxins have deleterious effects in consumption considering the global distribution chain, affecting major exporting countries. However, another study indicated that there was a potential risk of aflatoxin production if good storage practices were not employed (Zorzete et al., 2013). Recently, a review showed the importance of Brazil nuts for the Amazon rainforest, emphasizing on the social and environmental impact of its production, on the mycobiota contamination of seeds, and the presence of mycotoxins and related food safety aspects (Freitas-Silva & Venâncio, 2011). Therefore, there is a need a necessity to develop a method for the determination of aflatoxins in peanuts, however that determination of these toxins is not simple because they are found in complex matrices and have to be detected at very low concentrations. Most of the methods used for their determination use high-performance liquid chromatography (HPLC) together with solvent extraction with harmful solvents and multifunctional columns that are extremely expensive and matrix dependent (Ventura et al., 2004). Fluorescence detection is commonly used (Roth et al., 1995; Khayoon et al., 2010; Oliveira et al., 2009), but the limit of detection needs to be lowered by pre-column formation of hemiacetal derivatives with trifluoroacetic acid or post-column derivatization with bromine or iodine (Ventura et al., 2004). Some methodologies use liquid chromatography coupled with mass spectrometry (LC-MS) to determine these toxins in food (Huang et al., 2010) and medicinal herbs (Ventura et al., 2004). However, the LC-MS technique is expensive and available only in a few laboratories. HPLC with diode-array detection has also been developed using a second-order calibration with parallel factor analysis (PARAFAC) and applied for the simultaneous determination of aflatoxins B1, B2, G1 and G2 in pistachio nuts. This method offered the advantage of using solid phase extraction (SPE), of lower cost than the standard method of aflatoxin analysis (immune affinity column assay) as well as a unique and simple isocratic elution program for all samples and calibration transfer, thus saving both chemicals and time of analysis (Vosough et al., 2010).

Recently, a method for the simultaneous determination of aflatoxins B2 and G2 in peanuts was presented (Luna et al., 2013) that uses excitation–emission fluorescence data together with PARAFAC and the second-order standard addition method. The results of PARAFAC on a set of spiked and naturally contaminated peanuts indicated that the two aflatoxins could be successfully determined. The proposed method for determination of aflatoxins Af-B2 and Af-G2 was simple, fast...
and sensitive, with a limit of quantification much lower (<1.0 mg kg\(^{-1}\)) than the required legal limit of aflatoxins in peanut samples (20 mg kg\(^{-1}\) [Brasil, 2002]).

The aim of the present work is to compare the predictive ability of PARAFAC with other multi-way calibration methods, namely multilinear partial least squares with residual bilinearization (NPLS/RBL), and unfolded partial least squares (UPLS/RBL) with residual bilinearization to determine the aflatoxin B2 in the presence of G2 in peanut samples by spectrofluorimetry. In order to study whether the prediction results could be improved, and compared the precision and accuracy of the methodology, two other calibration methods suited to the second-order data were studied and compared with PARAFAC.

**2. Theory of Multi-way Calibration Methods**

2.1. Multi-way calibration methods

![Fig. 2. Schemes for decomposition of three-way data: a) unfolding method; b) PARAFAC; c) N-PLS method. (For definition of symbols refer to Section: Multi-way calibration methods).](image)

Multi-way calibration methods are particularly suitable for determination of analytes in complex samples, due to their ability to accurately quantify even in the presence of interferences not present in the calibration standards, a property known as the second-order advantage (Arancibia et al., 2008). It is possible because the interferents present in the unknown samples are modelled in the calibration step together with the calibration standards. Parallel factor analysis (PARAFAC), multilinear partial least squares (NPLS), and unfolded partial least squares (UPLS) are calibration methods that are widely used in the literature with multiway data (Olivieri & Escandar, 2014).
Actually, U-PLS is a first-order calibration method that applies PLS regression to the unfolded second-order data. In order to achieve the second-order advantage as PARAFAC does, the residual bilinearization (RBL) is often applied to allow UPLS and NPLS to deal with unexpected interferences (Braga et al., 2010). Figure 2 showed the schemes for decomposition of three-way data used in this work.

2.2 Processing of excitation-emission fluorescence matrices datasets

An excitation-emission fluorescence matrix (EEM) of a pure compound is obtained by recording the emission spectra at different excitation wavelengths of a pure standard. It is assumed that the measured data matrix for a single sample can be represented a bilinear model formed by the outer product of the vectors corresponding to the excitation and emission spectra of each compound. It is also assumed that the signals from the various constituents in the sample are additive (Elcoroaristizabal et al., 2014). Hence, the measured data can be represented by Eq. (1).

\[ \mathbf{R} = \sum_{k=1}^{K} c_k \mathbf{x}_k \mathbf{y}_k^T + \mathbf{E} \]  

(1)

where \( \mathbf{x}_k \) and \( \mathbf{y}_k \) are the excitation and emission profiles respectively, \( c_k \) is the concentration of constituent \( k \) in the sample and \( \mathbf{E} \) is an error term.

The EEMs matrices from different samples can be arranged in different ways, depending on the multi-way algorithm used for the analysis. They can be organized as a three-way data cube \( \mathbf{X} \) (PARAFAC) or into a trilinear model similar to PARAFAC (NPLS). In PARAFAC, the decomposition of the cube is carried out and then the scores of a matrix \( \mathbf{A} \) (samples) are regressed against the \( \mathbf{y} \) vector of the aflatoxin concentrations. In NPLS, the regression is directly performed following the PLS scheme. The inner relationship between the scores of \( \mathbf{X} \) and the concentrations \( \mathbf{Y} \) is based on a PLS model (Eq. 2):

\[ \mathbf{U} = \mathbf{T} \mathbf{B}_{PLS} + \mathbf{E} \]

(2)

where \( \mathbf{U} \) and \( \mathbf{T} \) are the scores of \( \mathbf{X} \) and \( \mathbf{Y} \), respectively, both having the maximal covariance; and \( \mathbf{B}_{PLS} \) is the matrix of regression vectors. The principal feature of the algorithm is that it produces score vectors that, in a trilinear sense, have maximum covariance with the explained part of the dependent variable. Therefore, the model is unique, like PARAFAC (Granato & Ares, Ed., 2014) but it is not fitted in the least squares sense but according to the philosophy of PLS to maximize the covariance of the dependent and the independent variables. It is achieved by simultaneously fitting a multilinear model of the independent variables, and a regression model relating the two-decomposition models (Bro, 1998).

Another approach to calibration is to unfold the three-way data cube into a two-way array, i.e., into a rectangular data table of \( I \) vectorized samples, which can be processed by general multivariate calibration models. The unfolding of a three-way data array can be carried out in different ways. Theoretically, an \( I \times J \times K \) array will give rise to an \( (I \times J) \times K \) rectangular data table where \( I \) is the number of samples, \( J \) is the number of sensors in the excitation mode, and \( K \) is the number of sensors in the emission mode), and may be processed by conventionally chemometric methods, such as partial least squares (PLS) (Ni et al., 2011) (Granato & Ares, Ed., 2014). Since classical PLS
is used, the second-order advantage no longer exists, and interferences must be removed before predictions are calculated. It is achieved by means of a residual bilinearization step (RBL). Details about PARAFAC, UPLS/RBL, and NPLS/RBL algorithms can be found in the literature (Olivieri & Escandar, 2014).

2.3 Residual bilinearization

When unexpected constituents occur in the test data matrix \(X_{\text{test}}\), the sample scores obtained by projecting the unfolded \(X_{\text{test}}\) onto the PLS loadings are unsuitable for analyte prediction through Eq.3:

\[
y = \theta^T t
\]  

(3)

where \(t\) is the sample score vector, obtained as the projection of the unfolded data matrix for the test sample in the space defined by the calibration PLS loadings. The equation (3) is the usual predictive PLS expression when all test sample constituents are adequately modeled in the calibration step, i.e., if no unexpected components occurred in the test sample. To overcome this problem, a residual bilinearization procedure for linear dependency was can be used. RBL intends to model the residuals assuming that they can be arranged into a bilinear matrix. Specifically, the RBL procedure fits the sample data to the sum of two contributions: (1) the portion of the test data, which can be explained by the calibration PLS loadings, and (2) the contribution from the potential interferents, modeled by a certain number of principal components (\(N_{\text{RBL}}\)). The complete UPLS/RBL modeling equation involves a residual error term (Eq. 4) to be minimized by least-squares (Olivieri & Escandar, 2014):

\[
X_{\text{test}} = \text{reshape}(Pt_{\text{RBL}}) + B_{\text{RBL}}T_{\text{RBL}}^T + E_{\text{RBL}}
\]  

(4)

where the product \((B_{\text{RBL}}T_{\text{RBL}}^T)\) is the PCA (principal component analysis) model for the residual matrix \((X_{\text{test}} - \text{reshape}(Pt_{\text{RBL}}))\) with \(N_{\text{RBL}}\) principal components.

2.4 Comparison of the predictive ability of the second-order methods

The ordinary least squares (OLS) fit of the predictions versus some reference values is commonly used testing the trueness of the results provided by the analytical method. After the slope and intercept of the fitted line have been estimated, a test is applied to assess whether they are statistically different from 1 (slope) and 0 (intercept) (Franco et al., 2002). In this work, as described by Franco et al., (2002), the elliptic joint confidence region (EJCR) is calculated for both parameters (Eq. 5) as:

\[
N(a - \hat{a})^2 + 2(\sum x)(a - \hat{a})(b - \hat{b}) + (\sum x)^2(b - \hat{b})^2 = 2s^2F_{\alpha,2,N-2}
\]  

(5)

where \(N\) is the number of data points, \(s^2\) is the regression residual variance, the estimated values for the slope (\(\hat{b}\)) and intercept (\(\hat{a}\)) are obtained from linear regression, and \(F_{\alpha,2,N-2}\) is the critical F value with 2 and N-2 degrees of freedom at a given 100 \((1 - \alpha)\) confidence level, usually 95%. If the point \((1, 0)\) is inside the EJCR, it can be concluded that constant and proportional biases are absent.
2.5 Limitations of the second-order calibration methods

The second-order calibration methods can deal with potential interferences in real samples, in opposition to both zero-order and first-order calibrations. Potential interferences not included in the calibration set can be modeled, allowing us to quantify the calibrated analytes accurately, even in the presence of unknown constituents. In this context, a relevant property of the data is their multilinearity because this property is assumed by the underlying models of some algorithms available. When a three-way data array deviates from the multilinearity condition, because this requirement is violated, other non-multilinear algorithms should be applied. The selection of the proper algorithm depends on the particular cause provoking the loss of multilinearity. See the reference for more information (Olivieri et al., 2011).

3. Experimental Procedure

3.1. Chemicals and solvents

Stock solutions of aflatoxins – Crystalline aflatoxins Af-B2 and Af-G2 (1 mg each, Sigma Chemical Co., St. Louis, MO) were used to prepare stock solutions of individual aflatoxins by dissolving each aflatoxin in 100 mL acetonitrile for concentrations equal to 10 μg mL\(^{-1}\).

3.2. Preparation of aqueous standard solutions of aflatoxins

The calibration set was built with 18 solutions as follows: three blank solutions, five standard solutions for each aflatoxin and five mixed solutions with both aflatoxins (Table 1). All the aqueous standard solutions were stored in the dark at 4°C when not in use.

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3.3. Sample preparation

The peanut samples were obtained from commercial establishments. The sampling method used was that required by the European Union, 2003 (Official Journal of the European Union, 2003). The samples were collected from bags of 20 kg under vacuum/heat sealing. Incremental samples were taken from the bags and homogenized. Final portions of 1 kg were packed and immediately sent to the laboratory. Subsequently, 0.5 kg of the same batch of peanuts were grounded to obtain particle sizes smaller than 20 mesh, homogenized in their entirety and transferred to polyethylene containers with stoppers and stored in a freezer for a month.
3.4. Extraction, clean-up and analysis of aflatoxins

The procedure was based on extraction of 6 g of the peanut sample (raw shell) milled together with 1 g of sodium chloride in 30 mL of methanol 70% v/v. The extract was homogenized for one hour in a mechanical shaker. The extract was then brought to a filtration on qualitative filter paper. 15 mL of the extract was collected and mixed with 30 mL of deionized water. Then, the filtrate was passed through a glass microfiber membrane under vacuum. After this process, the extract should have a clear appearance. Otherwise, the last filtration step was repeated. With the aid of a glass syringe, 15 mL of the extract were transferred to an immunoaffinity column containing monoclonal antibody for aflatoxins B1, B2, G1, and G2. After the elution of the extract, the column was washed with two 10 mL portions of deionized water to remove possible residues coming from the extraction. Then, aflatoxins were isolated, purified, and concentrated in the column. Elution of aflatoxins was done with 1 mL of methanol in the column. The eluate was directly transferred to a quartz cuvette, and the aflatoxins were quantified by measuring fluorescence after derivatization reaction with 1 mL of bromine 0.003% v/v. All the spiked samples (solutions 19–29) were used to build the validation set (Table 2). The validation set followed a central composite design with axial points and a central point in triplicate.

Table 2 - Validation set of aflatoxins Af-B$_2$ and Af-G$_2$ (ng mL$^{-1}$).

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3.5. Preparation of spiked peanut samples

Uncontaminated peanut-based product samples were grounded and then split into four 6 g portions for each sample. Each sample was submitted to the extraction and clean-up process described aforementioned. After this, three of the portions of each extracted solution were spiked with aflatoxins to give final levels from 16 to 44 ng mL$^{-1}$ of Af-B$_2$ and Af-G$_2$, respectively whereas the remaining portion was treated as blank.

3.6. Instrumentation

A Lumex Panorama spectrofluorimeter recorded the fluorescence spectra. The excitation and emission slits were both maintained at 8 nm. All measurements were performed in 10 mm quartz cells at 25.0 ± 1.0°C.

3.7. Measurement and data analysis

The fluorescence spectra were recorded in the excitation range from 300 to 370 nm (step 2 nm) and in the emission range from 380 to 550 nm (step 1 nm). In order to avoid the presence of Rayleigh scattering, only the emission interval 427–550 nm was kept so that each data matrix
consisted of 36 emission wavelengths and 124 excitation wavelengths. The data were treated in MATLAB version 7.8. The PARAFAC calculations were carried out with the PLS toolbox (Eigenvector Research Inc., USA) and the N-PLS/RBL, and U-PLS/RBL calculations were carried out with MVC2_toolbox for MATLAB (Oliveiri et al., 2009). The PARAFAC calculations were done through a tensor \( X \) with the following dimensions: 29x36x124, which means 29 solutions and 36 excitation wavelength and 124 emission wavelengths, respectively. For the NPLS/RBL calculation, two separate sets were created: a calibration set was made as a tensor \( X_{\text{cal}} \) with the following dimensions: 18x36x124 as described before and a validation set as a tensor \( X_{\text{val}} \) with dimensions: 11x36x124. The same procedure was applied to build two separate sets: the calibration and validation sets for UPLS/RBL. After the matrices were vectorized, \( X \) (containing vectorized EEM matrices) and \( y \) (containing the concentration of analytes) from the calibration set were related through a PLS model. The calibration set was built as a matrix \( X_{\text{cal}} \) with the following dimensions: 18x4464 and \( y_{\text{cal}} \) containing the analyte concentrations for this set. The validation set was a matrix \( X_{\text{val}} \) with dimensions: 11x4464 and \( y_{\text{val}} \) containing the analyte concentrations for the validation set. This model was then used to predict new concentrations for unknown samples from the measured EEM matrices. Whenever unexpected interferences are found in new samples that were not present in the calibration set, the additional RBL step allowed an accurate prediction of the analytes’ concentration.

4. Results and Discussion

The original excitation spectrum was from 300 to 370 nm with a step of 2 nm, and emission spectrum was from 427 to 550 nm with a step of 1 nm.

Figure 3 shows a perfect match between the excitation and emission loadings of aflatoxins Af-B2 and Af-G2 and the normalized emission vectors of standard solutions. Therefore, the deconvolution of the excitation-emission fluorescence data with PARAFAC allowed a precise identification of the
analytes. A comparison of the different second-order calibration methodologies should be done through the elliptic joint confidence region (EJCR) for slope and intercept after regressing the predictions of each model against the reference values.

The validation set samples containing the Af-B2 and Af-G2 were prepared and evaluated with PARAFAC, U-PLS/RBL, and N-PLS/RBL algorithms. The selection of PARAFAC factors for these samples was also carried out through the analysis of the core consistency. The results obtained established that the number of total components required by PARAFAC in the samples with the studied interference was two. On the other hand, when U- and N-PLS/RBL algorithms were applied to the validation set samples, in addition to the number of latent variables estimated, these samples required the introduction of the RBL procedure to overcoming the presence of the Af-G2 interferent.

In figure 4 the prediction concentrations corresponding to the application of N-PLS/RBL, U-PLS/RBL, and PARAFAC respectively, are shown to the same validation set samples.

In figure 5, the ellipses of the EJCR analyses are shown for the slope and intercept of the corresponding plots. The ellipses corresponding to N-PLS/RBL, U-PLS/RBL and PARAFAC, include the theoretically expected point (1,0) suggesting a high-quality prediction This latter fact demonstrates that, in the system under study, it is appropriate to maintain the tridimensional nature of the data for the quantitative analysis in order successfully to quantitate the analyte.
Fig. 5. Elliptical joint regions (at 95% confidence level) for the slope and intercept of the regression of N-PLS/RBL (solid line), U-PLS/RBL (dashed line), and PARAFAC (dotted line) results. Black cross marks the theoretical (intercept = 0, slope = 1) point.

Therefore, Fig. 5 showed that all the tested methodologies offered accurate and precise results for the determination of aflatoxin Af-B2 in peanut samples. The size of the ellipse is mostly controlled by the standard error of the regression, and it gives an idea of the precision of the method. A higher precision method gives a smaller ellipse. The calculated root mean square errors of prediction (RMSEP) were 0.38, 2.03 and 2.25 ng.mL⁻¹ for aflatoxin B2 using PARAFAC, NPLS/RBL, and UPLS/RBL, respectively. These numbers corroborated with the results obtained in Figure 5. Therefore, PARAFAC is the most precise method for the determination of the aflatoxin Af-B₂ in peanut samples.

Finally, an F-test was performed with 95% confidence level (p = 0.05), considering the null hypothesis that there is no significant difference in errors for the determination of Af-B2 in peanuts given by the models PARAFAC and N-PLS/RBL, and PARAFAC and U-PLS/RBL (Domingos et al., 2013). For the F-test, the following expression was used:

\[ F = \frac{(\text{RMSEP}_i)^2}{(\text{RMSEP}_j)^2} \]  

(6)

where RMSEP is the root mean square of the prediction (validation), and the subscripts \(i\) and \(j\) represent the model that have the largest and lowest RMSEP values, respectively. The degree of freedom in the F-test was 7 for all models. The F-test results were 35.06 and 28.54 for the first and second comparisons. Both calculated F-values were higher than 3.79, which is the critical F-value (with confidence level of 95%). Once more, these results confirmed again PARAFAC methodology
was the most precision method to quantify Af-B2 in peanuts in presence of Af-G2 using excitation-emission matrices.

5. Conclusions

The proposed methodology was adequate for the determination of aflatoxins B2 in the presence of G2 for all three chemometric models studied (PARAFAC, NPLS/RBL, and UPLS/RBL). PARAFAC showed a slight better performance when compared with the other methods. This fact can be confirmed because the methodology was able to determine the aflatoxin B2 in peanuts even in the presence of an equivalent concentration of the aflatoxin G2 as described in the experimental part. Therefore, even with very complex sample matrix, the analytical performance of the multi-way method coupled with spectrofluorimetry shows that they are suitable to determine Af-B2 in peanut samples using excitation-emission matrices. This methodology could be a real alternative to the traditional of analysis, showing advantages in terms of sensitivity and reduce the use of organic solvents.

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