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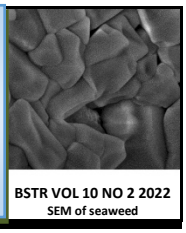

Limits of Detection Determination of Aflatoxin B1 using the Optical Waveguide Lightmode Spectroscopy via the Four-Parameter Logistic Model

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ABSTRACT

Mycotoxins are harmful secondary metabolites generated by a variety of fungi, and they may be found in a vast array of food and feed commodities and processed meats from animals fed infected meal. Numerous mycotoxins are extremely resistant and survive food processing, entering the food chain and posing a concern to human health. The "optical waveguide lightmode spectroscopy" (OWLS) method was used to detect aflatoxin B1 in plant sample matrices. The calibration curve for the detection of aflatoxin B1 utilizing "optical waveguide lightmode spectroscopy" (OWLS) displayed a sigmoidal shape; hence, the 5-PL or 4-PL model should be used to fit the data rather than a linear model. Using error function analysis with functions such as AICc, HQC, BIC, RMSE, adjR^2, Bias Factor, and Accuracy Factor, the 5-PL and 4-Pl models are distinguished inconsistently. The overlapping confidence intervals of the LogEC50 values suggested that the two techniques did not differ much, and the 4-PL model was selected due to its smaller number of parameters. The Limits of Detection for aflatoxin B1 value based on the 4-PL equation was 8.787 ng/mL with the 95% confidence interval from 5.728 to 13.100. In this study, the use of the 4-PL model was successful and was able to represent the entire date curve, not only the linear section. The linear component is crucial as a handy and swift approach for assessing the sensitivity of a developed biosensor technology and is often a more beneficial method for field applications when a quick and straightforward evaluation is required.

INTRODUCTION

Aspergillus flavus and *Aspergillus parasiticus* are two of the most common types of mold that create aflatoxins; these toxins are found in soil, hay, and cereals, among other places. In the 1960s, when the mold *Aspergillus flavus* was shown to be responsible for turkey X sickness and cancer in rainbow trout fed on peanut and cottonseed diets, the word "aflatoxin" was developed to describe the substance. Acute or chronic exposure to these compounds can lead to toxicity caused by aflatoxin, which manifests itself in a variety of ways. Hepatotoxicity, immunotoxicity, and teratogenicity can all result from chronic exposure, and hepatocellular carcinoma is particularly common in third-world nations. The FDA in the United States recognizes aflatoxins as an inevitable food contamination [1–4].

The International Agency for Research on Cancer has classified several types of aflatoxins, including aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) produced by both *A. flavus* and *A. parasiticus*, and aflatoxin M1 (AFM1) found in the fermentation broth of *A. parasiticus* and also produced when infected liver metabolizes AFB1 and AFB2 (IARC). Numerous studies on people, farm animals, and laboratory model species have uncovered species-specific characteristics of aflatoxin poisoning including its symptoms, biomarkers, and techniques for mitigation. One of the most common types of mycotoxins, aflatoxins are created through agricultural practices such growing, harvesting, storing, and processing. There is evidence that aflatoxin M1 can be passed on through breast milk [1–8]..

Bioligand binding to target receptors often exhibits sigmoidal profile when plotted on a semi log plot. This profile is often overlooked by many researchers which often resort to strong transformation to the linear form via log-log plot, which disturbs the error structure and skew the confidence interval estimation [9–12]. In ligand binding assay there are other non-linear regression curve-fitting methods which include cubic, quadratic, quartic, exponential, cubic spline, log-logit, bi-rectangular hyperbola, rectangular hyperbola (with and without a linear term), bi-exponential, two-parameter exponential, Gaussian, two site competition and Brain-Cousens among others. However, the sigmoidal profile is often best fitted to the four- or five parameter logistics equation [13]. The four-parameter logistic (4-PL) function are quite similar to the linear logit-log model and find widespread use in practice (a 4-PL curve transforms to a straight line in logit-log space). The 4-PL paradigm, like the lo-git-log model, has limitations when it comes to modeling asymmetric data. Similar to what was mentioned before, it has been proven that some approximations of the mass action model are nearly comparable to the logit-log model.

This whole mass action model approximated, like the 4-PL, fails to account for asymmetric data. Adding a fifth parameter to the 4-PL model allows for the regulation of the curve's asymmetry. The fitting procedure for this model, known as the five-parameter logistic (5-PL) model, is straightforward and is available in commercial software packages like GraphPad^{Tm} and Origin^{TM}. Numerous dose-response curves from a wide range of immunoassay and bioassay technologies have been fitted using both the 5-PL and 4-PL.

The lack-of-fit error that arises when the 4-PL is fitted to asymmetric dose-response data is almost eliminated by the 5-PL model due to the added flexibility provided by its asymmetry parameter. The 5-PL model strikes a great compromise between the extremes of overparameterized models, that might fit data closely however at the cost of a huge range in the predictions, and underparameterized models, which suffer from high lack-of-fit errors. The extent of asymmetry, the position of the transition zone, the length of the transition region, and the overall length of the transition zone are the other four factors. For functions having fewer than five parameters, it is extremely difficult to obtain a satisfactory fit to asymmetric sigmoidal dose-response data.

Aflatoxin B1 detection using optical waveguide lightmode spectroscopy (OWLS) was previously reported. A sigmoidal calibration curve was evident, however the curve could not be fit into any of the existing sigmoidal models. The purpose of this research is to standardize the data by reshaping it using a 4-PL model and a 5-PL model, and then to calculate the Limits of Detection (LOD).

Processing of Data

In this study, data from a published work by Adányi et al. [14] showing the calibration curve for Aflatoxin B1 in Figure 2 was used. The data was processed using Webplotdigitizer 2.5 software [15], which converts scanned figures into comma-separated data. This software has been widely used by researchers and is known for its reliability [16,17].

Four parameter logistics modelling

A non-linear regression using the four- (**Eqn 1**) and five- (**Eqn 2**) parameter logistic equations [18] was utilized to fit the curve based on least square fitting as follows;

$$y = Bottom + \frac{(Top-Bottom)}{1+10^{(LogEC_{50}-x)Hillslope}} \quad \text{Eqn. 1}$$

$$y = Bottom + \frac{(Top-Bottom)}{\left(1+10^{(LogEC_{50}-x)Hillslope}\right)^{S}} \quad \text{Eqn. 2}$$

Where,

The mass (arbitrary unit) is represented by y, and the concentration of Aflatoxin B1 (ng/mL) is represented by x. The top and bottom refer to the maximum and minimum responses in mass (arbitrary unit), respectively. The Log EC_{50} value signifies aflatoxin B1 levels that creates a 50% signal response, and the Hillslope (Hill coefficient) represent slope-like parameter. The S parameter represents the symmetry. The models were fitted using the PRISM software (v 5.0) from www.graphpad.com. The limit of detection (LOD) was determined based on the pooled standard deviation [9–11,13] instead of the blank value or the lowest concentration of aflatoxin B1 used. These values were then interpolated using the sigmoidal dose-response 4-PL or 5-PL equations to determine the corresponding concentration of aflatoxin B1, including the confidence interval.

Statistical analysis

Statistics functions such as adjusted coefficient of determination (R^2), Root-Mean-Square Error (RMSE), corrected AICc (Akaike Information Criterion), bias factor and accuracy factor (BF, AF) using the same set of experimental data, models with varying numbers of parameters were compared to one another to see if there was a significant difference in terms of fitness. The RMSE allows number of parameters' penalty and was calculated using Eqn 3, where n illustrates the number of experimental data, where else p is the number of parameters calculated by the model and experimental data and values predicted by the model are Ob_i and Pd_i, respectively [19].

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(Pd_i-Ob_i)^2}{n-p}} \quad \text{(Eqn. 3)}$$

In linear regression, the best fitting model was determined by R^2 or coefficient of determination. However, in nonlinear regression, the R^2 does not give a comparative analysis where the number of parameters between models is different. To overcome this, adjusted R^2 was used to calculate the quality of the nonlinear models. In the adjusted R^2 formula, S_y^2 is the total variance of the y-variable and RMS is Residual Mean Square (Eqns. 4 and 5).

$$Adjusted\ (R^2) = 1 - \frac{RMS}{S_Y^2} \quad \text{(Eqn. 4)}$$

$$Adjusted\ (R^2) = 1 - \frac{(1-R^2)(n-1)}{(n-p-1)} \quad \text{(Eqn. 5)}$$

Various statistical models can be evaluated for a given range of experimental data using the Akaike Information Criterion (AIC). Alternatively, AICc (the corrected AIC) should be used for data sets with numerous parameters or few data point values. [20]. The AICc was calculated based on the following Eqn. 6.

$$AICc = 2p + n1n\left(\frac{RSS}{n}\right) + 2(p+1) + \frac{2(p+1)(p+2)}{n-p-2} \quad \text{(Eqn. 6)}$$

AICc provides information about the disparities in the number of parameters and the fitting between two models. The smallest AICc value would indicate the best fitting between the models [20]. A further information-theory-based approach to statistics is the Bayesian Information Criterion (BIC; Eqn. 7). The number of parameters is punished more harshly by this error function than it is by AIC [21].

$$BIC = n.\ln\frac{RSS}{n} + p.\ln(n) \quad \text{(Eqn. 7)}$$

The Hannan–Quinn information criterion, often known as theHQC, is an additional error function approach that relies on the information theory (Eqn. 8). In contrast to the AIC, the HQC exhibits a high level of consistency because the equation contains the ln ln n term. [22];

$$HQC = n \times ln\frac{RSS}{n} + 2 \times p \times ln(\ln n) \quad \text{(Eqn. 8)}$$

To determine the validity of the models, both BF and AF were used. The Bias Factor should be set to 1 to achieve a correlation of 1 between the predicted and observed values. If the Bias Factor (as shown in Equation 9) is greater than 1, it indicates a fail-safe model, and if it is less than 1, it indicates a fail-negative model. If Accuracy is less than 1, it means that the prediction will be less accurate (Eqn. 10).

$$Bias\ factor = 10\left(\sum_{i=1}^{n} log\frac{(Pd_i/Ob_i)}{n}\right) \quad \text{(Eqn. 9)}$$

$$Accuracy\ factor = 10\left(\sum_{i=1}^{n} log\frac{|(Pd_i/Ob_i)|}{n}\right) \quad \text{(Eqn. 10)}$$

RESULT AND DISCUSSION

Standard curves in ligand-receptor binding assays are typically nonlinear and sigmoidal in nature. The 4-PL or, less commonly, the 5-PL model is the best option for fitting this type of curve [23]. To achieve a good fit between experimental and calculated data, it is necessary to modify the parameters of the curve model and fit the raw data to the 4-PL curve. This is often represented by a line running through the experimental data. In the original work the researchers obtained a sigmoidal profile but chose to use a linear regression model, which resulted in the equation y =−0.358 lg x + 0.843 and an R^2 value of > 0.993 [14]. They reported a detection limit of between 0.5 and 10 ng/mL. The results in **Figs. 1** and **2** show the sigmoidal curve obtained using the 4-PL equation for the calibration curve and the 4-PL and 5-PL equations on the same graph. The sigmoidal profile obtained was typical, and the correlation coefficient value of 0.996 indicated a good fit.

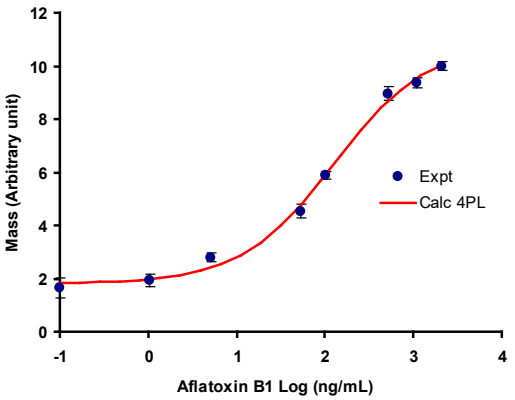

Fig. 1. Calibration curve for the determination of aflatoxin B1 modelled according to the four-parameter logistic equation.

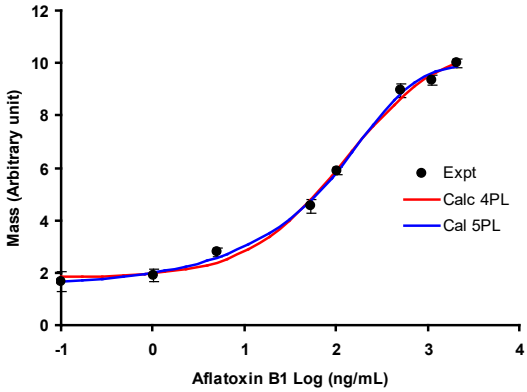

Fig. 2. Calibration curve for the determination of aflatoxin B1 modelled according to the four- and five-parameter logistic equations.

The result of the error function analysis shows that the simpler 4-PL model is more reliable having smaller AICc, BIC and HQF values whilst the other error functions such as RMSE, R^2, adjR^2, BF and AF values indicated that the 5-PL model is superior to the 4-PL. To settle this issue the logEC$_{50}$ values for both models are compared. The LogEC$_{50}$ value for the 4-PL model was 2.104 ng/mL (95% confidence interval or C.I. of 1.988 to 2.300) while the 5-PL model shows a LogEC$_{50}$ value of 1.992 ng/mL (95% C.I. of 1.881 to 2.116). As the 95% confidence interval overlap, the LogEC$_{50}$ values were deemed not significantly different [24], and when this occur, based on Occam's razor, the model having a lower number of parameter should be chosen instead [18].

Table 1. Error function analysis of the 4-PL and 5-PL models.

Model	p	RMSE	R^2	adR^2	AICc	BIC	HQC	BF	AF
4-PL	4	0.287	0.996	0.991	22.48	-17.20	-19.66	0.99	1.04
5-PL	5	0.239	0.998	0.993	75.25	-20.35	-23.43	1.00	1.03

The 4-PL equation resulted in an LOD value of 8.787 ng/mL with the 95% confidence interval from 5.728 to 13.100. This falls within the estimated range of 0.5 to 10 ng/mL reported in the original study. When a curve exhibits a clearly sigmoidal profile, it is recommended to calculate the LOD value using the 4-PL method. Therefore, the LOD value obtained through 4-PL modeling should be used for reporting purposes.

In this study we report on the use of an adjusted coefficient of determination (adjR^2) instead of the classical R^2. This is because the classical coefficient of determination R^2 does not take into account the number of parameters of an equation and is inaccurate to represent comparison between models having different number of parameters. R^2 is defined as "the coefficient of multiple determination, measures the percentage of the variation in the dependent variable which is explained by variations in the independent variables taken together" [25].

To compensate for this deficiency, the adjusted R^2 (adjR^2) term is introduced. In contrast to traditional R^2, adjusted R^2 takes into account the total number of instances and variables in the model. Adding more variables always increases the regular R^2, regardless of whether or not they improve the model's specification. Coefficient of determination adjusted for sample size and set of independent variables, as stated by Hair et al. The coefficient of determination will almost always increase when more independent variables are included in the model, but the adjusted coefficient of determination can decrease if the additional independent variables offer insufficient explanation or if the number of degrees of freedom is too small.

When comparing equations with varying numbers of independent variables or samples sizes, this statistic is quite helpful. The standard error of estimate (SEE) is a statistical measure of the variance in the predicted values that can be used to create confidence intervals around an expected value. It is the standard deviation of the statistical sampling distribution and is calculated as the sample standard deviation of the means. For example, the standard error of the mean is the sample standard deviation of the means. Hair et al. state that the SEE is a useful indicator of how much one sample differs from another in terms of the value of a test statistic [26]. A normal distribution is "the anticipated distribution of projected values that would occupy numerous samples of the data," which is equivalent to the standard deviation of a variable around its mean [27].

CONCLUSION

In conclusion, the calibration curve for the detection of aflatoxin B1 using the "optical waveguide lightmode spectroscopy" (OWLS) exhibited the sigmoidal patent and either the 5-PL or the 4-PL model should be used to fit the data instead of a linear model. Error function analysis using functions such as AICc, HQC, BIC, RMSE, adjR^2, Bias Factor, Accuracy Factor shows mixed results in distinguishing between the 5-PL and 4-Pl models. The overlapped confidence interval of the LogEC50 values indicated that both methods were not significantly different from each other, and 4-PL model was chosen based on it having a fewer number of parameters. In this analysis, the 4-PL model was successful in modeling the entire curve rather than just the linear portion. The linear portion is significant because it provides a quick and easy way to evaluate the sensitivity of a developed biosensor method and is often more practical for field applications where a fast and simple assessment is required.

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