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Chemometric Approach Based on Accuracy Profile and Data Chronological Distribution as a Tool to Detect Performance Degradation and Improve the Analytical Quality Control for Aflatoxins' Analysis in Almonds Using UPLC−MS/MS

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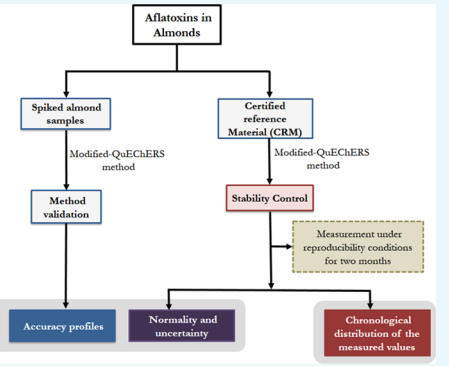
ABSTRACT: One of the main objectives of routine laboratories is the development of simple and reliable methods as well as meeting fit-for-purpose criteria for regulatory surveillance. In this study, the accuracy profiles and the evaluation of the distribution of results in the case of aflatoxins in almonds have been performed using ultraperformance liquid chromatography−tandem mass spectrometry (UPLC−MS/MS). The method consists of designing the experiment and using certified reference material (CRM) to evaluate the bias, to calculate the combined uncertainty, and to construct the control charts. Good sensitivity (limit of quantifications (LOQs) 0.34−0.5 μg/kg) and recovery (between 82 and 107%) were achieved. The proposed method was successfully tested with a proficiency test in almond powder with acceptable z scores ($-2 \leq z \leq 2$). The results provided direct evidence for the proper functioning and stability of the whole analytical protocol, allowing acceptable combined uncertainty.



1. INTRODUCTION

Aflatoxins (AFs) are a group of mycotoxins that are secreted as a result of the secondary metabolism of molds in the field or during storage. More than 300 secondary metabolites have been identified, but only about 30 have real toxic properties.[1] AFs are ubiquitous in almonds and have been reported in many countries. *Aspergillus flavus* and *Aspergillus parasiticus* are the main producers of the AFs. Among this latter group, Aflatoxin B1 (AFB1) is classified as carcinogenic to humans and animals.[1] For this reason, regulations have established the maximum levels (MLs) of mycotoxins in food. For instance, the MLs set by the European Union (EU) for nut products intended for direct human consumption or use as an ingredient in foodstuffs are 2 and 4 μg/kg for AFB1 and total AFs (AFB1 + AFB2 + AFG1 + AFG2), respectively.[2]

In the literature, a large number of analytical methods are available for the determination of AFs in almonds. Most methods are based on chromatographic separation such as liquid chromatography with fluorescence detection[3,4] or with mass spectrometry detection, where AFs are analyzed together with other mycotoxins.[5,6] However, appropriate sample preparation prior to analysis is needed in the case of complex matrixes such as almonds (high lipid content). The sample treatment for the determination of AFs in nuts involves, in most cases, immunoaffinity column clean-up (IAC) to reduce the matrix interferences and consequently to achieve high sensitivity.[7] Although IAC has become a very advantageous clean-up step due to its high selectivity for some mycotoxins, it is expensive, time-consuming, and requires stable antibodies as well as large volumes of the solvent. AFs have been also determined in nuts using solid-phase microextraction (SPME)[8] and dispersive liquid−liquid microextraction (DLLME).[5] The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, a combination of extraction and clean-up steps, has been increasingly applied to mycotoxins due to its ease of use and suitability for extraction from complex matrices.[9−11]

Chemical analysis can be defined as a sequence of elementary operations that are statistically independent of each other. The result of analysis should be considered as a continuous random variable and that the mean and the standard deviation are considered as a statistical description of distribution of experimental values. Any chemical analysis requires procedures for method development, calibration, and validation of results. The quality approach aims to ensure the reliability and traceability of results. In this study, the

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Table 1. UPLC−MS/MS Parameters for the Analysis of AFs in the ESI$^+$/MRM Mode[a]

mycotoxins	molecular formula	molecular weight [g/mol]	RT [min]	parent ion [m/z]	daughter ion [m/z]	dwell time [ms]	cone voltage [V]	collision energy [V]
AFB1	$C_{17}H_{12}O_6$	312	6.09	313.2	285.1(Q)*	25	5	24
					241.1(q)*			36
AFB2	$C_{17}H_{14}O_6$	314	5.80	315.2	287.1(Q)	25	20	26
					259.1(q)			30
AFG1	$C_{17}H_{12}O_7$	328	5.53	329.2	243.1(Q)	25	20	25
					283.1(q)			25
AFG2	$C_{17}H_{14}O_7$	330	5.23	331.2	245.1(Q)	25	50	30
					257.1(q)			30

[a](Q)*, quantification product ions; (q)*, confirmation product ions.

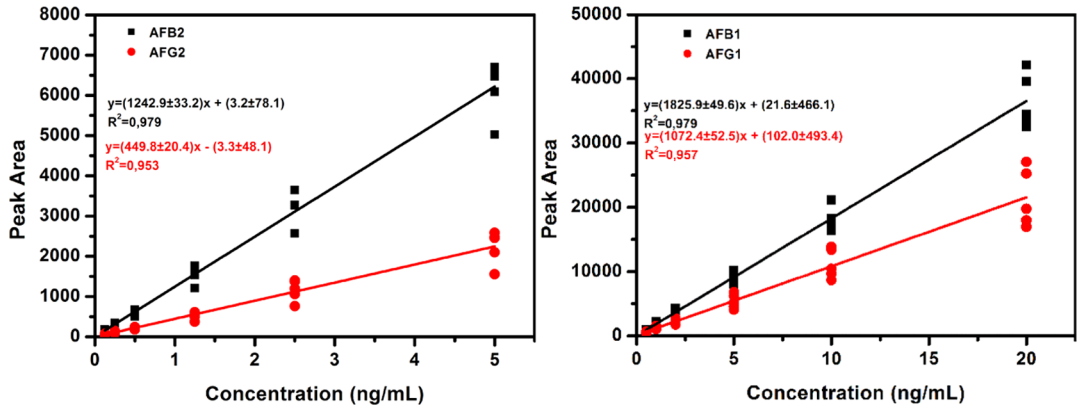

Figure 1. Representation of the peak area as a function of six concentration levels for the five analytes studied under reproducibility conditions (n = 5).

experimental protocols and the chemometric methods used to validate our analytical method are described. The approach was applied to the determination of aflatoxins in almonds by ultraperformance liquid chromatography coupled with tandem mass spectrometry. More specifically, the objective of this paper was to statistically evaluate the validity of this assay by means of accuracy profiles and data chronological distribution and to describe the procedures for estimation of biases, calculation of uncertainty through the use of certified reference material (CRM), and the method of standard additions, encompassing both trueness (bias) and reproducibility.

2. RESULT AND DISCUSSION

2.1. Ultraperformance Liquid Chromatography−Tandem Mass Spectrometry (UPLC−MS/MS) Method. Analyses were performed in multiple reaction monitoring (MRM) and positive polarity. MS parameters and the retention times are shown in Table 1. According to the criteria for MS detection and confirmation,[12] four points for each compound were selected (two MRM transitions) and monitored for quantification and confirmation. Also, the retention time of the analyte in the spiked sample corresponded to that of a calibration standard injected in the same run within a tolerance of 2.5%. The peak area ratios from the different transitions recorded for both the standard and sample (spiked sample) were within the tolerances fixed by EU criteria.

2.2. Method Validation. *2.2.1. Linearity Evaluation, Matrix Effect, and Limit of Quantification (LOQ).* Linearity of the regression model was confirmed after applying a lack-of-fit (Fisher-test) based on the analysis of variance (ANOVA). Calculations of the mean, variance, standard deviation of the five measurements, relative standard deviation (RSD) values were conducted. The full results are illustrated in Figure 1. Also, all RSD values are less than 20%, which is the value accepted in our protocol. The variability related to the matrix effects (evaluated for individual concentration) causes low signal suppression and enhancement but it is not very important for the AFs in almonds (data not shown). Therefore, the calibration standards were prepared in organic solvents for quantification. To estimate the LOQ, we used the standard deviation (SD) observed on the responses under repeatability conditions of a pseudo-blank sample. LOQ represents 10 times SD, which all ranged from 0.34 to 0.5 μg/kg in almonds. The LOQs estimated in the real almond sample were suitable for quantitative determination at EU legislation levels established at 2 and 4 μg/kg for AFB1 and total AFs, respectively.[2]

2.2.2. Normality. On the basis of 20 results obtained after 20 independent analyses (Table 2), the first idea of their distribution can be obtained by calculating a few statistical parameters, including the mean, variance, standard deviation, and then the p-order centered moments of the distribution (with p = 2, 3, 4), a p-order centered moments being defined by the following relation: $m_p = \lim_{n\to\infty}\left(\frac{\sum_{i=1}^{n}(x_i-\mu)^p}{n}\right)$. The same relationship can be used to calculate the p-order centered moment when n is large enough. The estimation of moments of orders 2, 3, and 4 then allows us to calculate the Fisher eccentricity and flattening coefficients, $g_1 = \sqrt{n}\frac{(\sum_{i=1}^{n}(x_i-\bar{x})^3)^2}{\sqrt[3]{(\sum_{i=1}^{n}(x_i-\bar{x})^2)^3}} = \frac{m_3}{\sqrt[3]{m_2^3}}$ and

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Table 2. Results Obtained for the Four AFs and AF_T (19 Independent Analyses) in the Same CRM (Almond Powder)

aflatoxins in almonds powder (random variable $X(\mu g/kg)$)						Fisher coefficients						Shapiro–Wilk test (n = 19)			
measurement	AFB1	AFB2	AFG1	AFG2	AFT	statistical parameter	AFB1	AFB2	AFG1	AFG2	AF_T		W_{obs}	$W_{0,99}$	$W_{0,95}$
1	1.476	5.607	4.296	2.331	13.710	mean	1.98	4.53	4.17	2.37	13.06	AFB1	0.899	0.863	0.901
2	2.352	5.592	4.377	2.487	14.808	standard deviation	0.35	0.92	0.69	0.52	1.37	AFB2	0.958	0.863	0.901
3	2.007	5.787	4.368	2.790	14.952	estimation of variance	0.12	0.84	0.48	0.27	1.89	AFG1	0.897	0.863	0.901
4	1.494	6.183	4.596	2.856	15.129										
5	1.875	2.628	6.132	1.482	12.417	centered moment (p = 2)	0.11	0.75	0.43	0.24	1.70	AFG2	0.982	0.863	0.901
6	2.367	3.819	3.600	1.695	11.481										
7	2.325	3.864	3.714	1.668	11.571										
8	2.472	3.726	4.437	2.595	13.230	centered moment (p = 3)	−0.01	0.05	0.21	0.01	−0.30	AF_T	0.942	0.863	0.901
9	2.019	4.968	4.461	1.908	13.356										
10	1.539	3.585	4.125	1.800	11.049										
11	2.115	4.464	4.185	2.562	13.326	centered moment (p = 4)	0.02	1.45	0.94	0.14	5.91				
12	1.512	3.993	2.982	2.262	10.749										
13	1.515	4.029	3.105	2.598	11.247										
14	2.103	4.530	4.308	2.049	12.990	Fisher eccentricity coefficient (g_1)	−0.27	0.08	0.74	0.08	−0.13				
15	1.872	4.482	4.497	2.169	13.020										
16	2.115	4.098	4.701	2.373	13.287										
17	1.695	4.224	3.414	3.411	12.744	Fisher flattening coefficient (g_2)	−1.34	−0.42	2.08	−0.56	−0.95				
18	2.310	5.625	3.702	3.075	14.712										
19	2.400	4.938	4.194	2.856	14.388										

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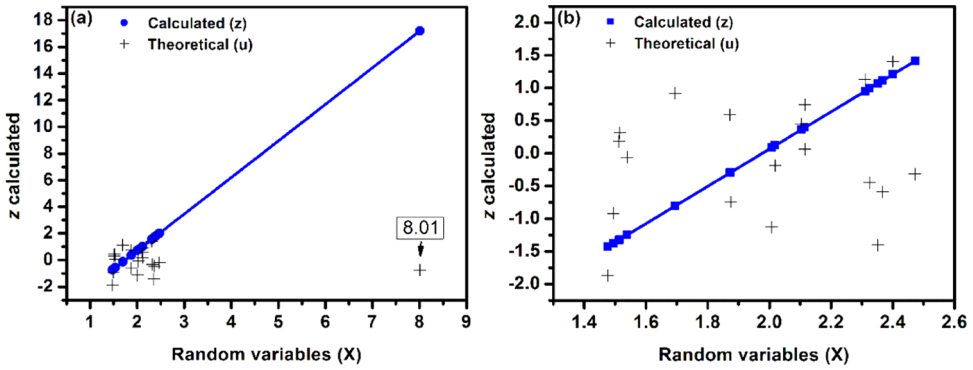

Figure 2. (a) Henry line with all measurements without elimination of outliers (n = 20, case of AFB1). (b) Henry line after deletion of the highest value of 8.01 μg/kg (n = 19, case of AFB1).

$g_2 = \frac{n\sum_{i=1}^{n}(x_i - \bar{x})^4}{(\sum_{i=1}^{n}(x_i - \bar{x})^2)^2} - 3 = \frac{m_4}{m_2^2} - 3$, respectively, which provide information on the fit of the distribution. We consider that there is a fit if $g_1 = 0$ and $g_2 = 0$. It appears, considering the values of g_1 and g_2, that the distribution of the 20 repetitions did not fit the normal distribution with the existence of extreme values ($g_2 > 0$) and probably higher than the mean ($g_1 > 0$). As expected from the examination of the Fisher coefficients, it can be seen that there are the highest values for all target analytes that need to be removed, after which the remaining 19 values are adjusted. However, the distribution of AFB1 stretches toward the negative values ($g_1 < 0$) and there is a greater frequency of observed distribution away from the mean ($g_2 < 0$). The result from the study of Fisher coefficients shows that these data sets do not follow the normal distribution.

The normality was also investigated using the Henry line (graphical method). The pairs of points (x_i, z_i) form a straight line (Figure 2), where z_i is the reduced central value associated with each experimental value x_i and theoretical reduced central value u_i. The pairs (x_i, z_i) are more or less well aligned according to the deviation of the real distribution from the theoretical one. By this means, it is possible to identify the points that slightly distort the graph and that are suspected to be outliers (e.g., AFB1 in Figure 2). This proves that it is important to use more convenient alternatives such as the Shapiro–Wilk test to confirm that hypothesis. Indeed, after the application of the Shapiro–Wilk test, the observed values (W_{obs}) were compared with the critical values (W_{crit}) at significance levels α = 0.01 and 0.05. Normal distribution of responses was confirmed ($W_{obs} > W_{crit}$) at α = 0.01 for all compounds but not at α = 0.05 in the case of AFB1 and AFG1.

2.2.3. Specificity and Confirmation of Positives. Examination of the chromatograms reveals that for the matrix tested (almond powder), there is no problem with peaks of interfering substances in the aflatoxin retention windows. The retention times obtained with the spiked samples in relation to the retention times of the standards [$(t_{STD} - t_{spiked\ S}) \times 100/t_{STD}$] are less than 2.5%.[12] Detection by mass spectrometry also requires the fragmentation of the AFs that give two ionic products each. The ratios of ion intensities obtained with the fortified almond samples are compared to those obtained with the standards. The deviations obtained are less than 30% set by the decision 2002/657/EC of the European Commission. The retention time (RT, ±2.5%) and ion ratio (IR, ±30%) variations measured in the spiked samples and those obtained from the calibration standards were within the permitted tolerances. Figure 3 illustrates the MRM chromatograms of spiked almond samples with AFs.

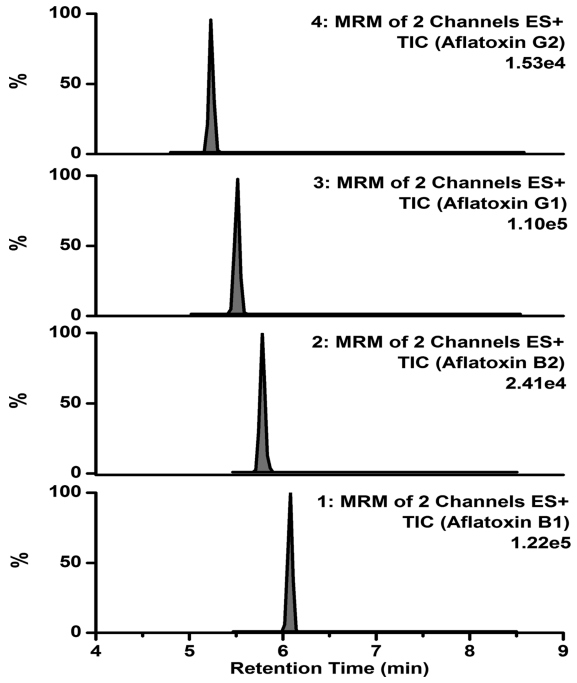

Figure 3. MRM chromatograms of spiked almond samples with AFB1 and AFG1 at a concentration of 2 μg/kg and AFB2 and AFG2 at 0.5 μg/kg.

The absence of significant matrix effects could be attributed to the sample treatment proposed in this study, which enables the majority of lipids to be separated.

2.2.4. Accuracy Profiles, Trueness, Recoveries, and Uncertainties. In Table 3, a limit of acceptability (20%) and a probability of tolerance (95%) were set. The results of the trueness show that the biases (systematic errors) are different from zero and are negative for the majority of compounds, which corresponds to an underestimation; the maximum bias is observed for AFB1 at level 1 (0.5 ng/mL) and reaches −5.96%, but it is not significant. Also, there is no orderly variation of the biases with analytes or concentrations.

Here, the number of measurements performed to estimate the mean concentration is 9, so the confidence interval is obtained with n = 9 (number of measurements), k (α;ν) = 2.31, and u_C being the combined uncertainty. The results

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Table 3. Accuracy Profiles of AFB1 and AFB2

compounds	AFB1						AFB2					
tolerance probability	95%						95%					
limit of acceptability (%)	20	20	20	20	20	20	20	20	20	20	20	20
level (ng/mL)	1	2	3	4	5	6	1	2	3	4	5	6
precision RSD (%)	13.11	5.70	3.11	1.58	1.02	3.11	8.08	8.72	12.72	6.35	3.72	12.72
biases (%)	−5.96	−3.50	−1.88	−0.73	0.77	−0.19	2.22	1.69	−1.58	−0.88	2.90	−0.16
trueness (%)	94.0	96.0	98.0	99.2	100.7	99.7	102.4	101.6	98.4	99.1	102.9	99.4
lower limit of the tolerance interval	0.3	0.8	1.8	4.8	9.7	19.8	0.025	0.05	0.3	1.05	2.2	4.8
upper limit of the tolerance interval	0.7	1.2	2.2	5.2	10.3	20.2	0.225	0.45	0.7	1.45	2.8	5.2

Table 4. Calculation of Repeatability, Within-Lab Reproducibility (RSD), Recovery, and Uncertainty (U)

compounds	spiking Level (μg/kg)	precision RSD (%)	biases (%)	recovery (%)	lower acceptability limit (%)	upper acceptability limit (%)	*U* (%)
AFB1	2.0	5.31	−0.47	100.2	70	110	11.50
	10	3.58	4.93	105.6	70	110	12.16
AFB2	0.5	8.06	6.40	107.4	50	120	21.16
	2.5	4.09	3.12	103.8	70	110	10.71
AFG1	2.0	6.78	−12.70	88.2	70	110	32.58
	10	1.80	−5.04	95.0	70	110	11.30
AFG2	0.5	9.48	−10.90	89.1	50	120	32.08
	2.5	7.43	−11.12	82.4	70	110	32.36

Table 5. Proficiency Test (PT) Results and the Estimated Uncertainties (U)

compound	mean (n = 19)	SD	RSD (%)	*U* (%)	measured value (μg/kg)	PT assigned value (μg/kg)	PT tolerance value (μg/kg)	*z*-score
AFB1	1.98	0.35	17.73	2.1	2.4	2.9	1.7	−0.58
AFB2	4.53	0.92	20.19	4.9	6.3	6.8	3.9	−0.25
AFG1	4.17	0.69	16.55	2.7	4.5	5.3	3.2	−0.5
AFG2	2.37	0.52	21.85	2.1	3.0	3.1	1.9	−0.11
AF_T	13.06	1.37	10.52	10.4	16.2	17.9	9.5	−0.36

obtained show that the intraday means are within the confidence intervals calculated. Also, we concluded that the instrumental performance and calibration remained stable during the 3 consecutive days of measurements, with an RSD below 15%.

We also intended to evaluate the accuracy profile of AFs in spiked almond samples as another possible approach. Recoveries were determined from the validation experiments analyzing almond samples spiked at 0.25 × ML, 1 × ML, 1.25 × ML, and 5 × ML. Calculations of the combined uncertainties can be performed (Table 4). On the one hand, the method accuracy profile can be considered to visualize the order of magnitude of method biases by taking into account the matrix effect. The relative biases calculated are considered acceptable as they are less than 13%. On the other hand, the results obtained show a variation in uncertainty that does not depend on the compound and concentration. The values range from 10.7 to 12.2% for concentrations of 2 and 10 μg/kg for AFB1, 2.5 μg/kg for AFB2, and 10 μg/kg for AFG1. However, for levels of 0.5 μg/kg AFB2, 2 μg/kg AFG1, 0.5 and 2.5 μg/kg AFG2, the uncertainties obtained are slightly higher and on the orders of 20 and 30%. These uncertainties remain low and satisfactory considering the number of steps integrated in the overall analytical protocol.

For recoveries, the results obtained are all within the required limits as performance criteria, with respect to the requirements of European Commission Regulation EC 401/2006[13] (70−120%). The accuracy profiles demonstrate that the method for the determination of the AFs is adapted to its fit for purpose and it is therefore valid without having to assign a correction factor for further measurements.

2.2.5. Use of Internal Quality Control (IQC). Performance evaluation is applied to continuous quantitative variables (X) (values in Table 2). To make a decision on the suitability of the method, and after participating in a proficiency test (PT) from BIPEA (Bureau Interprofessionnel d'Etudes Analytiques) for the determination of AFs in almond powder (BIPEA 3-1131-0084), the tolerance value is used to determine an interval around the assigned value. Within this interval, a result of measurement is considered acceptable. The detailed results of the PT organized by BIPEA (Bureau Interprofessionnel d'Etudes Analytiques) for CRM of almond powder are summarized in Table 5.

After that, a chronological distribution of measured values of AFs is performed in the same CRM under reproducibility conditions for two months (Figure 4), allowing to control the stability of the method over time and the estimation of the uncertainties.

On the basis of the results obtained, the measurement uncertainty was estimated at values below 11%, which is acceptable. Furthermore, this approach can also lead to the construction of the control chart (Shewhart charts) with the survey limit (SL) and control limit (CL) based on the measured values of CRM. This type of graph corresponds to the best representation of the results obtained by a laboratory. It is preferable to use at least 30 measurements, but this number of measurements was not carried out. Under these conditions, Student's t-values can be taken as 2 and 3, respectively. The graphs in Figure 4 display all measurement results and their associated deviations, namely, between the measured values, the true values of the CRM, and the SL and CL. All points were below the target values (PT assigned

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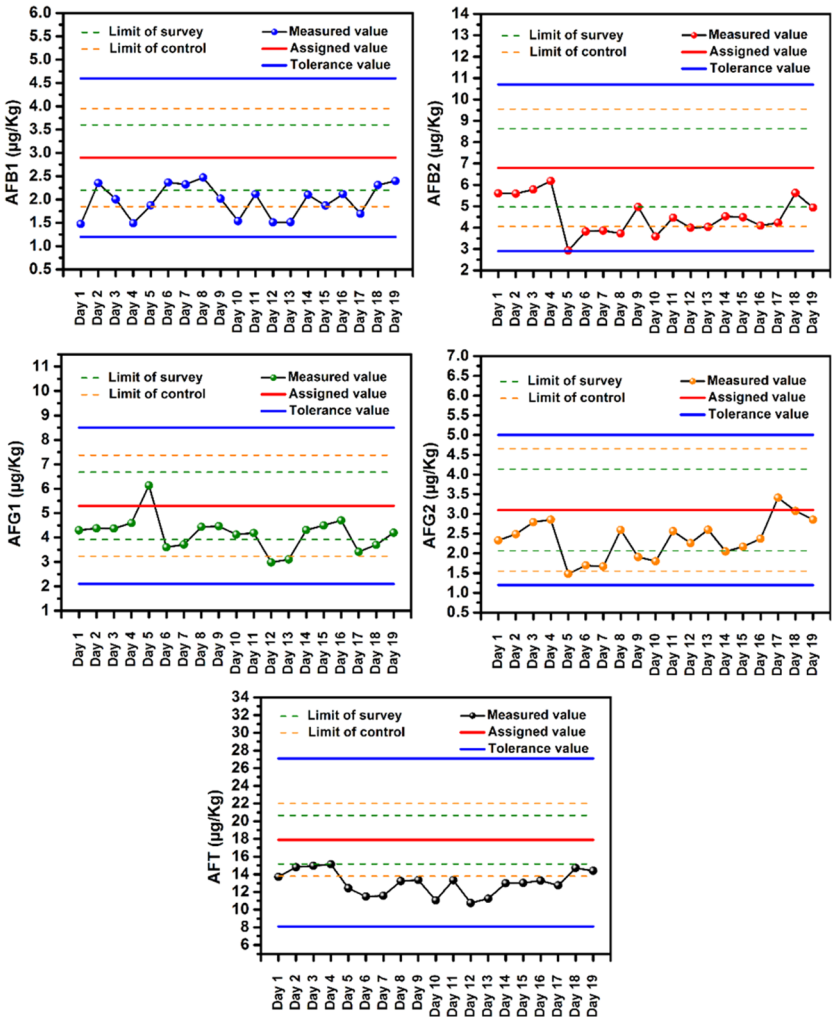

Figure 4. Chronological distribution of the measured values of AFs and total AFs in the same CRM of almond powder analyzed for two months. The red lines correspond to the PT assigned value, the blue lines mark the boundary of the tolerance value from BIPEA, and the green and orange lines represent the survey and control limits, respectively.

values) and within the acceptable interval, which confirms the results obtained during the PT. However, the interpretation should be considered carefully when a measurement goes outside of the control limits. Our results randomly oscillate on the lower side of the target value between the control limits and target values. This means that our analytical process is under control. Fortunately, only 6 measurements out of 76 were below the lower control limit, including 5 points of AFB1 and 1 point of AFB2. Besides, we attempt to apply the trend rules of these consecutive points according to the guideline ISO TS 13530, based on the general principles that could be applicable to other analyses.[14] For example, in the case of AFB2, AFG1, and AFG2, the graphs show two consecutive points between SL and CL. Therefore, it is important to ensure that the next value must be taken into account. Concerning the four analytes, no more than three consecutive points are increasing or decreasing, which is quite good. The variations observed are slightly above 20%, and this may be attributed to the reproducibility of UPLC−MS/MS measurements. Overall, these results show that the method described allows us to reach relatively low measurement uncertainties. Moreover, the sample treatment proposed could compensate for the possible variations related to the instability of the instrument or the possible losses that may occur during the various stages of analysis.

In the present study, normality was demonstrated, and this is important to ensure the validity of the use of classical statistics and their significance. We employed a linear statistical model, which allows us to estimate the systematic bias or uncertainty (systematic error) and the random uncertainty. The accuracy profile can be used to provide correction to the biases and the limits of acceptability, if necessary. The recovery varied from 82.4 to 107.4% for concentrations between 0.5 and 10 μg/kg and fulfilled the performance criteria defined in the EU regulation (2002/657/EC). The estimated measurement uncertainties were for all of the aflatoxins between 10.7 and 32.4%. The estimated biases do not significantly affect the method, contrary to what was initially thought from the examination of the raw data and given the long period of analysis (2 months). Besides, the construction of the control charts clearly indicates proper functioning and stability of the whole analytical protocol, as well as the possibility of evaluating the next quality control results.

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents. All organic solvents and acids were of high performance liquid chromatography

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(HPLC) or LC−MS analytical grade. Salts were of analytical grade. Methanol (MeOH) was purchased from Merck (Darmstadt, Germany), and acetonitrile (MeCN) was from CARLO EBRA Reagents (France). Formic acid was supplied by Sigma-Aldrich (Darmstadt, Germany). Anhydrous magnesium sulfate powder ($MgSO_4$) was purchased from AppliChem GmbH (Darmstadt, Germany), sodium chloride (NaCl) was from Merck (Darmstadt, Germany), and ammonium formate (HCO_2NH_4) was from LOBA Chemie (India). Ultrapure water was provided by SOLVACHIM (Casablanca, Morocco). A Minisart NY25 syringe filter with hydrophilic polyamide (nylon, 0.2 μm) was obtained from Sartorius.

Analytical standard solutions for AFB1 (2 μg/mL), AFB2 (0.5 μg/mL), AFG1 (2 μg/mL), AFG2 (0.5 μg/mL), and OTA (10 μg/mL) were purchased from Biopure (Tulln, Austria). The working standard solutions were prepared as follows: 0.5, 1, 2, 5, 10, and 20 ng/mL for AFB1, AFG1, and OTA; 0.125, 0.25, 0.5, 1.25, 2.5, and 5 ng/mL for AFB2 and AFG2.

Certified reference materials (CRMs) of almond powder (BIPEA 3-1131-0084) containing AFB1, AFB2, AFG1, and AFG2 were obtained from the Bureau Interprofessionnel des Études Analytique (BIPEA, France).

3.2. Extraction Procedure. AFs are extracted from ground almonds according to the QuEChERS method described previously with some modifications.[15] Briefly, in a 50 mL polypropylene tube, ground almonds (5 g) were extracted using a methanol−acetonitrile (15 mL) solution (60:40, v/v) and vortexed for 1 min using a VF2 Junkelkunkel (IKA-Labortechnik). Subsequently, anhydrous $MgSO_4$ (6.5 g) and NaCl (1.25 g) were added. After 1 min of shaking at room temperature, the mixture was centrifuged at 4000 rpm for 3 min on a refrigerated centrifuge (0 °C) Sigma 2-16 KL (Sigma GmbH, Germany). The extracts were then frozen overnight at −18 °C to separate the majority of lipids. Finally, 1.5 mL of upper organic phase was directly filtered through a NY syringe filter (0.2 μm) and injected into UPLC−MS/MS.

3.3. Instrumentation. Detection was performed using a UPLC Acquity H-class PLUS system, coupled with a TQ-S micro triple quadrupole mass spectrometer (Waters, Milford, MA). Chromatographic separation of AFB1, AFB2, AFG1, and AFG2 was carried out with an ACQUITY UPLC BEH C_{18} analytical column (1.7 μm, 2.1 mm × 100 mm) (Waters). The autosampler was set at 10 °C. The flow rate of the mobile phase was fixed to 0.45 mL/min, and the injection volume for the UPLC system was 2 μL. The column oven was maintained at 45 °C. The mobile phase consisted of eluent A (H_2O, 5 mM ammonium formate, 0.1% formic acid) and eluent B (MeOH). The gradient elution started at 98% eluent A for 0.25 min with a linear increase to 99% eluent B in 8 min. Then, the column was re-equilibrated with initial conditions for 2 min.

For MS/MS detection, the electrospray ionization (ESI) interface was used in positive polarity with the following settings: capillary voltage, 4 kV; ESI source temperature, 150 °C; desolvation gas temperature, 450 °C; cone gas flow, 1 L/h; and desolvation gas flow, 990 L/h. The acquisition of data was performed by applying the multiple reaction monitoring (MRM) mode with a dwell time of 0.025 s. Masslynx and Targetlynx V 4.2 software (Waters Corp., Milford, MA) were employed for data acquisition and processing.

3.4. Theory, Statistical Model and Data Processing. In this case, it is important to confirm the normality distribution of experimental values obtained, then evaluate the systematic error, random error, combined uncertainty, specificity of the method, detection and quantification limits, confidence interval of the measurement result, and finally construct the control chart. Statistics packages/software used in this work for data processing and plotting include Excel, MINITAB, and Origin.

3.4.1. Methods. 3.4.1.1. Linearity Evaluation, Matrix Effect, and Limit of Quantification (LOQ). The determination of the range of analysis allows us to check whether the technique is suitable for the concentrations of interest. The requirement for linearity applies to the relationship between the calculated and the introduced concentration and not to the response function, which is the relationship between the signal and the introduced concentration. To assess the linearity, calibration curves were prepared in an organic solvent and analyzed with three replicates (n = 3) on the same day. Six levels of calibration concentrations used were 0.5, 1, 2, 5, 10, and 20 ng/mL (AFB1 and AFG1) and 0.125, 0.25, 0.5, 1.25, 2.5, and 5 ng/mL (AFB2 and AFG2). In total, five consecutive injections on five different days were conducted with the same operator. The matrix effect (ME) was calculated for individual concentrations (2 ppb for AFB1 and AFG1, 0.5 ppb for AFB2 and AFG2) by dividing the area corresponding to the spiked extract sample with the area of a standard solution of the same concentration. The limits of quantification (LOQs) were estimated via the blank approach based on the standard deviation (SD) of the response as 10 times the SD.

3.4.1.2. Normality. To test the validity of normality, we performed 20 independent analyses (20 independent analyses including sample treatment and measurements from the same sample) on the certified reference material of almond powder (BIPEA 3-1131-0084). About 20 measurements represent indeed a minimum if we look at fitness testing for the normal distribution. For comparison, three approaches were considered: the use of Fischer's coefficient, the graphical method (Henry line), and the Shapiro−Wilk test.

3.4.1.3. Specificity. Specificity is the ability to establish the existence of the analyte in the presence of other components. It is the ability to demonstrate that the analyte being analyzed in the matrix is the analyte of interest. Specificity is based on an absence of interferences. The specificity could be estimated by the standard additions method by calculating the recovery rates.

3.4.1.4. Trueness Evaluation, Recovery, and the Expanded Measurement Uncertainty (U). Nine measurements of the AFs were carried out on three different days (three series, nine measurements, and three repetitions), which give three analyses over a one-day interval between each measurement for both calibration solutions (reference materials) and spiked samples. This makes it possible to appreciate the correction to be brought to the result obtained as well as the calculations of the measurement uncertainty. It is a requirement under ISO/IEC 17025 that laboratories determine and make available the (expanded) measurement uncertainty, expressed as U, associated with analytical results.[16] Our analytical method with a few steps is applicable. It is relatively simple and excluded the use of solid-phase extraction (SPE) or immunoaffinity columns (IAC). Potential sources of uncertainties are the accuracy and reproducibility of LC−MS/MS measurements. For uncertainty measurements, we used the approach given by the following formula[17]

$$\text{result} = \text{true value} + \text{systematic error} + \text{random error}$$

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The measurement uncertainty, which characterizes the dispersion, is expressed as a standard deviation (SD), which is the square root of the sum of all variances associated with the error sources. Systematic error (bias) is the difference between the expected test result and the accepted reference value. To estimate of systematic error (systematic uncertainty), the accuracy profile method was applied to calibration solutions (reference materials) prepared in the solvent as well as to the fortified real samples. A correction factor could be obtained and applied to the measurement result if necessary. However, since the reference materials are only known with a certain uncertainty (u_{REF}), this must be also counted. This uncertainty is given in the form of the expanded uncertainty, U, as $U = k \times u_c$, generally, $k = 2$ (coverage factor) for a confidence interval of 95%. The random error is defined as the dispersion of the mean. Random uncertainty is given by the following equation: $u_R = \frac{SD}{\sqrt{n}}$, where n is the number of measurements. The uncertainty can be calculated by other ways[16] on the basis of a certified reference material (CRM) analyzed over a period of 2 months. The CRM must have the same matrix as the samples. This implies equality of variances and can also be tested by the method of standard additions. Under these conditions, SD is measured on the CRM. This gives the following combined uncertainty $u_C = \sqrt{u_{CRM}^2 + u_R^2}$

3.4.1.5. Confidence Interval. The confidence interval can be determined by applying Student's t test with combined uncertainty. The confidence interval is calculated as $\bar{x} \mp k(\alpha,v)u_C$, where $\bar{x}$ is the mean, k follows the Student's test at v degrees of freedom, $v = n - 1$, α is the confidence level, typically $\alpha = 0.05$ for $P = 0.95$, n is the sample size, and u_C is the combined uncertainty. The expanded uncertainty is given by $U_C = k \times u_C$. The survey (SL) and control (CL) limits are calculated according to the following formulas: SL = Assigned value(target) $\mp$ (2 × SD) and CL = Assigned value (target) $\mp$ (3 × SD).

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Notes

The authors declare no competing financial interest.

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