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Interlaboratory Precision Evaluation of an Immunoaffinity Column Clean-up HPLC Method for Determination of Aflatoxins B and G in Naturally Contaminated Corn Flour

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Abstract

This interlaboratory collaborative study evaluated the precision performance of a high-performance liquid chromatography (HPLC) method for the determination of AFB₁, AFB₂, AFG₁, AFG₂, and total aflatoxins (AFs) in naturally contaminated corn flour. Three naturally contaminated test materials representing different contamination levels were prepared, assessed for homogeneity and stability, and distributed to 11 participating laboratories. Each laboratory analysed the test items in triplicate under repeatability conditions using immunoaffinity column clean-up, post-column derivatization, and fluorescence detection. Participant results were statistically screened by analyte and contamination level to identify censored measurements, outlying values, and inconsistent laboratory performance before calculation of the final precision parameters. Precision estimates were obtained for AFB₁, AFB₂, and AFG₁ at levels A and B, and for all four aflatoxins at level C. AFG₂ could not be formally evaluated at levels A and B because the number of quantitative results was insufficient, mainly due to results reported as not detected or below the limit of quantification. Across the evaluable analyte–level combinations, repeatability coefficients of variation ranged from 3.17% to 10.25%, while reproducibility coefficients of variation ranged from 8.77% to 27.71%. Reproducibility variability was generally higher than repeatability variability, confirming the contribution of between-laboratory effects to total method variation. The results indicate that the HPLC method provided technically interpretable repeatability and reproducibility estimates for most analyte–level combinations in naturally contaminated corn flour, although low-level determination of AFG₂ remained limited by analytical quantifiability.

Keywords Aflatoxin; HPLC; corn flour; interlaboratory precision; reproducibility

1. Introduction

Cereals, belonging to the Gramineae family, constitute a major component of human and animal diets worldwide and represent one of the principal routes of exposure to mycotoxins, toxic secondary metabolites produced by certain filamentous fungi. Environmental conditions

during cultivation, harvesting, storage, and processing strongly influence fungal growth and subsequent mycotoxin production in cereal grains [1]. Among cereal-associated mycotoxins, aflatoxins (AFs) produced mainly by the fungal species, *Aspergillus flavus* and *Aspergillus parasiticus*, are of particular concern because of their



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high toxicity and widespread occurrence in cereals and cereal-based products [2, 3]. Based on their fluorescence characteristics under ultraviolet light, AFs are classified into B, G, and M groups. These compounds exhibit potent hepatotoxic, mutagenic, and carcinogenic effects and have been strongly associated with the development of hepatocellular carcinoma. Cereals such as corn, wheat, and rice are particularly susceptible to AF contamination during both pre- and post-harvest stages, making them important dietary sources of exposure for humans and animals. Furthermore, the metabolic conversion of aflatoxin B₁ (AFB₁) to aflatoxin M₁ (AFM₁) in lactating animals can lead to secondary contamination of milk and dairy products, thereby increasing the potential risk to consumers [4].

Several analytical approaches have been developed for the detection of AFs in food and feed. Chromatographic techniques, particularly high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), and liquid chromatography–mass spectrometry (LC–MS), remain the most reliable methods owing to their high sensitivity, selectivity, and analytical accuracy. Immunological methods such as enzyme-linked immunosorbent assay (ELISA) and lateral flow assays are widely employed for rapid screening; however, they may be affected by matrix interferences and limited quantitative precision. More recently, biosensor-based technologies have emerged as promising tools for rapid and portable detection. Despite these advances, chromatographic methods, especially HPLC, continue to be regarded as the reference techniques for confirmatory analysis [2, 4]. Consequently, regulatory frameworks and standardized analytical methods have been established to ensure reliable monitoring and control of aflatoxin contamination in food and feed. In particular, ISO 16050 specifies an HPLC-based method for the determination of AFB₁ and total AFs (AFB₁, AFB₂, AFG₁, and AFG₂) in cereals and cereal-derived products and is widely applied in official control and reference laboratories [5].

Despite the availability of standardized analytical methods, significant interlaboratory variability may arise from differences in sample preparation, derivatization procedures, and instrumental conditions, particularly at concentration levels close to regulatory limits. To evaluate the performance of the standardized HPLC method described in ISO 16050, an interlaboratory collaborative study was conducted using naturally contaminated cereal test materials containing different levels of AFB₁, AFB₂, AFG₁, and AFG₂. The test materials were distributed to participating laboratories, and the analytical results were statistically evaluated to assess method performance characteristics, including repeatability and reproducibility. The findings provide valuable information on the re-

liability of the method for the determination of aflatoxins in cereals and cereal-derived products.

2. Materials and Methods

2.1. Interlaboratory Study Design

The interlaboratory comparison study was designed and implemented according to the requirements of INSO 9001 [6] and ISO/IEC 17043 [7] guidelines for proficiency testing schemes. The study design, selection of test materials, contamination levels, sample preparation procedures, homogeneity and stability assessment, distribution conditions, and statistical evaluation criteria were established by an expert committee consisting of representatives from the National Standards Organization, the Research Institute of Standards, and specialists in mycotoxin analysis. A simultaneous participation model was adopted, in which identical test items were distributed to all participants at the same time.

2.2. Preparation of Test Materials

Naturally contaminated corn samples containing aflatoxins B and G were employed as test materials. Three distinct contamination levels (A, B, and C) were established to cover a broad concentration range representative of naturally occurring contamination. Levels A and B were obtained from individual naturally contaminated corn batches, whereas level C was prepared by blending four corn lots exhibiting comparable levels of contamination. All samples were ground, sieved to particle sizes below 600 µm, and homogenized using a mixer in accordance with reference material preparation guidelines [8]. The final materials were then automatically packaged in moisture-resistant polyethylene and aluminum laminate bags, each containing approximately 170 g of corn flour, to ensure stability and prevent moisture uptake during storage and distribution.

2.3. Participants and Distribution of Test Items

Eleven laboratories participated in the interlaboratory comparison study. Participating laboratories were accredited according to ISO/IEC 17025 [9] or recognized as collaborating laboratories and had previously demonstrated satisfactory performance in proficiency testing programs for aflatoxin determination. Each laboratory received three coded test samples representing the three contamination levels. The test materials were sealed at ambient laboratory temperature and distributed simultaneously to the participating laboratories by postal service at ambient temperature without refrigeration, together with analytical instructions and reporting forms. The homogeneity and stability of the test materials were assessed as described in Section 2.4.

2.4. Homogeneity and Stability Assessment

Homogeneity and stability studies were conducted according to Iranian National Standard, INSO13283 [10] and ISO 13528:2022 [11]. For each contamination level, ten randomly selected samples were analyzed in duplicate to evaluate between-sample variability. Stability was assessed by storing selected samples under laboratory and frozen conditions and comparing analytical results over the testing period. The between-sample standard deviation (Ss) and differences between homogeneity and stability measurements were calculated to confirm that the prepared materials were sufficiently homogeneous and stable throughout the interlaboratory study.

2.5. Reagents, Standards

All reagents and solvents, including methanol, acetonitrile, phosphate-buffered saline (PBS, pH 7.4), sodium chloride, potassium bromide, and nitric acid, were of analytical or HPLC grade and were purchased from Merck (Darmstadt, Germany). Immunoaffinity columns specific for AFB₁, AFB₂, AFG₁, and AFG₂ were obtained from Aokin (Berlin, Germany). A mixed AF standard solution containing AFB₁, AFB₂, AFG₁, and AFG₂ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Calibration solutions at seven concentration levels were prepared by appropriate dilution of the stock standard solution with methanol.

2.6. Sample Preparation

Sample preparation and aflatoxin analysis were performed in accordance with Iranian National Standard No. 6872 [12] and ISO 16050 method [5]. Briefly, 50 g of the ground sample was accurately weighed and mixed with 5 g of sodium chloride. Subsequently, 200 mL of methanol–water (80:20, v/v) extraction solvent was added, and the mixture was shaken on a mechanical shaker for 30 min. The extract was filtered through Whatman filter paper. A 20 mL aliquot of the filtered extract was diluted with 130 mL of deionized water to obtain 150 mL of diluted extract. The diluted extract was then passed through a glass microfiber filter (GF, 1.6 µm pore size). A 70 mL portion of the filtered solution was used for immunoaffinity cleanup.

Immunoaffinity columns specific for AFB₁, AFB₂, AFG₁, and AFG₂ were allowed to equilibrate to room temperature and conditioned with 10 mL of phosphate-buffered saline. Subsequently, 70 mL of the diluted extract was passed through the column at a flow rate of approximately 2–3 mL/min. After sample loading, the column was washed with 10 mL of deionized water and dried under positive air pressure.

Extracted aflatoxins were eluted with 1.5 mL of HPLC-

grade methanol and collected in a clean amber vial. The eluate was subsequently diluted with 1.5 mL of deionized water and thoroughly mixed using a vortex mixer. Finally, 100 µL of this solution was injected into the HPLC for further separation and detection of aflatoxins.

2.7. HPLC Conditions and Quantification

Aflatoxins were determined by reversed-phase HPLC coupled with fluorescence detection. Chromatographic separation was performed using a C18 column, maintained at 40 °C. The mobile phase consisted of water, methanol, and acetonitrile (6:3:2, v/v/v) and was delivered in isocratic mode at a flow rate of 1.0 mL/min. Prior to use, the mobile phase was filtered through a membrane filtration system and degassed by sonication. Post-column derivatization was performed either by an electrochemical derivatization system (Kobra cell) or by a photochemical derivatization device (UV cell). For laboratories employing the Kobra cell system, 120 mg of potassium bromide and 350 µL of 4 M nitric acid were added per liter of mobile phase to generate bromine in situ for post-column derivatization. Fluorescence detection was carried out at an excitation wavelength of 365 nm and an emission wavelength of 435 nm.

Calibration curves were constructed by plotting chromatographic peak area against analyte concentration. The resulting curves showed acceptable linearity, with coefficients of determination (R^2) greater than 0.98. The exact concentration ranges used by the participating laboratories were not collected as part of the interlaboratory dataset.

2.8. Recovery Assessment

Method accuracy was evaluated through recovery experiments. A blank corn sample was spiked with mixed aflatoxin standards at a concentration equivalent to 5 µg/kg of AFB₁. This spiking level was selected based on the maximum tolerance limit specified by the Iranian national standard for aflatoxins in corn and was considered representative of a regulatory-relevant concentration for evaluating method recovery [13]. Spiked samples were processed together with routine samples and analyzed under identical conditions. According to INSO 6872, recovery values between 70% and 110% were considered acceptable [12]. Reported results were corrected for recovery according to the analytical procedure before statistical evaluation.

2.9. Quantification

The analytical results were calculated according to Eqs. (1) and (2). The amount of analyte in the sample extract was determined from the calibration curve using Eq. (1).

$$W_{smp} = \frac{(Signal_{smp} - b)}{a} \quad (1)$$

where W_{smp} is the amount of analyte in the sample extract (μg), $Signal_{smp}$ is the chromatographic peak area of the analyte in the sample, a is the slope of the calibration curve, and b is the intercept. The analyte concentration in the sample was calculated according to Eq. (2).

$$C_{sample} = \frac{W_{smp} \times V_{Ext.solvent} \times V_{Dil} \times V_{Elut} \times V_{Inj.std}}{W_s \times V_{Ext.aliquot} \times V_{passed} \times V_{Inj.smp}} \quad (2)$$

where C_{sample} is the analyte concentration in the sample ($\mu\text{g}/\text{kg}$), $V_{Ext.solvent}$ is the extraction solvent volume (200 mL), V_{Dil} is the final diluted extract volume (150 mL), V_{Elut} is the final eluate volume (3 mL), W_s is the sample weight (50 g : 0.05 kg), $V_{Ext.aliquot}$ is the aliquot volume of the extract (20 mL), V_{passed} is the volume of diluted extract passed through the immunoaffinity column (70 mL), and Inj_{smp} and Inj_{std} are the injection volumes of the sample and calibration standard, respectively.

2.10. Statistical Analysis

Statistical evaluation was performed according to ISO 13528:2022 [11] and Iranian National Standard No. 13283 [10]. Homogeneity and stability data were evaluated before distribution of the test items using between-sample standard deviations and comparisons of mean values. Participant results were then organized by contamination level and analyte and statistically assessed to estimate the interlaboratory precision of the analytical method.

Censored results, including values reported as not detected or below the limit of quantification, were identified before precision calculations. Outlier and consistency screening was performed using Cochran and Grubbs tests, together with Mandel h and k statistics. Cochran testing was used to assess excessive within-laboratory variation, whereas Grubbs testing was used to identify extreme laboratory mean values. Mandel h statistics were used to evaluate between-laboratory consistency, and Mandel k statistics were used to assess within-laboratory repeatability. Only acceptable and technically interpretable results were retained for calculation of the final repeatability and reproducibility parameters.

Random selection of test items for homogeneity and stability assessment was performed using Minitab software. Diagnostic statistical outputs, including histograms, normal probability plots, and Grubbs outlier plots, were also generated using Minitab.

3. Results and discussion

3.1. Overview of the interlaboratory study

This collaborative study was designed to evaluate the

performance of the HPLC method for the determination of AFB₁, AFB₂, AFG₁, and AFG₂ in cereal matrices and to estimate its interlaboratory precision parameters. Naturally contaminated corn flour was selected as the test material because it better represents routine analytical conditions than artificially spiked samples, particularly with respect to matrix–analyte interactions, extraction behavior, and the heterogeneous distribution of AFs in cereal matrices. Three contamination levels, designated as levels A, B, and C, were prepared and distributed to the participating mycotoxin laboratories.

A total of eleven laboratories participated in the study. Each laboratory received one test item from each contamination level and was requested to analyse the samples in triplicate under repeatability conditions. The target analytes were AFB₁, AFB₂, AFG₁, AFG₂, and total AFs. The results were statistically evaluated for each analyte and contamination level to identify inconsistent results, assess within- and between-laboratory variability, and calculate the final precision parameters. Because naturally contaminated aflatoxins may be unevenly distributed in cereal matrices, homogeneity and stability assessments were required to ensure that the observed variability mainly reflected method performance rather than test-material inconsistency.

3.2. Homogeneity and stability of the test materials

The homogeneity and stability of the naturally contaminated corn flour test materials were evaluated before distribution to the participating laboratories. Homogeneity was assessed using the between-sample standard deviation (S_s), whereas stability was evaluated by comparing the overall mean obtained in the homogeneity study (X) with the mean obtained in the stability study (Y). The evaluation criteria were based on the standard deviation for proficiency assessment, also referred to as the target standard deviation (S or σ_{pt}). Where relevant and where sufficient uncertainty information was available, an uncertainty-based acceptance criterion was also considered. As summarized in Table 1, the homogeneity criteria were satisfied for all analyte–level combinations for which a formal evaluation could be performed. For AFG₂ at level A, the target standard deviation was not available; therefore, a formal criterion-based homogeneity conclusion was not assigned. However, the observed between-sample standard deviation was low ($S_s = 0.0056$), suggesting limited between-sample variation.

Stability was confirmed for AFB₁ at level A and for all analytes at levels B and C, according to either the primary acceptance criterion or the uncertainty-based criterion. In contrast, for AFG₁, AFB₂, and AFG₂ at level A, the reported results did not demonstrate fulfillment of a formal sta-

Table 1. Homogeneity and stability assessment of aflatoxin-contaminated corn flour test materials

Row	Test Level	Measurand Aflatoxin	Homogeneity Evaluation				Stability Evaluation			Standard Deviation S
			S_s	$S_s = 0$	$S_s \leq 0.3\sigma_{pt}$	$S_s <$	$ X-Y $	$ \bar{x}-\bar{y} \leq 0.3\sigma_{pt}$	$ \bar{x}-\bar{Y} \leq 0.3\sigma_{pt} + 2\sqrt{(u^2(X)+u^2(Y))}$	
1	A	B ₁	0.107	--	×	--	0.267	--	×	0.568
2		G ₁	0.065	--	--	×	0.599	--	--	0.136
3		B ₂	< 0	×	×	--	0.096	--	--	0.057
4		G ₂	0.006	--	--	--	0.099	--	--	--
5	B	B ₁	0.125	--	×	--	0.006	×	--	0.812
6		G ₁	< 0	×	×	--	0.033	--	×	0.276
7		B ₂	0.061	--	×	--	0.097	--	×	0.102
8		G ₂	0	×	×	--	ND	×	--	--
9	C	B ₁	0.179	--	×	--	0.258	×	--	2.158
10		G ₁	0.210	--	×	--	0.666	--	×	0.787
11		B ₂	0.115	--	--	×	0.154	--	×	0.284
12		G ₂	0.106	--	×	--	0.034	--	×	0.078

S_s : between-sample standard deviation
 S or σ_{pt} : standard deviation / proficiency-test standard deviation
 X : overall mean of the homogeneity-test results
 Y : overall mean of the stability-test results
 $u(x)$: repeatability uncertainty of the homogeneity results
 $u(y)$: repeatability uncertainty of the stability results

bility criterion. Therefore, these low-level stability results should be interpreted with caution and should not be used as the sole basis for a definitive stability claim unless additional supporting evidence, such as uncertainty estimates or replicate stability data, is available. Overall, the results indicate that the prepared materials were suitable for use in the interlaboratory comparison, although stability verification at low contamination levels remained particularly important.

3.3. Screening and statistical evaluation of participant results

After receipt of the participant results, the data were organized by contamination level and analyte. Because each laboratory analysed the distributed test items in triplicate under repeatability conditions, laboratory-level means and within-laboratory standard deviations were calculated for each analyte–level combination. The corresponding Mandel h and k statistics and screening remarks are provided in Supplementary Tables S1A–S1C. Before calculation of the final precision estimates, the re-

sults were screened to identify censored measurements, outlying values, and inconsistent laboratory performance. Results reported as ND or below the LOQ were treated as censored before statistical evaluation. The LOD and LOQ values reported by each participating laboratory were not collected within this study, as the interlaboratory evaluation focused on the comparability and precision of reported quantitative results. The screening included Cochran and Grubbs tests, together with Mandel h and k statistics, to evaluate within-laboratory variation, extreme laboratory means, and between-laboratory consistency. Only acceptable and technically interpretable results were retained for calculation of repeatability and reproducibility parameters. The number of retained laboratory datasets for each analyte–level combination is reported as p in Table 2. The number of acceptable quantitative results differed among analytes and contamination levels. At level A, sufficient quantitative results were available for AFB₁, AFB₂, and AFG₁, whereas AFG₂ was strongly affected by censored measurements, with most laboratories reporting values below the limit of quantification. Therefore, AFG₂

Table 2. Interlaboratory precision estimates for individual aflatoxins at contamination levels A, B, and C.

Level	Analyte	<i>p</i>	Mean (µg/kg)	<i>S_r</i> (µg/kg)	<i>r</i> (µg/kg)	<i>CV_r</i> (%)	<i>S_R</i> (µg/kg)	<i>R</i> (µg/kg)	<i>CV_R</i> (%)
A	AFB ₁	10	2.867	0.187	0.525	6.53	0.594	1.663	20.71
	AFB ₂	10	0.296	0.026	0.073	8.78	0.061	0.172	20.72
	AFG ₁	9	1.011	0.075	0.210	7.40	0.143	0.401	14.17
	AFG ₂	—	ND	—	—	—	—	—	—
B	AFB ₁	6	3.602	0.175	0.490	4.85	0.316	0.884	8.77
	AFB ₂	7	0.488	0.035	0.099	7.21	0.075	0.210	15.38
	AFG ₁	7	1.161	0.056	0.158	4.86	0.127	0.357	10.98
	AFG ₂	—	ND	—	—	—	—	—	—
C	AFB ₁	10	9.706	0.308	0.861	3.17	2.172	6.082	22.38
	AFB ₂	11	1.068	0.102	0.287	9.59	0.296	0.829	27.71
	AFG ₁	10	3.590	0.162	0.452	4.50	0.878	2.459	24.46
	AFG ₂	11	0.301	0.031	0.086	10.25	0.082	0.229	27.17

p: number of accepted laboratory datasets;

Mean: mean concentration of the accepted results;

S_r: repeatability standard deviation;

r: repeatability limit (2.8S_r);

CV_r: repeatability coefficient of variation (100S_r/Mean);

S_R: reproducibility standard deviation;

R, reproducibility limit (2.8S_R);

CV_R: reproducibility coefficient of variation (100S_R/Mean).

ND: not determined because sufficient quantitative results were not available.

at level A was not suitable for formal precision estimation. At levels B and C, higher contamination levels generally produced more interpretable quantitative results, although differences among analyte–level combinations remained evident in terms of within-laboratory variation, between-laboratory consistency, and the number of accepted results.

The laboratory-level evaluation was used as a diagnostic screening step and was not intended to rank or compare participating laboratories. Because acceptability could differ according to analyte, concentration level, quantifiability, and within-laboratory variation, decisions to retain or exclude results were made for each analyte–level combination based on statistical and analytical suitability for precision estimation.

3.4. Interlaboratory precision: repeatability and reproducibility

The final repeatability and reproducibility estimates for

the retained analyte–level combinations are summarized in Table 2. At level A, precision parameters could be calculated for AFB₁, AFB₂, and AFG₁, whereas AFG₂ was not evaluated because the available results were predominantly below the limit of quantification. The repeatability coefficients of variation (CV_r) ranged from 6.53% to 8.78%, while the reproducibility coefficients of variation (CV_R) ranged from 14.17% to 20.72%. Among these analytes, AFG₁ showed the lowest between-laboratory variability, whereas AFB₂ showed the highest within-laboratory variability.

At level B, precision estimates were based on six accepted laboratory datasets for AFB₁ and seven datasets for AFB₂ and AFG₁, with three replicate measurements per laboratory. Although these datasets allowed estimation of within- and between-laboratory variability, the resulting precision estimates are less robust than those based on all 11 participating laboratories and should therefore be interpreted with caution. The CV_r values ranged from

4.85% to 7.21%, and the CV_R values ranged from 8.77% to 15.38%. Compared with level A, the level B results generally showed lower relative variability, particularly for AFB₁ and AFG₁. However, these estimates should be interpreted with some caution because they were based on a smaller number of accepted laboratories. AFG₂ could not be evaluated at this level because sufficient quantitative results were not available.

At level C, precision estimates were available for all four aflatoxins. Within-laboratory variability remained comparatively low for AFB₁ and AFG₁, with CV_R values of 3.17% and 4.50%, respectively, but was higher for AFB₂ and AFG₂ (9.59% and 10.25%). In contrast, CV_R values ranged from 22.38% to 27.71%, with the highest value observed for AFB₂. The marked difference between CV_R and CV_T at this level indicates that between-laboratory variation contributed more strongly to the overall variability than repeatability within individual laboratories. The precision characteristics depended on both analyte and contamination level. Repeatability was consistently better than reproducibility, as expected in an interlaboratory study. The results also show that increased analyte concentration did not uniformly reduce between-laboratory variability; although repeatability generally improved for AFB₁ and AFG₁, reproducibility variability remained substantial at level C. Accordingly, interpretation of method precision should consider the number of accepted laboratories, analytical quantifiability, and the relative contributions of within- and between-laboratory variation.

3.5. Method performance, limitations, and applicability for standardization

The precision estimates presented in Table 2 provide the basis for interpreting the practical performance of the method under interlaboratory conditions. The final precision dataset showed that the method provided usable repeatability and reproducibility estimates for most analyte–level combinations. Precision parameters were obtained for AFB₁, AFB₂, and AFG₁ at levels A and B, and for all four aflatoxins at level C. In contrast, AFG₂ could not be formally evaluated at levels A and B because the number of quantitative results was insufficient, mainly due to results reported as not detected or below the limit of quantification. This finding shows that the main limitation for AFG₂ at low contamination levels was analytical quantifiability rather than only statistical inconsistency. Across the evaluable analyte–level combinations, reproducibility variability was generally higher than repeatability variability. This pattern is expected in interlaboratory studies and indicates that between-laboratory factors contributed substantially to total method variation. Po-

tential sources of this variability include sample preparation, immunoaffinity clean-up efficiency, derivatization conditions, calibration practices, chromatographic performance, and analyst-dependent procedural differences. Therefore, strict adherence to the standardized procedure remains essential when the method is implemented across different laboratories.

Taken together, the results support the applicability of the HPLC method for the determination of AFB₁, AFB₂, AFG₁, AFG₂, and total AFs in cereal matrices, particularly when analyte concentrations are above the practical quantification limits of the participating laboratories. The study also highlights that low-level determination of AFG₂ requires careful attention, because censored or non-quantifiable results may prevent formal precision estimation.

4. Conclusion

This interlaboratory collaborative study evaluated the precision performance of an HPLC method for the determination of AFB₁, AFB₂, AFG₁, AFG₂, and total AFs in naturally contaminated corn flour. The test materials were assessed for homogeneity and stability before distribution, and participant results were statistically screened before calculation of the final repeatability and reproducibility parameters.

The method provided technically interpretable precision estimates for most analyte–level combinations. Reproducibility variability was generally higher than repeatability variability, confirming that between-laboratory factors contributed substantially to total method variation. This finding emphasizes the importance of consistent implementation of the analytical procedure, particularly with respect to sample preparation, immunoaffinity clean-up, derivatization, calibration, and chromatographic analysis. Low-level determination of AFG₂ remained the main limitation because sufficient quantitative results were not available at levels A and B, with most laboratories reporting results as ND or below the LOQ. However, the method showed reliable performance for AFG₂ determination at higher concentration levels. Nevertheless, the results provide useful interlaboratory precision estimates for the application of the HPLC method to the determination of AFs in naturally contaminated corn flour. Its applicability to other cereal matrices should be evaluated in future interlaboratory studies.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- [1] A. Mousavi Khaneghah, I. Eş, S. Raeisi, Y. Fakhri, Aflatoxins in cereals: State of the art, *J Food Saf*, 2018, 38(6), e12532. <https://doi.org/10.1111/jfs.12532>.
- [2] V.O. Okechukwu, O.A. Adelusi, A.P. Kappo, P.B. Njobeh, M.A. Mamo, Aflatoxins: Occurrence, biosynthesis, mechanism of action and effects, conventional/emerging detection techniques, *Food Chem*, 2024, 436 137775. <https://doi.org/10.1016/j.foodchem.2023.137775>.
- [3] H. Dai, S. Liang, D. Shan, Q. Zhang, J. Li, Q. Xu, C. Wang, Efficient and simple simultaneous adsorption removal of multiple aflatoxins from various liquid foods, *Food Chem*, 2022, 380 132176. <https://doi.org/10.1016/j.foodchem.2022.132176>.
- [4] M. Xue, Z. Qu, A. Moretti, A.F. Logrieco, H. Chu, Q. Zhang, C. Sun, X. Ren, L. Cui, Q. Chen, Y. An, C. Li, H. Zhong, Z. Cao, F. Wang, Y. Sun, L. Wang, J. Hou, C. Zhang, M. Yang, Y. Ding, Y. Yao, P. Li, Y.-G. Zhu, Aspergillus Mycotoxins: The Major Food Contaminants, *Advanced Science*, 2025, 12(9), 2412757. <https://doi.org/10.1002/advs.202412757>.
- [5] International Organization for Standardization (ISO), ISO 16050:2003, Foodstuffs — Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products — High-performance liquid chromatographic method, 2003, p. 30.
- [6] Iran National Standards Organization, (INSO-ISO 9001) Quality management system requirements, Institute of

Standards and Industrial Research of Iran, Iran, 2018.

[7] International Organization for Standardization (ISO), ISO/IEC 17043: Conformity assessment — General requirements for the competence of proficiency testing providers, 2023, p. 30.

[8] International Organization for Standardization (ISO), ISO/TR 33402: Good practice in reference material preparation, 2025, p. 30.

[9] International Organization for Standardization (ISO), ISO/IEC 17025: General requirements for the competence of testing and calibration laboratories, 2017, p. 30.

[10] Iran National Standards Organization, INSO 13283: Statistical methods for use in proficiency testing by interlaboratory comparison, Institute of Standards and Industrial Research of Iran, Iran, 2024.

[11] International Organization for Standardization (ISO), ISO 13528, Statistical methods for use in proficiency testing by interlaboratory comparison, 2022, p. 30.

[12] Institute of Standards and Industrial Research of Iran, ISIRI 6872: Food and feed stuffs Determination of aflatoxins B&G by HPLC method using immunoaffinity column clean up-Test method Institute of Standards and Industrial Research of Iran, Iran, 2011.

[13] Iran National Standards Organization, INSO 5925: Food and feed-Maximum tolerated level of mycotoxins., Institute of Standards and Industrial Research of Iran, Iran, 2020.

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