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Chemometric Validation of a High-Performance Liquid Chromatography Method to Detect Ochratoxin A in Green Coffee

Andrea Mancusi [1], Serenella Seccia [2], Alessandra Izzi [3], Daniele Coppola [3], Mario Tessieri [2], Antonello Santini [2] and Irene Dini [2,*]

[1] Department of Food Microbiology, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute 2, 80055 Portici, Italy; andrea.mancusi@izsmportici.it

[2] Department of Pharmacy, University of Napoli Federico II, Via Domenico Montesano 49, 80131 Napoli, Italy; seccia@unina.it (S.S.); mario.tessieri@unina.it (M.T.); asantini@unina.it (A.S.)

[3] Laboratorio Chimico Merceologico (LCM), Corso Meridionale, 80143 Napoli, Italy; a.izzi@si-impresa.na.camcom.it (A.I.); d.coppola@si-impresa.na.camcom.it (D.C.)

* Correspondence: irdini@unina.it

Abstract: Green coffee beans and their derivatives are employed in dietary supplements, pharmaceuticals, and cosmetic formulations owing to their antioxidant properties and secondary metabolites, which may play a role in preventing metabolic syndrome. Mycotoxins, particularly ochratoxin A (OTA), present a considerable contamination risk and are regulated under strict guidelines established by the European Union and various food safety authorities. This study validates a reverse-phase HPLC method with a fluorimetric detector for quantifying OTA in green coffee beans, following the ISO 17025:2018 standard. The OTA recovery rate (≥70%) met European Union (EU) Regulation No. 2023/2782. The calibration curve showed perfect linearity (r-value = 1). As EU legislation requires, the application field detected OTA levels between 3 μg/kg and 5 μg/kg. Precision (sr = 0.0073) and accuracy (±0.76 μg/kg) were statistically acceptable, with a 95% confidence level indicating reliable results.

Keywords: food supplements; nutraceuticals; contaminants; analytical validation; metrological approach; ochratoxin A dosage; green coffee beans

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1. Introduction

The coffee plant belongs to the *Rubiaceae* family. Coffee is cultivated in over fifty nations and ranks among the most frequently traded agricultural commodities globally [1]. The total coffee bean production worldwide is about 10,035,576 tons annually. According to FAOSTAT, Brazil (3,700,231 tons annual production) and Vietnam (1,763,476 tons annual production) are the world's coffee production leaders, followed by Colombia, Indonesia, Ethiopia, Peru, Honduras, India, Uganda, Guatemala, Mexico, and Nicaragua [2]. The raw coffee beans that have not been fermented and processed constitute green coffee. Green coffee beans' complex nutritional profile and bioactive compound content are responsible for many beneficial effects on human health. Green coffee contains carbohydrates (55.0–65.5%), nitrogen compounds (11.0–15.0%), lipids (10.0–18.0%), minerals (3.0–5.4%), purine alkaloids (0.8–4.0%), chlorogenic acids (6.7–9.2%), and, in lower percentages, non-volatile aliphatic acids (quinic, citric, and malic acids), phenols (e.g., chlorogenic acid), diterpenes, caffeine, and trigonelline [3]. Green coffee and its derivative products (e.g., beverages, dietary supplements, additional foods, and nutraceuticals) have high antioxidant power. They can reduce body weight, improve blood pressure, plasma lipids, and cognitive functions [4],

control type 2 diabetes and alleviate symptoms of Parkinson's disease, prevent cardiovascular illnesses, promote gut well-being, and diminish the likelihood of developing cancer and depression [5]. Microfungi and their secondary metabolites, known as mycotoxins, particularly ochratoxin A (OTA) and aflatoxins, can contaminate green coffee beans and their derived products [6].

Contamination can occur in green coffee (both conventional and organic) during picking, storage, and transport [7]. OTA is generated by microfungi of the *Penicillium* genus (e.g., *Penicillium verrucosum)* and *Aspergillus* genus (e.g., *A. ochraceus*, *A. alliaceus*, *A. niger*, *A. carbonarius*, and *A. glaucus*) [8,9]. The temperature and humidity conditions may affect the growth of these microfungi [10]. OTA can induce nephrotoxicity, hepatotoxicity, neurotoxicity, teratogenic, and immunosuppression [11], has been classified as carcinogenic, and is included in the 2B group by the International Agency for Research on Cancer (IARC) [12]. The FAO/WHO Expert Committee on Food Additives considers the OTA-tolerable weekly intake as low as 112 ng/kg of body weight [13]. The highest allowable limit in roasted and ground coffee is 3 μg/kg, and 5 μg/kg in instant coffee. However, no specific limits are imposed for green coffee beans that are still unprocessed [14]. Nevertheless, controlling the OTA levels at various stages of coffee production (e.g., picking, washing, fermentation, and roasting) is crucial to decreasing the possible toxicity of the final products.

Various techniques have been reported for the detection of ochratoxin A in foods, including TLC (thin-layer chromatography) [15], HPLC (high-performance liquid chromatography) using a fluorescence detector (FLD) [16], HPLC with mass detector (HPLC-MS and HPLC-MS/MS) [17], immunoaffinity columns associated with a fluorometer (fluorometric kits) [18], gas chromatography coupled with mass spectrometry (GC-MS) [6], capillary electrophoresis linked to laser-induced fluorescence detector (CE-LIF) [19], enzyme-linked immunosorbent assay (ELISA) [6], molecular imprint polymers (MIPs) [20], micellar electrokinetic capillary chromatography (MEKC) [21], immunosensing test [22], fluorescence polarization immunoassay (PFIA) [23], isotope dilution method [24], and radioimmunoassay (RIA) [25], biosensors, and aptamers (single-stranded oligonucleotides designated in vitro to fix with high specificity and affinity to targets) [26]. However, as many of these tests are not validated, their results may be considered as not fully guaranteed.

This work validates, with a metrological approach, an analytical determination that uses HPLC-FLD to dose the OTA in green coffee beans.

The method validation is fundamental to trace data and prevent inaccurate dosages, which may lead to negative commercial impact and health risks. The process of validation is both costly and wasteful of time. The scientific community must increasingly focus on validating advanced analytical procedures for quality control in food to prevent redundant testing and save time. Validation's primary goal is to assess the uncertainty, which is a "marker" of how well the results align with the intended purpose and traceability. It provides a quantifiable measure of the results' reliability and accuracy.

The factors that must be validated to ensure the accuracy and reliability of scientific measurements and experiments to guarantee that the results are accurate, precise, and relevant to the research objectives include measuring range, robustness, specificity, and sensitivity [27]. The measuring range is the spectrum of the measured values where the precision and accuracy are deemed acceptable. The robustness refers to a method's capacity to resist significant changes in the final results due to intentional variations during its implementation. The intermediate repeatability tests, such as control charts, can provide insights into the method's robustness and may indicate the need for a dedicated robustness estimation study. The specificity indicates the method's capability to evaluate chemical parameters amidst possible interfering substances quantitatively. The sensitivity provides

the method's skill to distinguish minor differences in the measurement's concentration, quantity, or characteristics.

2. Materials and Methods

2.1. Samples

Columbia supreme green coffee beans (1 kg), bought at the local Italian market, were used for the method validation. The samples were kept at −20 °C up to the time of use.

The authors retain a selection of the analyzed samples for reference.

2.2. Solvents and Chemicals

Liquid chromatography (LC)-grade solvents (acetonitrile, deionized water, glacial acetic acid) and $NaHCO_3$ were acquired from Merck (Darmstadt, Germany).

Phosphate buffer saline (PBS) was made using potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate (1.16 g), sodium chloride (8.00 g), and 900 mL of deionized water. After dissolving the reagents, the pH of the solution was brought to 7.4 with a 0.1 mol/L HCl solution. The final volume of the solution was 1 L. Solvents and reagents used to prepare PBS were obtained from Merck (Darmstadt, Germany). OTA standard was bought from Merck (Darmstadt, Germany).

2.3. Green Coffee Sample Preparation

The green coffee bean samples (weight 15.0 g, accuracy ± 0.1g) were extracted with 150 mL of MeOH/$NaHCO_3$ (1/1, *w*/*w*) thirty minutes under stirring [28]. The extract was filtered on extra-fast pleated filter paper (Colaver. Milan, Italy). Successively, 50 mL of the filtrate was subjected to centrifugation f for 15 min (T = 4 °C) at 1300 rpm in a Thermo Fisher Scientific centrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

The extract (10 mL) was mixed with 30 mL of phosphate buffer saline (PBS) [28] and subjected to affinity chromatography (Ochraprep, R-Biopharm AG, Darmstadt, Germany).

2.4. Purification on Immunoaffinity Columns

Immunoaffinity columns (Ochraprep No. RBRP14/RBRP14B; R-Biopharm AG. Darmstadt, Germany) were brought to room temperature before conditioning and connected to the vacuum system (Vac-Man® Laboratory Vacuum Manifold, Promega, Milan, Italy). Five ml of PBS was flowed (2–3 mL/min), followed by 40 mL of the diluted extract (flow rate 5 mL/min). Subsequently, the immunoaffinity column was washed with 10 mL of PBS and 10 mL of deionized water (approximately 1–2 drops/second) and dried by applying a gentle vacuum with Visiprep (Supelco, Merck, Darmstadt, Germany) for 5–10 s.

2.5. Ochratoxin Dosage

The identification and dosage of OTA were obtained with an HPLC Shimadzu (Tokyo, Japan) equipped with Kinetex C18 column (250 × 4.6 mm × 5 µm) (Phenomenex, Torrance, CA, USA), a thermostat compartment for the column (T = 40 °C), a fluorimetric detector RF-20Axs (λ excitation = 333 nm, λ emissions = 454 nm), and LabSolutionLc Shimadzu 5.60 SP2 software for data management (Shimadzu, Tokyo, Japan). Finally, an isocratic flow (1.0 mL/min) comprising acetonitrile: water: acetic acid 49:51:1 (*v*/*v*/*v*) and injection volume 10 (µL) was employed.

2.6. Method Validation

Method validation was performed according to the requirements of the ISO 17025:2018 [27]. Selectivity was determined by chromatographing green coffee bean samples in an immunoaffinity column (Ochraprep; R-Biopharm AG, Darmstadt, Germany) before the HPLC

run and checking the possible presence of interfering signals with the OTA retention time (t_R).

The calibration curve was performed in triplicate analyses of five OTA standard solutions (0.1 µg/kg–0.5 µg/kg–1 µg/kg–2 µg/kg–5 µg/kg). These values were chosen based on the restrictions established by the existing EU regulations regarding coffee.

Linearity, LOD, LOQ, measuring range, and calibration uncertainty were obtained from the calibration curve.

Linearity was verified by measuring the calibration curve regression coefficient and confirmed metrologically by residual analysis.

Residuals (e_i) were obtained by

$$e_i = Y_i - \grave{Y}_i.$$

Here, $\grave{Y}_i$ = experimental value and Y_i = predicted value obtained by the calibration line.

Standardized residuals were obtained by subtracting the average from the observed value and dividing by the standard deviation.

Studentized residuals were obtained according to the following relationship:

$$\text{Studentized residual} = \frac{\text{residual}}{\text{ds(residual)} * \sqrt{(1 - \text{h} - \frac{1}{n})}}$$

Method precision was determined by calculating the relative standard deviations (RSD%). It was acquired by repeating five consecutive HPLC analyses of the spiked samples (0.1 µg/kg; 0.5 µg/kg; 1 µg/kg; 2.5 µg/kg; 5 µg/kg) on the same day and after one week.

$$\text{RSD\%} = \frac{S * 100}{x}$$

The Shapiro–Wilk test assessed the data normality. The probability test's level was p = 95%.

$$\text{W} = \frac{\sum_{i=1}^{n} a_i x(_i)}{\sum_{i=1}^{n} (x_i - \overline{x})^2}$$

$x(i)$ = smallest estimated sample's value; x_i = estimated sample's value; $\overline{x}$ = arithmetic mean of the sample estimate's values.

The Huber test was used to determine the median absolute deviation.

In the Huber test, after having arranged the data in ascending order, the data's median, the difference between each data and the obtained median (D_i), the differences' median (D_m), and the product $D_m \times 4.5$ were calculated. The probability test's level was p = 95%.

$$\text{Anomalous values} = \frac{D_i}{D_m} \geq 4.5$$

Accuracy and recovery were found by repeating ten consecutive HPLC analyses of the spiked samples obtained by injecting OTA (3.33 µg/kg) into the green coffee sample after it was left to rest overnight so that the OTA interacted with the green coffee matrix.

The accuracy was expressed as bias%:

$$\text{Bias\%} = \frac{\textit{Measured value} - \text{True value}}{\text{True value}} * 100$$

The recovery was expressed as a percentage according to the following relationship:

$$\text{Recovery\%(R\%)} = \frac{\text{C2} - \text{C1}}{\text{C3}} * 100$$

C_2 was the average OTA concentration in spiked samples; C_1 was the average OTA concentration in samples before addition. It was initially set to 0 because the original samples did not contain ochratoxin A. C_3 was the ochratoxin A concentration added (Ca = 3.33 µg/kg).

The measurement of the uncertainty was determined by studying all significant uncertainty sources to estimate the expanded uncertainty (uncertainty of the measurement method) [29].

Type A and B uncertainties were determined in accordance with EURACHEM/CITAC guidelines [30].

Type A was calculated based on 10 repeated HPLC analyses of the 2 µg/kg sample.

$$\text{U Type A} = \sqrt{\frac{\text{variance}}{\text{Degrees of freedom}}}$$

Type B uncertainties [30] (Table 1) considered were the following:

Table 1. Type B uncertainties considered in this work.

	U(mr)	U(ct)	U(200t)	U(1000t)	U(m)
Uncertainty	0.020	0.0036	0.0019	0.0015	0.00022

U(mr): Uncertainty linked to the reference material.

U(ct): Uncertainty linked to the calibration curve (five levels of standards were considered).

$$\text{U(ct)} \frac{Sx/y}{b} * \sqrt{1/\text{n} + 1/\text{m}}$$

Here,

S = Residual's standard deviation.

n = Points used for the calibration line.

m = Readings taken for each sample.

U(200t) = Uncertainty linked to a 200 µL pipette. It was found by taking into account repeatability and a certificate of calibration.

U(1000t) = Uncertainty linked to a 1000 µL pipette. It was found by taking into account repeatability and a certificate of calibration.

U(p) = Uncertainty linked to a 10 mL pipette was determined by taking into account repeatability and certificate of calibration.

U(m) = Uncertainty linked to a 10 mL flask. It was found by taking into account repeatability and a certificate of calibration.

$$\text{Resulting uncertainty } u_c\ (y) = \sqrt{u\ (X_1)]^2 + [u\ (X_2)]^2 + [u\ (X_3)]^2}$$

$$\text{Extended uncertainty } U(y) = k * u_c(y)$$

k is the coverage factor (values between 2 and 3 indicate a probability level of about 95%).

2.7. Statistical Analysis

ANOVA, Huber, and Shapiro–Wilk tests were performed with Statistica, Version 7.0 software (StatSoft, Hamburg, Germany).

3. Results

3.1. Method Selectivity

An immunoaffinity column (Ochraprep) was used to eliminate molecules capable of interfering with the ochratoxin A dosage.

The resolution of the reverse-phase column was evaluated by measuring the OTA's retention factor (k').

$$k^1 = \frac{t_R - t_D}{t_D}$$

t_R = Retention time;

t_D = Dead time.

For this purpose, the column's dead time (t_D) was theoretically and experimentally calculated.

Theoretically, it was obtained according to the following relation:

$$(t_D) = \frac{\text{dead volume}}{\text{flow}} = \frac{0.5 * 25\text{ cm} * (0.46)^2\text{ cm}^2}{1\text{ mL} * \text{min}^{-1}} = 2.65\text{ min}$$

The dead volume was reduced by 15%, considering that the columns with core–shell particles are more tightly packed than similar columns [31].

$$t_D = 2.65\text{ min} - 0.3975\text{ (15\% dead volume)} = 2.25\text{ min.}$$

OTA retention time (t_R) = 6.67 min.

The reverse-phase column dead time (1.68 min) was determined by injecting a solution containing uracil into the HPLC device.

The obtained chromatogram is shown in Figure 1.

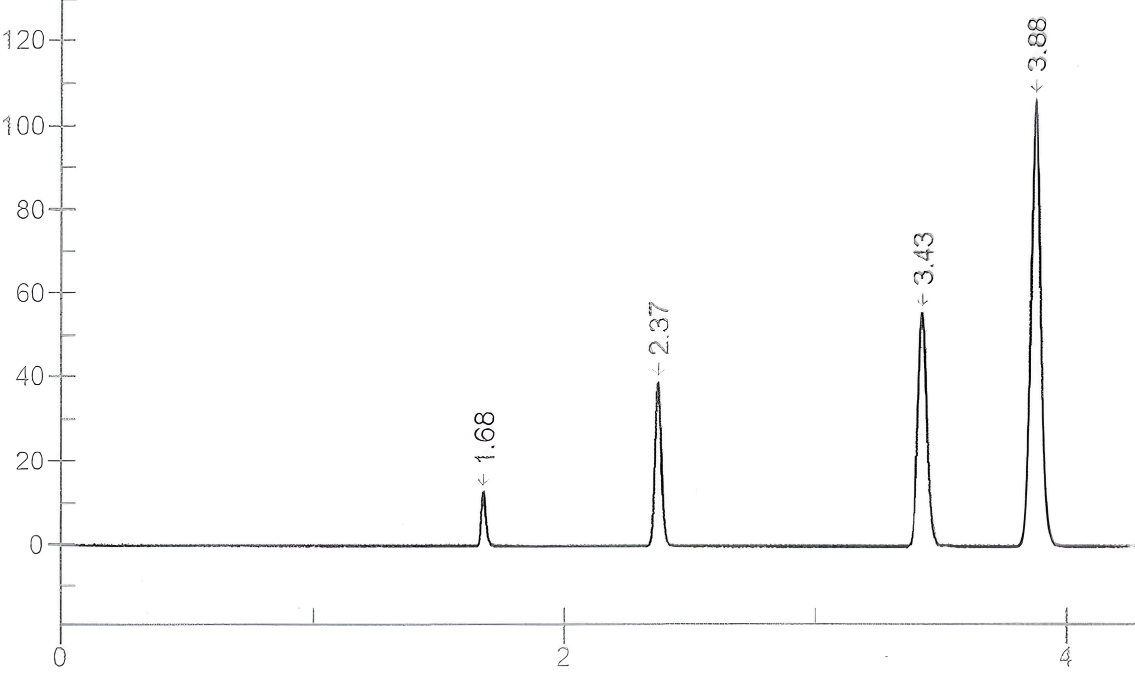


Figure 1. Chromatogram of the test solution (uracil (1.68 min), acetophenone (2.37 min), toluene (3.43 min), and naphthalene (3.88 min).

The ochratoxin A retention factor (6.67 min) was decided by injecting five concentrations of OTA standards (0.1 µg/kg–0.5 µg/kg–1 µg/kg–2 µg/kg–5 µg/kg) (Figure 2).

$$\text{The capacity factor } (k') = \frac{6.67\text{ min} - 1.68\text{ min}}{1.68\text{ min}} = 2.97$$

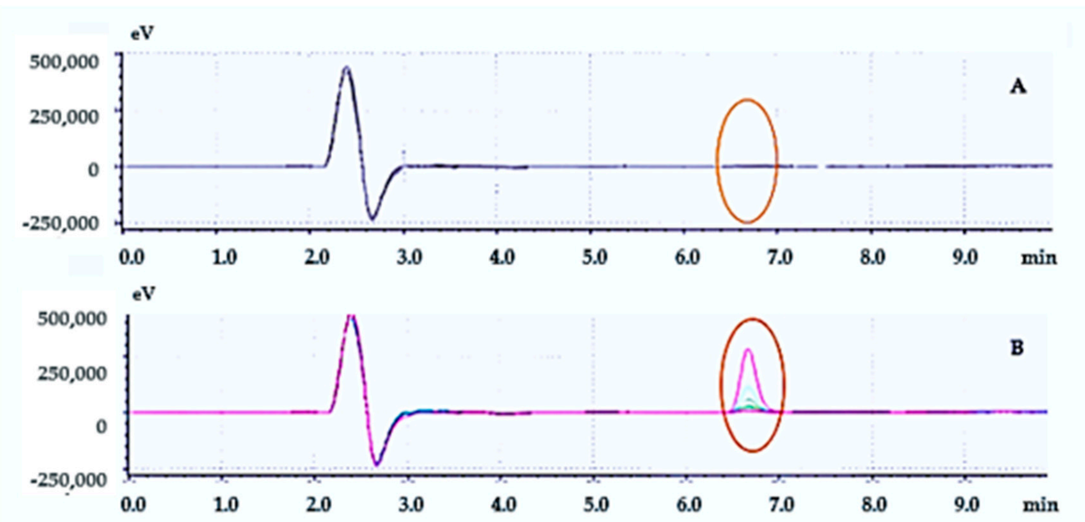
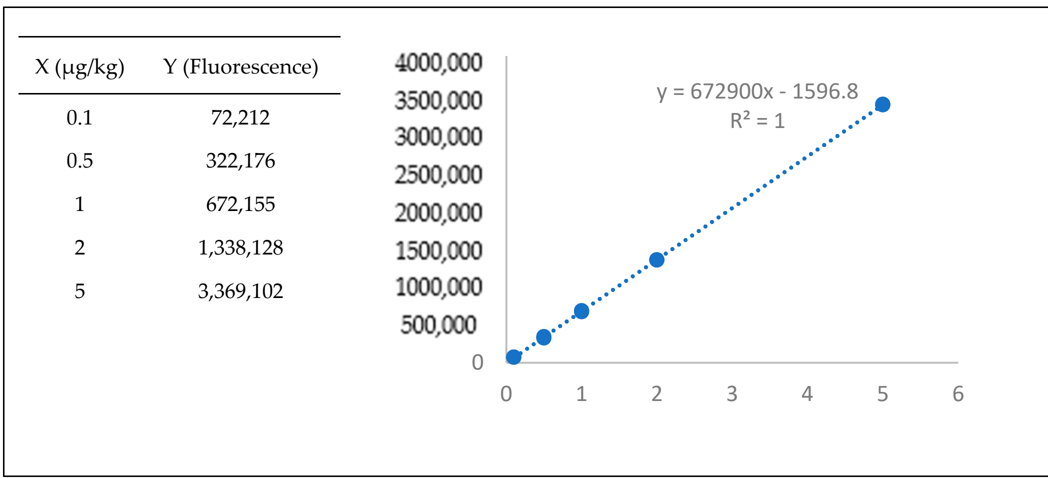


Figure 2. Chromatogram of the sample after Ochraprep chromatography (**A**) and chromatogram of the sample spiked with five concentrations (0.1 µg/kg–0.5 µg/kg–1 µg/kg–2 µg/kg–5 µg/kg) of OTA standards (**B**) after Ochraprep chromatography. In (**B**) the different colors indicate chromatograms obtained with different concentrations.

Selectivity was attributed to the absence of signals at the retention time. This is where the OTA signal appears when it detects a sample where OTA is absent.

3.2. Calibration Curve

Figure 3 reports the calibration curve obtained by measuring five OTA standard solutions.



X (µg/kg)	Y (Fluorescence)
0.1	72,212
0.5	322,176
1	672,155
2	1,338,128
5	3,369,102

Figure 3. Calibration curve obtained by measuring five replicates of five OTA standard solutions.

The curve's linearity was assessed through the regression coefficient (r) analysis (r = 1) and confirmed by the normally distributed residuals in the ANOVA test (Figure 4).

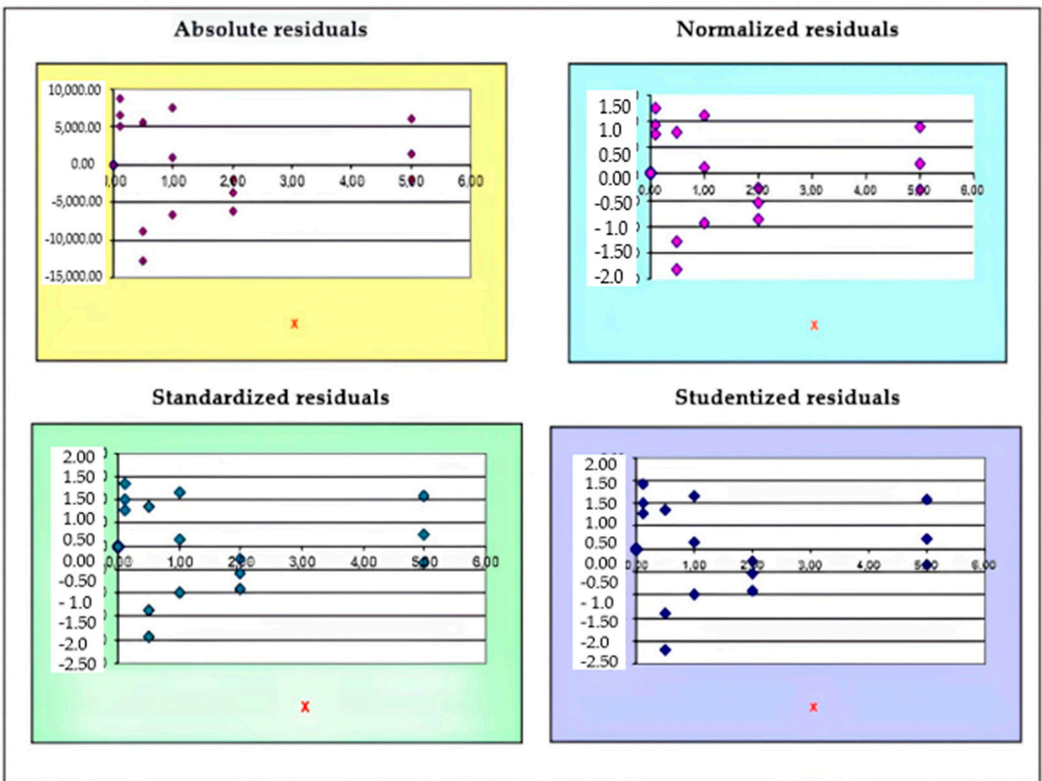


Figure 4. The ANOVA test determined residual distribution. ID = sample number; x = concentration; h_i = leverage coefficient; e_i = absolute residuals; e_{Ni} = normalized residuals; e_{Si} = studentized residuals; e_{ji} = standardized residuals.

More than 98% of the standardized residuals are in the range between − 2 and + 2. The homoskedasticity's violation was excluded as residuals fell in the interval + 3 − 3 [32].

3.2.1. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limits of detection (LOD) and limit of quantification (LOQ) were 3.3 σS and 10 σS, respectively (σ = relative standard deviation; S = slope of the standard curve).

The measuring range lower limit was calculated from the LOQ. The upper limit was retrieved from the maximum point of the calibration curve. The LOD value was 0.047 µg/kg, and the LOQ was 0.11 µg/kg.

3.2.2. Application Field

The application field refers to the interval between the minimum and maximum quantification thresholds within which the analytical method yields quantitative results characterized by adequate linearity, accuracy, and precision. The lower limit of quantification depends on the method's sensitivity. In this work, the range of application was from 0.11 µg/kg to 5 µg/kg.

3.3. Precision

Precision refers to how closely the results from different measurements of a uniform sample agree with one another when conducted under set conditions [33].

The intraday and interday precision, estimated below for five replicates, confirmed the test's reproducibility (Table 2).

Table 2. Intraday and interday precision.

	0.1 µg/kg	0.5 µg/kg	1 µg/kg	2 µg/kg	5 µg/kg
Intraday (RSD%)	1.1	1.3	1.4	1.5	1.7
Interday (RSD%)	1.5	1.5	1.7	1.8	1.9

The Shapiro–Wilk test was employed to verify if results were available according to a normal distribution (Gaussian) with true mean and variance.

In this work, W = 0.971 demonstrated the normal distribution. Subsequently, the Huber test was used to determine the possible presence of values that were different in an evident way from others (data aberrant or outliers) and residual homoscedasticity. No anomalous data in the Huber test were detected.

3.4. Accuracy and Recovery

Accuracy and recovery were evaluated in 10 fortified samples obtained by injecting ochratoxin A (3.33 µg/kg) into each green coffee sample.

The recovery was 76% (variation coefficient% = 5.47).

The accuracy was bias = −24%.

3.5. Uncertainties

Uncertainty elucidates the measurement's fluctuations. It expresses the degree of skepticism regarding the concentration achieved [27].

The uncertainties can be estimated by statistical means (type A) or evaluated by other means (type B).

The type A contribution was estimated as the relative deviation of the experimental parameter subject to measurement (Figure 4). In this specific case,

$$u(rip) = 0.0039.$$

The evaluation of category B is an assessment of the estimated input obtained differently to the treatment statistic of a series of observations. The standard uncertainty was evaluated based on a scientific judgment of all helpful information on the possible variability of x_i (uncertainties linked to the reference material, calibration curve, and related to the use of pipettes (200 µL, 1000 µL, and 10 mL), and of a flask (10 mL). The type B uncertainties allowed the calculation of the resulting and extended uncertainties.

Resulting uncertainty u_c (y) = 0.042;

Extended uncertainty U(y) = 0.084;

K = 2 (it corresponds to a confidence level of approximately 95.4%).

4. Discussion

Consumers are more concerned and searching for safe, high-quality food products. One of the main risks impacting food safety is the possible contamination due to mycotoxins. Ochratoxin A is the mycotoxin that most often contaminates green coffee beans. The European Community, with the EC regulation 1370/2022 [14], has set the maximum level of ochratoxin A at 3 µg/kg for roasted and ground coffee and at 5 µg/kg for instant coffee, but it has not set the maximum levels allowed in green coffee beans. Nowadays, green coffee is roasted to prepare coffee beverages. Nonetheless, its extracts are used to produce food supplements and nutraceuticals. Therefore, knowing the OTA's concentration in green coffee beans can help in understanding the concentration in processed products based on green coffee beans. Many analytical tests have been created to confirm the concentration

of ochratoxin A in coffee. However, robust validation of a method was still missing to the best of our knowledge; hence, it should be developed for official analyses.

This work validated a method based on reverse-phase HPLC with a fluorometric detector to determine ochratoxin A in green coffee.

Many research papers have reported the use of reverse-phase chromatography to determine OTA. This work employed an HPLC column packed with core–shell particles whose thin particle size distribution and little internal porosity guarantee better packing and column efficiency than other types of reverse-phase columns [31]. The capacity of the reverse-phase column to retain the ochratoxin A was considered adequate for experimental purposes as its capacity factor was ≅3. A k′ value greater than 1 indicates that the stationary phase sufficiently retains the solute [33].

Successively, the method was subjected to a validation process using objective evidence to confirm that it was suitable for its intended uses and to establish that any decisions based on the results could be taken safely. As outlined in ISO 17025:2018 [27], there are two different approaches to validating a method: one consists of interlaboratory comparisons, while the other relies on internal validation through a single laboratory study [34]. This work evaluated the linearity, LOD, LOQ, application field, accuracy, measurement uncertainties, recovery, and accuracy. For this purpose, a calibration curve was generated with five levels of a standard, and the linearity of the curve was evaluated, determining, in the first instance, the correlation coefficient (r) that indicates the degree of correlation between data (perfect if =1; good if >0.995). The correlation was considered excellent (r = 1) [35]. However, the regression coefficient could lead to errors because the points with greater concentration have a superior statistical "weight" compared to those with lower concentration. Therefore, the residuals analysis was carried out.

In ANOVA, the residuals are the differences between the actual values and those estimated according to the assumed model. The predicted values are subtracted from the observed values to obtain them. ANOVA requires residuals to assess model validity and detect discrepancies between the accepted model and data. Various deviations from ANOVA assumptions can be seen by studying the residuals, including heterogeneity, correlated errors, and outliers [32]. This work estimated the absolute, normalized, standardized, and studentized residuals. When performing the ANOVA test, we determine if the difference between the averages of the absolute residuals reflects a fundamental difference between the groups or is due to the random noise inside each group. The absolute residual is the difference between the equation's right- and left-hand sides when the calculated values are used as variables. They are negative for points under the regression line and zero for points that fall along it. The sum of all of the residuals should be zero. Normalized residuals are residuals divided by the standard deviation. The standardized residuals are normalized and scaled to mean = 0 and variance = 1. They test the normality assumption.

The studentized residuals are the difference between observed and predicted values divided by their standard deviation. The importance of studentized residuals lies in their ability to provide a standardized measure of a model's errors. They identify outliers and influential points in a dataset [32]. The residuals arranged according to a random relationship above and below the reference zero line confirmed the method's ability to give a signal directly proportional to the analyte's level in the sample. The interval between the minimal and the maximal amount of the samples, in which the analytical method provided quantifiable results (application's range = 0.11–5 µg/kg), was considered suitable to detect the OTA concentration tolerable by law in coffee (3–5 µg/kg).

The saturation levels of the extraction process and the detector determine the upper limit of quantification. Even though it makes sense to determine the total calibration range, evaluating linearity over a concentration that spans 50–150% of the assumed level or the

maximum residue limit in samples suffices. However, the minimum acceptable range should be determined based on the analysis scope [33]. The method's selectivity was achieved by passing the crude extracts through a column filled with monoclonal antibodies specifically targeting OTA, which can keep toxins in the sample within the gel suspension before chromatographing it using HPLC. The method precision was expressed as RSD (%) evaluating the intraday and interday data variability. It was obtained by repeating five tests with the same methodology on identical material, within the same laboratory, by the same operator, utilizing the same equipment, on the same day and after one week, and evaluating the standard deviation (sr), the data's distribution, and the presence of anomalous data. The low sr value indicated a low dispersion degree from the mean.

The Shapiro–Wilk test verified the normal data distributions. The Shapiro–Wilk test statistic (W) evaluates how well the standardized and ordered sample quantiles match the standard normal quantiles. The statistic can take a value between 0 and 1. $W = 1$ indicates a perfect match [27]. If W is $\cong$1, the normal hypothesis is confirmed. In the present work, $W = 0.971$, which demonstrated the data's normality.

The Huber test was used to check the presence of anomalous data, and it is recognized as one of the most robust tests for finding "anomalous" data. It employs an evaluation centered around the median, which remains unperturbed by extreme values within a sorted data series. The absence of $D_i \geq D_m \times 4.5$ demonstrated that no outliers were present. The homogeneity of variances (homoscedasticity) of the residues and the Huber test verified the test's linearity and sensitivity. The RSD values below 2 confirmed the test's precision.

Recovery and accuracy were evaluated in blank samples, found negative for OTA, spiked with 3.33 µg/kg ochratoxin A.

Recovery is the percentage of analyte retrieved from a sample, and assessing it is crucial for evaluating extraction efficiency. In this work, the recovery average of OTA in spiked samples was =76%, complying with Regulation (EU) 2023/2782 of the European Commission of 14 December 2023 [36], amending Regulation (EC) no. 401/2006 regarding the performance criteria for OTA in foods [37].

The accuracy of the analytical assay depends on how closely the concentration indicated by the assay matches the true value. The accuracy reflects the impact of systematic errors on the result. It evaluates the method's robustness and the ability to remain largely unaffected by intentional variations during implementation [27].

Finally, the uncertainties evaluation allowed the reliability of the results to be established. The uncertainty is a parameter linked to the result that describes the variability of values that could reasonably be ascribed to the measurand. It represents the maximal gap between a measured concentration and the actual value found when evaluating a sample with a specific level of significance.

In this work, the gap between $y - uc(y)$ and $y + uc(y)$ covers approximately 95% of the values ascribed to Y, of which y is an estimate. Therefore, the probability distribution defined by the measurement outcome y and the uncertainty composite standard uc(y) is approximately normal (Gaussian), and uc(y) is a valid approximation of the standard deviation of y [38].

5. Conclusions

The proposed work used a metrological approach to validate an existing reverse-phase HPLC fluorimetric method [28] to measure the amount of ochratoxin A, one of the foremost dangerous mycotoxins contaminating green coffee beans. It brings further data to confirm that the test is suitable for the use for which it was designed because it determines the OTA levels in coffee permitted by law (measuring range = 0.11 µg/kg to 5 µg/kg). The results showed that the method's recovery respects the performance criteria for mycotoxins

reported in the European Commission Regulation (EU) 2023/2782 (European Regulation (EU) 2023/2782). The absence of substances with retention times similar to OTA makes the method selective for experimental purposes. The reliability of the results was estimated with a probability of 95% based on the expanded uncertainty calculation. The straightforward nature of the method, along with its limited environmental footprint resulting from the moderate use of solvents, indicates its potential for application in routine analyses. Validation procedures are both time-consuming and expensive. The scientific community must enhance its endeavors in validating analytical methods to develop precise, accurate, and selective tests. Such advancements are essential for producing reliable and consistent results across various conditions, thereby ensuring food quality.

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