Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Original Article

Comparing determination methods of detection and quantification limits for aflatoxin analysis in hazelnut

Ümit Şengül*

Giresun University, Central Research Laboratory, Giresun, Turkey

ARTICLE INFO

Article history:
Received 18 December 2014
Received in revised form
22 March 2015
Accepted 24 April 2015
Available online 29 May 2015

Keywords:
aflatoxin
hazelnut
limit of detection
limit of quantification
method validation

ABSTRACT

Hazelnut is a type of plant that grows in wet and humid climatic conditions. Adverse climatic conditions result in the formation of aflatoxin in hazelnuts during the harvesting, drying, and storing processes. Aflatoxin is considered an important food contaminant, which makes aflatoxin analysis important in the international produce trade. For this reason, validation is important for the analysis of aflatoxin in hazelnuts. The limit of detection (LOD) and limit of quantification (LOQ) are two important parameters in validation. In this study, the LOD and LOQ values have been determined using the Association of Official Agricultural Chemists (AOAC) Method 991.31, which is one of the most viable high-performance liquid chromatography analysis methods in the analysis of aflatoxin in hazelnuts. Several approaches can be used to calculate LOD and LOQ values. In this study, to calculate the LOD and LOQ values, the visual evaluation (empirical) method, the signal-to-noise method, and calibration curve approaches were applied. The most appropriate approaches were compared. Our conclusion is that the visual evaluation method provided much more realistic LOD and LOQ values.

1. Introduction

Hazelnuts (*Corylusavellana*) are hard-peel fruits that are grown for worldwide trade. Hazelnuts are produced principally in Turkey, Italy, the United States, and Spain (550,000 tons, 110,000 tons, 25,000 tons, 18,000 ± tons, respectively, per year) followed by France, Greece, and Portugal [1] and are an important export product. Hazelnut plants generally grow in temperate climate zones with a relatively high humidity and a high rainfall rate. Adverse climatic conditions result in the formation of molds during the harvesting, drying, and storing processes of hazelnuts. Aflatoxin is one of the mycotoxins that can be generated by these molds [2–4].

Aflatoxins B1, B2, G1, and G2 (AFB1, AFB2, AFG1, and AFG2, respectively) are toxic metabolites generated by *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins have been reported to be associated with acute liver damage, liver cirrhosis, induction of tumors, and teratogenic effects [5–7]. The toxic effects include acute hepatitis, immunosuppression, and

* Giresun University, Central Research Laboratory, 28100 Giresun, Turkey.
E-mail address: usengule@yahoo.com.
http://dx.doi.org/10.1016/j.jfda.2015.04.009

hepatocellular carcinoma. In humans, the risks associated with aflatoxin consumption are well documented, and the International Agency for Research on Cancer has designated aflatoxin as a human liver carcinogen [8–10]. A major problem associated with hazelnut production is the formation of aflatoxin-producing molds. Because aflatoxin is a serious global problem, studies of the prevention, detection, and improvement of analytical test methods continue [11]. The European Commission has set limits for the maximum levels of total aflatoxin and AFB1 allowed in hazelnuts: 4 μg/kg (total aflatoxins) and 2 μg/kg (AFB1) [12]. For this reason, the analysis of the amount of aflatoxin in hazelnuts is important.

The purpose of an analytical method is the delivery of a qualitative and/or quantitative result with an acceptable uncertainty level. Therefore, theoretically speaking, "validation" really indicates "measuring uncertainty". In practice, method validation is performed by evaluating a series of method-performance characteristics, such as precision, trueness, selectivity/specificity, linearity, operating range, recovery, limit of detection (LOD), limit of quantification (LOQ), sensitivity, ruggedness/robustness, and applicability [13]. LOD and LOQ are two important parameters in quantitative analysis. The definition of LOD is defined by the United States Pharmacopeia as "a parameter of limit tests. It is the lowest concentration of the analyte that can be detected, but necessarily not quantitated, under the stated experimental conditions". In contrast, LOQ is defined as a parameter of quantitative assays for low levels of compounds in sample matrices. The LOQ is the lowest concentration in a sample that may be measured with an acceptable level of accuracy and precision under the stated experimental conditions [14–19].

Several approaches are used to calculate detection limits, which are the most important parameters of validation. These approaches are the visual evaluation, signal-to-noise, standard deviation of the blank, and calibration curve methods [14–17,20]. Because even a very small amount of aflatoxin in food is very dangerous for human health, analysis for this toxin must be very accurate and precise. This requires that analysis be performed via a validated standard method. LOD and LOQ are two important parameters of validation. Each laboratory uses different LOD and LOQ calculation methods for aflatoxin analysis with a high-performance liquid chromatography (HPLC). Our purpose in this study is to compare these calculation methods. To accomplish this, among the LOD and LOQ calculation methods, the visual evaluation, signal-to-noise, and calibration curve methods are examined, and the results are compared.

2. Materials and methods

In this study, the LOD and LOQ values were determined with three different calculation approaches by using the AOAC Method 991.31 "Aflatoxins in Corn, Raw Peanuts, and Peanut Butter Immunoaffinity Column (Aflatest) Method" [21]. The aflatoxin analysis was performed on an Agilent 1100 Model HPLC instrument (Agilent Technologies, Barcelona, Spain). For this purpose, three independent experiments were performed for each calculation approach in different time frames.

2.1. Materials

Toxin-free hazelnut samples were used in the study. In total, 10 kg of the hazelnut sample was ground to homogeneity, and 10 samples from different sample points were taken to verify the processes before analysis was performed. Aflatoxin was not found in any sample. Experiments were conducted with samples of this toxin-free blank. The samples were stored at −18°C in a freezer.

2.2. Standards and chemicals

A standard solution of aflatoxin (Aflastandard, R-Biopharm, Madrid, Spain) was used to prepare spike samples and to calibrate devices. The aflatoxin stock standard is sold in a 1000 μg/L concentration in a methanol solution. It consists of 250 μg/L AFG1, G2, B1, and B2 type aflatoxins. In the calibration curve methods, a standard solution of aflatoxin was dissolved in methanol (Supelco, Bellefonte, PA, USA), which contains 2600 μg/L aflatoxin (AFG1, AFB1 = 1000 μg/L; AFG2, AFB2 = 300 μg/L), was used in conjunction with this standard. HPLC gradient grade methanol and acetonitrile, nitric acid 65%, potassium bromide, and sodium chloride were purchased from Merck (Darmstadt, Germany). AflaTest-P immunoaffinity columns (IAC) with a 1 mL volume were purchased from VICAM (Milford, MA 01757, USA) for cleanup and isolation of aflatoxins extracted from hazelnut samples.

2.3. Instrument and chromatographic conditions

Analysis was performed using an HPLC 1100 series (Agilent Technologies, Barcelona, Spain) instrument fitted with an auto-sampler and a fluorescence detector.

- RP-HPLC column: ODS–2
- HPLC mobile phase: water-acetonitrile-methanol (6:2:3) (v/v/v) per liter of the mixture with 119 mg of potassium bromide and 350 μL nitric acid added
- Flow rate: 1.0 mL/min
- Wavelength: excitation: 360 nm; emission: 430 nm
- Injection volume: 100 μL
- Column temperature: 20–25°C
- Cobra cell: current source 100 μA set

2.4. Procedure

2.4.1. Visual evaluation (empirical methods)

The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The quantitation limit at which the analyte can be quantified with acceptable accuracy and precision is generally determined [16,17]. The concentration of spike samples are determined as follows. The concentration was gradually reduced after adding an aflatoxin standard of 1 μg/kg from each toxin in 4 μg/kg total aflatoxin (AFG2, G1, B2, and B1) to selected blank sample nuts. The peaks at concentrations under 1 μg/kg total aflatoxin were not observed. Therefore, this concentration is taken as the minimum detectable concentration. The

spike samples were prepared with 1 μg/kg total aflatoxin containing 0.25 μg/kg of each toxin. A 25 g blank sample was weighed into a blender jar. Then, 250 μL of a 10-fold diluted stock standard solution was added into the weighed 25 g blank sample to include 1 μg/kg aflatoxin, and 10 sample pieces were prepared in this manner. Therefore, the lowest detectable levels of the enriched (spike) samples were obtained. The samples were examined by HPLC after extraction according to AOAC method 991.31. The averages of 20 analysis results and their standard deviations and LOD and LOQ values were obtained according to Eqs. (1) and (2) for each experiment by analyzing 10 samples with two injections at a time in the HPLC under the chromatographic conditions mentioned above [17,22].

$$LOD = 3 \times SD + B_{ave} \quad (1)$$

$$LOQ = 10 \times SD + B_{ave} \quad (2)$$

SD: standard deviation of measurements
B_{ave}: average concentration of spike samples

2.4.2. *Signal-to-noise method*

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3:1 or 2:1 is generally considered acceptable for estimating the detection limit. A typical signal-to-noise ratio is 10:1 for a quantitation limit [16,17]. In this study, this ratio was found by comparing the average peak height values of the 10 samples containing 1 μg/kg total aflatoxin and the noise peak-to-peak (Nptop) average value of the 10 blank samples, using the Agilent 1100 HPLC ChemStation software.

2.4.3. *Based on the calibration curve*

A specific calibration curve should be studied using samples containing an analyte in the range of LOD or LOQ. The residual standard deviation of a regression line or the standard deviation of the y-intercepts of regression lines may be used as the standard deviation [16,17]. In this method, LOD and LOQ values were calculated by both the residual standard deviation of a calibration curve [Eq. (3)] [23] and the y-intercepts of regression lines using Agilent ChemStation 1100 software. In three independent experiments, two different aflatoxin standards were used. In the first two, an aflatoxin solution containing 250 μg/L of each toxin, or 1000 μg/L in total, was used, and in the other study, an aflatoxin standard containing 2600 μg/L concentration in total (AFG1, AFB1 = 1000 μg/L; AFG2, AFB2 = 300 μg/L) was used. First two calibration curves were estimated aflatoxin standard solutions at concentrations of 0.0150 μg/L, 0.250 μg/L, 0.750 μg/L, 1.50 μg/L, 2.50 μg/L, 3.75 μg/L, and 5.00 μg/L for each aflatoxin (AFG1, B1, G2, B2) and the other calibration curve was estimated 1.00 μg/L, 2.00 μg/L, 4.00 μg/L, 8.00 μg/L, 12.0 μg/L, 16.0 μg/L, and 20.0 μg/L for AFB1 and AFG1, and 0.300 μg/L, 0.600 μg/L, 1.20 μg/L, 2.40 μg/L, 3.60 μg/L, 4.80 μg/L, and 6.00 μg/L for AFB2 and AFG2, respectively. Based on the average of these three experiments, the LOD and LOQ values were determined according to equations (4) and (5) for the calibration method [16,17].

$$S_{res} = \sqrt{\frac{\sum (Y - Y_{est})^2}{n - 2}} \quad (3)$$

Y: the observed values for the dependent variable
Y_{est}: predicted values

$$LOD_{calibration} = 3 \times (\sigma/s) \quad (4)$$

$$LOQ_{calibration} = 10 \times (\sigma/s) \quad (5)$$

σ = the standard deviation of the response or standard deviation of y-intercepts
s = the slope of the calibration curve

3. Results and discussion

In this study, AOAC Official Method 991.31 was used in the aflatoxin analysis of hazelnut samples, and LOD and LOQ values were determined for four aflatoxin types (AFG2, G1, B2, and B1). Visual evaluation, signal-to-noise, and calibration curve methods [15–17,20] compared in this study are recommended by the International Conference of Harmonization (ICH) for the determination of LOD and LOQ except for the standard deviation of the blank method, as the blank method does not include the negative effects caused by aflatoxin and analyte signals. Instead, it only evaluates ground noise. The aflatoxin standard was used for the other methods, and the LOD and LOQ values include both ground noise and analyte signals.

Fig. 1 shows the HPLC chromatogram of aflatoxin in a spike sample containing 0.25 μg/kg from each toxin (AFG1, G2, B1, and B2) in 1 μg/kg totals. The AFG2, G1, B1, and B2 toxins had a retention time of 7.113 minutes, 8.322 minutes, 9.617 minutes, and 11.342 minutes, respectively.

Regardless of the device and method, LOD and LOQ determination processes are usually based on taking six or 10 times the standard deviation for LOQ and taking three times the standard deviation for LOD. In all three methods, the calculations were performed by taking three times the standard deviation for LOD and 10 times the standard deviation for LOQ.

For the visual evaluation approach, the LOD and LOQ values calculated from the results of three independent analyses of spike samples prepared with aflatoxin standard (0.25 μg/kg for each type, 1 μg/kg in total) and averages of these values, their standard deviation, and recovery rate are shown in Table 1. In this approach, the LOD and LOQ values are calculated from the results of the lowest concentration of the samples that can be detected by HPLC. The peaks obtained by HPLC are normally sharp, symmetrical, and completely separated from other analyte peaks. However, because the peaks obtained from the samples enriched with the empirical method with a total of 1 μg/kg aflatoxin are examined at very low concentrations, these peaks are wide, nonsymmetrical, small, and not well separated. For this reason, the reproducibility of the analysis results is very low. The average results for AFG2, G1, B2, and B1 were lower than 0.25 μg/kg. The

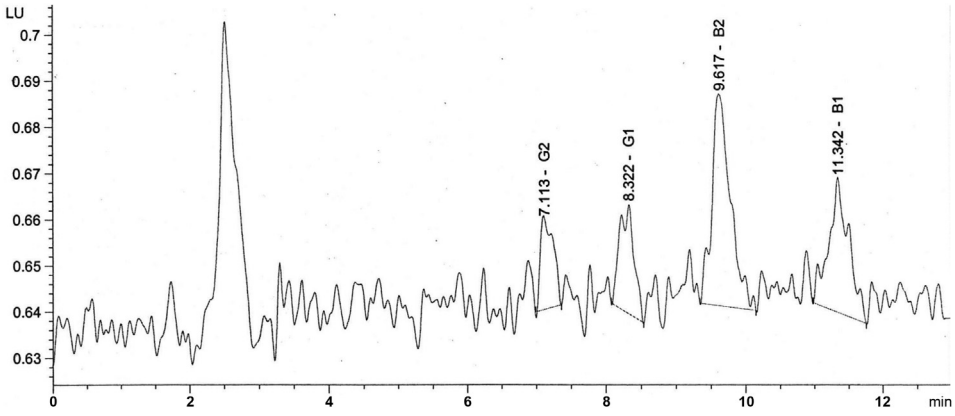

Fig. 1 – High-performance liquid chromatography chromatogram of aflatoxin in a spike sample.

reason for this is that because the aflatoxin standard is added at a very low concentration when preparing the spike sample, it greatly affects the rate of recovery more, and the rate of recovery thus decreases. This decrease in recovery and the differences in the results cause increases in the standard deviation, high rates of LOD and LOQ values in the empirical formula, and differences between parallel tests. When the averages of the results are considered, the highest LOD value is 0.242 μg/kg for AFB1, and the highest LOQ value is 0.396 μg/kg for AFG1. The lowest LOD and LOQ values are for AFB2 and AFG2. The LOD and LOQ values of AFG1 and AFB1 are higher. However, this situation cannot be generalized. The results among the aflatoxin types differed among the three independent experiments.

The S/N method values are given in Table 2, and these values were calculated by taking the averages of the Nptop values of blank samples with peak rate averages in the analysis outputs of three independent experiments conducted with spike samples prepared with a total of 1 μg/kg aflatoxin. In total, 10 blank samples were analyzed with two injections at a time for the analysis of blank samples. The noise (Nptop) result averages and the standard deviations [$S_{N(ptop)}$] are given in Table 2.

As can be seen in Table 2, using the S/N method, both the LOD and LOQ values of AFG2, G1, B2, and B1 are nearly the same. The LOD and LOQ values are lower than the empirical method values. The LOD and LOQ values of AFG1 and AFB2 are slightly higher than the others. However, the standard deviation of both values is quite high relative to the others. Although the values in the first and third experiments are nearly the same, the very high AFG1 and AFB2 values increased their averages and standard deviations.

The first two calibration curves estimated by injecting triplicate aflatoxin standard solutions at concentrations range 0.015–5.0 μg/L of AFB1, B2, G1, and G2 and the other calibration curve was made at a concentrations range of 1–20.0 μg/L for AFB1 and AFG1, and 0.30–6.00 μg/L range for AFB2 and AFG2, respectively. The regression analysis results can be seen in Table 3. Linear correlation coefficient (R^2) was found

Table 1 – Results of analysis committed three different times with visual evaluation (empiric) methods.

		Aflatoxins (μg/kg)			
		AFG2	AFG1	AFB2	AFB1
First working	Mean	0.1651	0.1748	0.1654	0.1893
	SD[a]	0.0170	0.0303	0.0185	0.0234
	Recovery %[b]	66.06	69.92	66.14	75.72
	LOD (1)	0.2163	0.2658	0.2208	0.2596
	LOQ (1)	0.3355	0.4780	0.3502	0.4235
Second working	Mean	0.1504	0.1391	0.1400	0.1710
	SD[a]	0.0150	0.0166	0.0101	0.0129
	Recovery %[b]	60.16	55.62	56.00	68.40
	LOD (2)	0.1955	0.1887	0.1703	0.2096
	LOQ (2)	0.3007	0.3046	0.2410	0.2998
Third working	Mean	0.1554	0.1745	0.1804	0.1760
	SD[a]	0.0281	0.0232	0.0130	0.0269
	Recovery %[b]	62.16	69.8	72.16	70.4
	LOD (3)	0.2397	0.2442	0.2195	0.2566
	LOQ (3)	0.4364	0.4067	0.3107	0.4448
LOD (mean)		0.2171	0.2329	0.2036	0.2419
LOD (SD)		0.0221	0.0398	0.0288	0.0280
LOQ (mean)		0.3575	0.3964	0.3006	0.3894
LOQ (SD)		0.0705	0.0872	0.0553	0.0783

[a] SD = standard deviation.
[b] % recovery = (level found/level added) × 100.

Table 2 – Results of analysis committed three different times with signal/noise methods[a,b].

	Aflatoxins (μg/kg)			
	AFG2	AFG1	AFB2	AFB1
LOD (1)	0.1002	0.0998	0.0987	0.0970
LOD (2)	0.1136	0.1229	0.1228	0.1000
LOD (3)	0.1022	0.0996	0.1040	0.0930
LOQ (1)	0.3340	0.3327	0.3290	0.3234
LOQ (2)	0.3787	0.4096	0.4068	0.3331
LOQ (3)	0.3405	0.3320	0.3466	0.3101
LOD (mean)	0.1053	0.1074	0.1082	0.0967
LOD (SD)	0.0059	0.0109	0.0103	0.0029
LOQ (mean)	0.3511	0.3581	0.3608	0.3222
LOQ (SD)	0.0200	0.0364	0.0333	0.0094

[a] Mean noise (ptop): 0.0140 μg/kg.
[b] $SD_{Noise(ptop)}$: 0.0033 μg/kg.

Table 3 – Regression analysis results obtained from the study of three different calibrations.

	Compound	Regression equation	R^2	RSD	SDy-int
First working	AFG2	$y = 48.1311x + 5.6554e^{-3}$	0.99993	0.0571	0.1755
	AFG1	$y = 48.4527x - 2.1677e^{-2}$	0.99993	0.0580	0.1697
	AFB2	$y = 123.7841x - 1.5268e^{-1}$	0.99990	0.1766	0.1664
	AFB1	$y = 80.3513x - 2.2701e^{-1}$	0.99927	0.3079	0.1820
Second working	AFG2	$y = 36.63024x + 5.4241e^{-2}$	0.99990	0.0511	0.1226
	AFG1	$y = 35.40368x + 8.0184e^{-2}$	0.99991	0.0476	0.1139
	AFB2	$y = 97.03741x + 5.3658e^{-2}$	0.99999	0.0409	0.1252
	AFB1	$y = 60.75359x + 5.2022e^{-2}$	0.99998	0.0381	0.1140
Third working	AFG2	$y = 28.10929x - 2.6692e^{-2}$	0.99983	0.0280	0.0538
	AFG1	$y = 26.85874x - 1.9323e^{-2}$	0.99997	0.0369	0.5962
	AFB2	$y = 79.48473x - 2.2182e^{-2}$	0.99997	0.0341	0.0530
	AFB1	$y = 50.09731x - 1.2030e^{-1}$	0.99993	0.1071	0.5914

R^2 = linear correlation coefficient; RSD = residual standard deviation for each; SDy-int = standard deviation of y-intercepts.

above 0.999 for all aflatoxin types. RSD values are below 10% except for two results.

The LOD and LOQ values were determined for the calibration method by taking the average of the results of the three experiments. The LOD and LOQ values were determined with two different calibration curve methods. The LOD and LOQ values calculated from a residual standard deviation of a regression line are given in Table 4, and the values calculated from the standard deviation of the y-intercepts of regression lines are given in Table 5.

The residual standard deviation of a regression line method includes mistakes resulting from background changes of different concentrations that are in the calibration curve, and it is important to take that into account. When the RSD values in Table 3 are considered, nearly identical data are observed. For example, the RSD values of AFB2 and AFB1 in the first experiment and the RSD values of AFB1 in the third experiment are all very high. The high RSD value of AFB1 results in a situation in which the LOD and LOQ values listed in Table 4 and their standard deviations for AFB1-type toxins are very high. The certainty rates of the AFG2 and AFG1 type aflatoxins are higher than the values for AFB2 and AFB1 in Table 5.

The LOD and LOQ values were calculated from the standard deviation of the y-intercepts of regression lines. The LOD and LOQ values of AFG2 and AFG1 are very high in the first two experiments, and the LOQ and LOQ values of AFG1 and AFB1 are high in the last experiment. This may be the result of using a different aflatoxin standard in the last experiment. The differences between values decrease the certainty rate and result in high standard deviation values. When the average values are considered, the LOD and LOQ values of AFG1 and AFB1 aflatoxins are high for both methods, although the values do not follow the same trends as the calculations using the residual standard deviation of a regression line approach, as the certainty rates of the LOD and LOQ values calculated using the standard deviation of the y-intercepts of regression lines are much worse than those obtained using the other method.

The summary of the three examined methods is shown in Table 6. The highest LOD and LOQ values were obtained using the empirical method. The LOD values of the S/N method are slightly lower than those of the empirical method, and their LOQ values are nearly same. The LOD and LOQ values of calibration curve method are quite lower than the other two methods. The certainty rates of the other methods are low, except for that of the S/N method.

The LOD and LOQ values have also been determined in some literature studies focusing on the determination of aflatoxin in foods. In a study conducted by Riordan and Wilkinson [24], the same method (AOAC 991.31) was used in aflatoxin determination in spices, and the LOD and LOQ values were determined by taking a LOD three times that of the noise value and a LOQ six times the level of noise in spike

Table 4 – LOD and LOQ values calculated from residual standard deviation of a regression line.

	Aflatoxins (μg/kg)			
	AFG2	AFG1	AFB2	AFB1
LOD (1)	0.0036	0.0036	0.0043	0.0115
LOD (2)	0.0042	0.0040	0.0013	0.0019
LOD (3)	0.0030	0.0041	0.0012	0.0064
LOQ (1)	0.0119	0.0120	0.0143	0.0383
LOQ (2)	0.0140	0.0134	0.0042	0.0063
LOQ (3)	0.0099	0.0137	0.0043	0.0213
LOD (mean)	0.0036	0.0039	0.0023	0.0066
LOD (SD)	0.0006	0.0003	0.0018	0.0048
LOQ (mean)	0.0119	0.0130	0.0076	0.0220
LOQ (SD)	0.0020	0.0009	0.0058	0.0160

SD = standard deviation.

Table 5 – LOD and LOQ values calculated from standard deviation of y-intercepts of regression lines.

	Aflatoxins (μg/kg)			
	AFG2	AFG1	AFB2	AFB1
LOD (1)	0.0109	0.0105	0.0040	0.0068
LOD (2)	0.0100	0.0096	0.0039	0.0056
LOD (3)	0.0057	0.0666	0.0020	0.0354
LOQ (1)	0.0363	0.0350	0.0133	0.0227
LOQ (2)	0.0333	0.0320	0.0130	0.0187
LOQ (3)	0.0190	0.2220	0.0067	0.1180
LOD (mean)	0.0089	0.0289	0.0033	0.0159
LOD (SD)	0.0023	0.0267	0.0010	0.0138
LOQ (mean)	0.0296	0.0963	0.0110	0.0531
LOQ (SD)	0.0075	0.0889	0.0030	0.0459

SD = standard deviation.

Table 6 – Summary of LOD and LOQ determination by different methods.

		Visual evaluation (Empirical methods)		Signal-to-noise S/N		Calibration curve			
						RSD of a regression line		SD of y-intercepts of regression lines	
	Compound	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LOD (μg/kg)	AFG2	0.2171	0.0221	0.1053	0.0059	0.0036	0.0006	0.0089	0.0023
	AFG1	0.2329	0.0398	0.1074	0.0109	0.0039	0.0003	0.0289	0.0267
	AFB2	0.2036	0.0288	0.1082	0.0103	0.0023	0.0018	0.0033	0.0010
	AFB1	0.2419	0.0280	0.0967	0.0029	0.0066	0.0048	0.0159	0.0138
LOQ (μg/kg)	AFG2	0.3575	0.0705	0.3511	0.0200	0.0119	0.0020	0.0296	0.0075
	AFG1	0.3964	0.0872	0.3581	0.0364	0.0130	0.0009	0.0963	0.0889
	AFB2	0.3006	0.0553	0.3608	0.0333	0.0076	0.0058	0.0110	0.0030
	AFB1	0.3894	0.0783	0.3222	0.0094	0.0220	0.0160	0.0531	0.0459

SD = standard deviation; RSD = residual standard deviation.

samples. The LOD values of the AFG2, G1, B2, and B1 aflatoxins were found to be 0.21 μg/kg, 0.09 μg/kg, 0.02 μg/kg, and 0.10 μg/kg, respectively. The LOQ values of the AFG2, G1, B2, and B1 aflatoxins were found to be 0.30 μg/kg, 0.50 μg/kg, 1.00 μg/kg, and 0.20 μg/kg, respectively. In a study conducted by Iqbal et al [25] on aflatoxins in peppers, the LOD and LOQ were 0.05 μg/kg and 0.53 μg/kg for AFB1 and AFG1, respectively, and 0.1 μg/kg and 0.60 μg/kg for AFB2 and AFG2, respectively. The LOD was calculated with a signal-to-noise ratio (S/N = 3) and LOQ (used S/N = 10). In a study conducted by Fu et al [26] with the S/N method for aflatoxin analysis in corn and peanut samples, the LOD and LOQ values were 0.32 μg/kg and 1.07 μg/kg for AFB1 and AFG1, respectively, and 0.19 μg/kg and 0.63 μg/kg for AFB2 and AFG2, respectively. These values were higher than ours. In a study conducted by Leong et al [27] with the S/N method for aflatoxin analysis in hazelnuts using HPLC, the LOD and LOQ values were 0.3 μg/kg and 0.6 μg/kg for AFG1 and AFB1, respectively, and 0.1 μg/kg and 0.4 μg/kg for AFG2 and AFB2, respectively. The AFG1 and AFB1 values were higher than ours, and the values found for AFG2 and AFB2 are nearly the same as ours. In the analysis of aflatoxin in the literature, the S/N method is generally used. In a study conducted by Arzandeh et al [28] on raw peanut kernels samples, the LOD and the LOQ were estimated using 3 × SD and 10 × SD, respectively, and calculated using seven times the injection standards with the lowest concentration that could be detected by HPLC. The LOD values were found to be 0.03 μg/kg, 0.01 μg/kg, 0.09 μg/kg, and 0.06 μg/kg, and the LOQ values were 0.10 μg/kg, 0.04 μg/kg, 0.30 μg/kg, and 0.20 μg/kg for AFB1, B2, G1, and G2, respectively. In an analysis by Martins et al [29] on nut samples with the S/N method using liquid chromatography-tandem mass spectrometry, the LOD values for AFG2, G1, B2, B1 were 0.1 μg/kg, 0.075 μg/kg, 0.75 μg/kg, and 0.05 μg/kg, respectively, and the LOQ values for AFG2, G1, B2, and B1 were 0.3 μg/kg, 0.2 μg/kg, 0.2 μg/kg, and 0.15 μg/kg, respectively [29]. These values are lower than ours. Thus, LOD and LOQ are two quantities that have accuracies that depend on the sensitivity of the device used.

If we compare the examined methods, the lowest values were obtained with the calibration curve method. This method is much easier and less time consuming in terms of analysis than the other two methods. However, only calibration solutions can be used in this method, and because these solutions are prepared from a stored aflatoxin standard solution, this method does not include errors that can result from the analysis method and sample matrix; instead, it only includes errors that can result from the device and from the preparation of the calibration solutions. For this reason, lower values are obtained. The other two methods include all errors that can result from the device, calibration, samples matrix, and method. The S/N method is a preferred method compared with the other methods, as can be seen in the literature. The best results were obtained with the S/N method in terms of reproducibility. The empirical method is the best method in terms of LOD definition. The general definition of LOD is the lowest concentration of an analyte that can be detected. When the empirical method is applied, the lowest concentration that can be detected is obtained by starting with the analyte concentration that we can certainly detect and then decreasing the concentration. The calculations are performed using this lowest concentration. For this reason, the values obtained are completely reflective of the definition of LOD. Although the S/N noise method is appropriate using this definition, it also includes direct blank experiments. The last two methods do not reflect this definition among the visual evaluation, signal-to-noise, and standard deviation of the blank and calibration curve methods suggested by the Conference on Harmonization (ICH 2006) guideline. These last two methods may be preferred in terms of their convenience.

4. Conclusion

Visual evaluation (empirical methods) and signal-to-noise approaches are the most appropriate approaches for LOD and LOQ determination. LOD and LOQ are the most important validation parameters in the analysis of hazelnut samples by HPLC. Because the values with the highest uncertainty rate among the values measured are added in the calculations, if we compare the values in Table 6, we can see that the visual evaluation method is the most appropriate of all the approaches for the analysis of LOD and LOQ and this method provided much more realistic LOD and LOQ values.

Conflicts of interest

The author has no conflict of interest relevant to this article.

REFERENCES

[1] Bacchetta L, Aramini M, Bernardi C. In vitro propagation of traditional Italian hazelnut cultivars as a tool for the valorization and conservation of local genetic resources. Hort Science 2008;43:562–6.
[2] Miletic R, Mitrovic M, Rakicevic M. Contrasting fruit properties of hazelnut cultivars grown on different rootstocks. Acta Hort 2009;845:283–6.
[3] Krska R. Mycotoxins. Anal Bioanal Chem 2009;395:1203–4.
[4] Luque MI, Rodriguez A, Andrade GMJ, Bermúdez E, Córdoba JJ. Development of a multiplex PCR method for the detection of patulin-ochratoxin A- and aflatoxin-producing molds in foods. Food Anal Method 2013;6:1113–21.
[5] Milhome MAL, Lima CG, De Lima LK, Lima FAF, Sousa DOB, Nascimento RF. Occurrence of aflatoxins in cashew nuts produced in northeastern Brazil. Food Control 2014;42:34–7.
[6] Baltaci C, Ilyasoglu H, Yuksel F. Single-laboratory validation for the determination of aflatoxins B1, B2, G1 and G2 in food based on immunoaffinity column and liquid chromatography with post column derivatization and fluorescence detection. Food Anal Method 2011;6:36–44.
[7] Anukul N, Vangnai K, Mahakarnchanakul W. Significance of regulation limits in mycotoxin contamination in Asia and risk management programs at the national level. J Food Drug Anal 2013;21:227–41.
[8] Binder EM, Tan LM, Chin LJ, Handl J, Richard J. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim Feed Sci Technol 2007;137:265–82.
[9] Wang J, Ogata M, Hirai H, Kawagishi H. Detoxification of aflatoxin B1 by manganese peroxidase from the white-rot fungus phanerochaetesordida YK-624. FEMS Microbiol Lett 2011;314:164–9.
[10] Chen YC, Liao CD, Lin HY, Chiueh LC. Survey of aflatoxin contamination in peanut products in Taiwan from 1997 to 2011. J Food Drug Anal 2013;21:247–52.
[11] Ozay G, Seyhan F, Yılmaz A, Whitaker TB, Slate AB, Giesbrecht F. Sampling hazelnuts for aflatoxin: Uncertainty associated with sampling, sample preparation, and analysis. J AOAC Int 2006;89:1004–11.
[12] European Commission Regulation (EC). No. 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. OJ 2001:L77/6.
[13] Taverniers I, De Loose S, Van Bockstaele ME. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trends Anal Chem 2004;23:535–52.
[14] United States Pharmacopeia. Validation of compendial methods, Twenty-Sixth Revision, National Formulary. 21st ed. Rockville, MD: The United States Pharmacopeial Convention Inc.; 2003.
[15] The fitness for purpose of analytical methods – A laboratory guide to method validation and related topics. In: Magnusson B, Örnemark U, editors. Eurachem Guide. (2nd ed. 2014). UK: LGC; 1985. p. 20–9.
[16] NATA Technical Note 17. Guidelines for the validation and verification of quantitative and qualitative test methods. Australia: National Association of Testing Authorities; 2013. p. 17–8.
[17] International Conference on Harmonization. Harmonized tripartite guideline, validation of analytical procedures, text and methodology. ICH Q2R1. 2006. p. 12–4.
[18] Brutto RL, Patel T. Method validation. In: Kazakevich Y, Lobrutto R, editors. HPLC for pharmaceutical scientists. New Jersey: Wiley; 2007. p. 483.
[19] Muscarella M, Lo Magroa S, Palermo C, Centonze D. Validation according to European Commission Decision 2002/657/EC of a confirmatory method for aflatoxin M1 in milk based on immunoaffinity columns and high performance liquid chromatography with fluorescence detection. Anal Chim Acta 2007;594:257–64.
[20] Vial J, Jardy A. Experimental comparison of the different approaches to estimate LOD and LOQ of an HPLC method. Anal Chem 1999;71:2672–7.
[21] AOAC Method 991.31. Aflatoxins in corn, raw peanuts, and peanuts and peanut butter (immunoaffinity column method). J AOAC Int 1991:74–81.
[22] Shrivastava A, Gupta VB. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. CYS 2011;2:21–5.
[23] Ott RL, Longnecker M. An introduction to statistical methods and data analysis. 5th ed. California: Duxbury Press; 2001.
[24] O'Riordan MJ, Wilkinson MG. A survey of the incidence and level of aflatoxin contamination in a range of imported spice preparations on the Irish retail market. Food Chem 2008;107:1429–35.
[25] Iqbal SZ, Bhatti IA, Asi MR, Bhatti HN, Sheikh MA. Aflatoxin contamination in chillies from Punjab Pakistan with reference to climate change. Int J Agr Biol 2010;13:261–5.
[26] Fu Z, Huang X, Min S. Rapid determination of aflatoxins in corn and peanuts. J Chromatogr A 2008;1209:271–4.
[27] Leong YH, Ismail N, Latif AA, Ahmad R. Aflatoxin occurrence in nuts and commercial nutty products in Malaysia. Food Control 2010;21:334–8.
[28] Arzandeh S, Selamat J, Lioe H. Aflatoxin in raw peanut kernels marketed in Malaysia. J Food Drug Anal 2010;18:44–50.
[29] Martins M, Pacheco AM, Lucas ACS, Andrello AC, Appoloni CR, Xavier JJM. Brazil nuts: Determination of natural elements and aflatoxin. Acta Amazon 2012;42:157–64.