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Original Article

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

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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

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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