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## Aflatoxin Contamination in Hazelnut Oil Obtained from Hazelnuts Containing High Levels of Aflatoxin

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### ABSTRACT

In this study, the issue of whether the aflatoxin in high contaminated hazelnut has been passed to the hazelnut oil during production or not has been investigated. The oil and oil cake of the hazelnut samples that contained aflatoxin at a high level were obtained for the study. The aflatoxin concentrations in hazelnut, hazelnut oil and oil cake were measured, and how much of the aflatoxin in the hazelnut was passed into the oil and oil cake has been determined. Aflatoxin analysis was performed using AOAC (Association of Official Analytical Chemists Method): 991.31 method, which is one of the validated method used in aflatoxin analysis in hazelnuts. The highest aflatoxin concentration in hazelnut oil has been determined as AFB1: 0.93, AFG1: 0.52, AFB2: 0.47 and AFG2: 0.21  $\mu\text{g kg}^{-1}$ . At the end of the study, it was determined that although the hazelnuts of which the hazelnut oil was obtained contained aflatoxin at a very high level, it was passed to the oil at very low levels below the maximum limits defined by the European Union, and almost all of it remained in the oil cake. Aflatoxin in hazelnut is passed to hazelnut oil at very low amount.

Keywords: Aflatoxin; Hazelnut; Hazelnut oil; Hazelnut oil cake

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### 1. Introduction

Hazelnut (*Corylus avellana* L.) is cultivated on the coast of the Black Sea of Turkey and in Southern Europe. Turkey is the biggest producer of hazelnut in the world, accounting for 75% of total world production (Aktaş et al 2011; Baltacı et al 2012). Hazelnut contains sterols, tannins, essentials minerals, free phenolic acids, sugars, organic acids and phenolic compound. Additionally, hazelnut contains tocopherols and other bioactive polyphenols, which exhibit a beneficial effect

on human health, reducing oxidative stress and risk of cancer, stroke, inflammation, and other neurodegenerative diseases (Schmitzer et al 2011). It has a rich nutritional source with 65% oil, 14% protein, and 16% carbohydrates. More than 90% of its oil consists of unsaturated fatty acids, especially oleic ( $C_{18:1}$ , 80%) and linoleic ( $C_{18:2}$ , 12%) acids (Özkal et al 2005). Hazelnut is also used in production hazelnut oil due to the high fat content. Hazelnut oil is also used for several purposes such as cooking, salad dressings, and flavoring ingredients,

among others. Hazelnut fatty acid composition is very similar to that of olive oil. Oleic (C 18:1) and linoleic (C 18:2) are the main fatty acids in both oils (Parcerisa et al 2000). Hazelnut oil also contained two to three times more  $\alpha$ -tocopherol than olive oil (Benitez-Sánchez et al 2003; Alaşalvar et al 2009).

The contamination of foodstuffs with aflatoxin is a major problem worldwide. Keeping food and feed ingredients away from mould is one of the major difficulties encountered in cultivated areas, especially in humid regions. The Black Sea Region is an area with high rainfall and a climate that is hot in summer and warm in winter. This feature increases the growth of mould in food.

Mycotoxins are those secondary metabolites of fungi which are associated with certain disorders in animals and humans (D'Mello & Macdonald 1997). Aflatoxins (AFs) are considered one of the main types of mycotoxins produced by different species of toxigenic fungi, especially *Aspergillus flavus* and *Aspergillus parasiticus* species. The most important aflatoxin types are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2). Among them, AFB1 shows the highest toxicity (Milhome et al 2014; Özlüoymak 2014; Asghar et al 2016). Mycotoxin contaminated grains and oil seeds are toxic and carcinogenic to humans and animals. The primary target organ of aflatoxin in toxicity and carcinogenicity with acute toxicity, immunosuppressive, mutagenic, teratogenicity and carcinogenic properties is the liver (Binder et al 2007). Informing producers and consumers that aflatoxin causes serious health problems is vital. The European Commission has set maximum permissible limits  $10 \mu\text{g kg}^{-1}$  for total aflatoxin and  $5 \mu\text{g kg}^{-1}$  for AFB1 in hazelnut (EC 2010). Informing producers and consumers that aflatoxin causes serious health problems is vital.

Hazelnut 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 (Miletic et al 2009; Lavkor & Biçici 2015). Studies into contamination of aflatoxin in nuts and nut

products in literature have accelerated with the detection of aflatoxin and aflatoxigenic moulds (Aycicek et al 2005; Gürses 2006; Bircan et al 2008; Basaran & Ozcan 2009; Baltacı et al 2012). Numerous studies have reported high incidence of aflatoxins contamination in edible oils such as olive oil (Daradimos et al 2000; Papachristou & Markaki 2004; Cavaliere et al 2007; Ferracane et al 2007), peanut oil (Elzupir et al 2010; Yang et al 2011), sesame oil (Elzupir et al 2010; Idris et al 2010), blended oil (Yang et al 2011), groundnut (Idris et al 2010; Mariod & Idris 2015), cottonseed oils (Idris et al 2010) and sunflower (Elzupir et al 2010; Mariod & Idris 2015) in worldwide.

Studies on aflatoxin in hazelnut oil are inadequate in Turkey and in other countries. The use of hazelnut oil as edible oil is becoming widespread as an alternative to olive oil in terms of the nutrients it contains. Hazelnuts that are not sold or consumed in the market are generally preferred for oil production. The probability of the existence of aflatoxin is high in these hazelnuts. The purpose of this study is to determine the rate of the aflatoxin that is passed to oil during the production of hazelnut oil, and to determine the amount of aflatoxin that remains in the oil cake after production.

## 2. Material and Methods

### 2.1. Materials and reagents

Methanol, acetonitrile (ACN), hexane, nitric acid 65%, potassium bromide and sodium chloride HPLC graded and purchased from Merck (Darmstadt, Germany). Immunoaffinity columns (Aflaprep P07) with 1 mL volume were purchased from R-Biopharm Rhone Ltd. (Darmstadt, Germany). Standard solution of aflatoxin (Aflastandard, R-Biopharm) was used in the preparation of calibration curves and recovery experiments. The stock standard of aflatoxin is sold as a  $1000 \text{ ng mL}^{-1}$  concentration of a methanol solution. It consists of  $250 \text{ ng mL}^{-1}$  AFG1, AFG2, AFB1, and AFB2 type aflatoxins. Ultra-pure waters was produced by Sartorius Arium Pro VF (Goettingen, Germany).

## 2.2. Samples

All of five hazelnut samples containing aflatoxin were taken from Food Control Laboratory, Ordu, Turkey. Hazelnut samples of at least 1 kg were transported to the laboratory in sterile polyethylene bags under cold conditions and preserved at -20 °C until the experimental process could be conducted. All samples were analysed individually (without subsampling) for aflatoxin content tests. Firstly, aflatoxin analyses were carried out on hazelnut samples. The oils and oil cake of these samples were obtained using hexane in the soxhlet apparatus. Amounts of aflatoxin were determined in the hazelnut oil and oil cake. All experiments were performed with at least three replicates.

## 2.3. Aflatoxin analysis and extraction process

The analysis was performed according to AOAC Official Method 991.31:2000 (AOAC 991:31), which has international validity in aflatoxin analysis (AOAC 1991).

Each of hazelnut, hazelnut oil and oil cake samples (25 g) were taken in a blender jar, 5 g of sodium chloride and 125 mL of ACN/H<sub>2</sub>O (70:30, v:v) were added to it. After blended for 2 min at high speed, the extract was filtered through Whatman No. 4 filter paper (Whatman International, Maidstone, UK). 15 mL was removed and 30 mL of water was added. It was mixed thoroughly and the extract was filtered through Whatman No. 4 filter paper. Finally, 15 mL of the reconstituted extract were passed through the Immunoaffinity columns (IAC) at a flow rate of 2 mL min<sup>-1</sup>. After passed to two aliquots of 10 mL ultrapure water through the column, AFs bound to the specific antibody were slowly released using 1 mL of methanol and diluted with 1 mL ultrapure water in HPLC vials. Vial was fully mixed in Vortex and made suitable for the high-performance liquid chromatography (HPCL).

## 2.4. Instrument and chromatographic conditions

Analysis was performed using a HPLC 1100 series (Agilent Technologies, Barcelona, Spain) fitted with an auto-sampler and a fluorescence detector operated at an excitation wavelength, of 360 nm

and emission wavelength of 430 nm. HPLC mobile phase was a mixture of water-acetonitrile-methanol (6:2:3, v:v:v) with a flow rate of 1.0 mL min<sup>-1</sup>. The chromatographic reverse phase HPLC separation was performed on a ODS-2 column. Column temperature was 25 °C. The injection volume was 100 µL.

## 2.5. Validation of the analytical method

In the analysis of hazelnut, hazelnut oil and oil cake samples, the method of AOAC 991.31 that is a valid method in aflatoxin analysis was used (AOAC 1991). The retention times of the standard and samples were respectively AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>, 14.5 min., 12.2 min., 10.6 min and 9.0 min. with 2% standard deviation. Peaks were found to be quite symmetrical and sharp. There was no interference.

### 2.5.1. Linearity

Linearity was estimated by diluting the total aflatoxin standard stock solutions at concentrations of 0.10, 0.50, 1.0, 2.5, 5.0, 10.0 and 20.0 ng mL<sup>-1</sup>. The concentration of the samples is within the range of calibration. Samples exceeded the calibration range were reread by being diluted. Linear correlation coefficient (R<sup>2</sup>) was found above 0.999 for all aflatoxin types. The residual standard deviation (RSD) values were below 1%. The values of the calibration curves are shown in Table 1.

**Table 1- Summary of calibration curve parameters**

Aflatoxin	Regression equation	R <sup>2</sup>	RSD%
AFG <sub>2</sub>	Y= 6.67775x-1.82801e <sup>-1</sup>	0.99941	0.38583
AFG <sub>1</sub>	Y= 6.44608x-2.05951e <sup>-1</sup>	0.99967	0.26338
AFB <sub>2</sub>	Y= 11.43629x-1.36371e <sup>-2</sup>	0.99916	0.47764
AFB <sub>1</sub>	Y= 8.85254x-1.77944e <sup>-1</sup>	0.99957	0.41328

R<sup>2</sup>, linear correlation coefficient; RSD, residual standard deviation

### 2.5.2. Accuracy and precision

Recovery, repeatability analysis was conducted in a hazelnut oil sample that did not contain toxin by adding standard addition from aflatoxin obtained from R-Biopharm Rhone that were spiked with 0.5, 1.0 and 2.5 µg kg<sup>-1</sup> of each aflatoxin. All spike

samples were kept at room temperature for at least 1 hour before analysis. Spike samples were studied as three parallels and three injections each (ICH 2006). From this point, the recovery was calculated and the accuracy of method was found according to Equation 1. Repeatability was used for precision, and the relative standard deviation (RSD) of the results was calculated according to Equation 2 and repeatability was found. Recovery rates ranged from 90.7-102.6% as AFG1>AFB1>AFG2>AFB2.

These values are within the acceptable values of AOAC and the Codex Alimentarius. The AOAC guideline for the acceptable recovery at the 10 µg kg<sup>-1</sup> level is 70-125% and the Codex acceptable recovery range is 70-110% for a level of 10-100 µg kg<sup>-1</sup>, and 60-120% for a level of 1-10 µg kg<sup>-1</sup> (Codex Alimentarius 1993; AOAC 2013). RSD percentage values were quite low. These results show that this method is suitable for aflatoxin analysis in hazelnut samples.

$$\text{Recovery}(\%) = (\text{Recovered concentration} / \text{Infected concentration}) \times 100 \quad (1)$$

$$\text{Relative standard deviation}(\%) = (\text{Standard deviation} / \text{Mean}) \times 100 \quad (2)$$

### 2.5.3. Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) and limit of quantification (LOQ) were calculated according to signal to noise (S/N) method. LOD and LOQ determined as signal to noise (S/N) ratio of 3 and 10 respectively (Şengül 2016). The results of repeatability, recovery, LOD and LOQ are given in Table 2.

## 3. Results and Discussion

In this study, five hazelnut samples that contained aflatoxin at high levels were examined. The oil was extracted from each hazelnut sample and the oil, oil cake and hazelnut samples were determined for aflatoxin existence by HPLC. The analyses were made in accordance with the AOAC 991:31, which is a validated method (AOAC 1991). Aflatoxin

**Table 2- Validation studies in hazelnut oil samples**

Aflatoxin	Spiking level (µg kg <sup>-1</sup> )	Repeatability (mean±SD <sup>a</sup> ) (µg kg <sup>-1</sup> )	Recovery (%) (mean±SD)	RSD <sub>R</sub> <sup>b</sup> (%)	LOD (µg kg <sup>-1</sup> )	LOQ (µg kg <sup>-1</sup> )
AFG2	0.50	0.443±0.012	88.58±2.42	2.74	0.0492	0.1642
	1.00	0.749±0.013	74.87±1.30	1.74		
	2.50	1.892±0.102	75.68±4.09	5.40		
AFG1	0.50	0.409±0.015	81.87±3.02	3.69	0.0528	0.1761
	1.00	0.710±0.011	70.97±1.15	1.63		
	2.50	1.870±0.026	74.81±1.04	1.39		
AFB2	0.50	0.446±0.017	89.22±3.37	3.77	0.0484	0.1615
	1.00	0.786±0.005	78.63±0.49	0.62		
	2.50	2.193±0.052	87.73±2.07	2.36		
AFB1	0.50	0.366±0.020	73.27±3.96	5.41	0.0590	0.1968
	1.00	0.648±0.007	64.85±0.74	1.15		
	2.50	1.750±0.016	70.00±0.66	0.94		

<sup>a</sup>SD, Standard deviation; <sup>b</sup>RSD<sub>R</sub>, Relative standard deviation

results on the samples are shown in Table 3. When the results are examined, it is seen that aflatoxin is passed to the oil at a very low level when the oil was extracted from the hazelnut samples. The highest aflatoxin concentration was detected in the AFG1 among the hazelnut samples analyzed in the study, and AFB1, AFG2 and AFB2 follow this. In the hazelnut oil, the highest concentration was found in AFB1; and AFG1, AFB2 and AFG2 follow this. The rate of the transition of the aflatoxin to the oil is at 5.83% for AFB2, 4.38% for AFB1, 3.44% for AFG2 and 1.88% for AFG1. It was observed that aflatoxin remained in oil cake at a great rate. The maximum aflatoxin concentrations in the analyses of hazelnut oils were as follows; AFB1: 0.93; AFG1: 0.52; AFB2: 0.47; and AFG2: 0.21  $\mu\text{g kg}^{-1}$ . When the amount of the aflatoxin in the oil cake was examined, it was observed that the aflatoxin values

in the oil cake were very high. Almost all of the aflatoxin remained in the oil cake. We can claim that the aflatoxin in the hazelnut is not passed to the oil and almost all of it remains in the oil cake.

The aflatoxin limits in foods vary according to countries and their economic conditions. According to the European Union, the maximum aflatoxin limits permitted in vegetables oil are 2  $\mu\text{g kg}^{-1}$  AFB1 and 4  $\mu\text{g kg}^{-1}$  for total aflatoxin (EC 2010). The values that we found in hazelnut oil are below these limit values.

Since no studies were detected in the literature on determining aflatoxin in hazelnut oil, the results were compared with the results of the studies that were conducted to determine aflatoxin in other oils. When the literature results were examined, it is observed that some oil types are contaminated more with aflatoxin.

**Table 3- The values aflatoxin in hazelnut, hazelnut oil and hazelnut oil cakes samples**

Sample no		Hazelnut ( $\mu\text{g kg}^{-1}$ )		Hazelnut oil ( $\mu\text{g kg}^{-1}$ )		Hazelnut oil cake ( $\mu\text{g kg}^{-1}$ )	
		mean $\pm$ SD	RSD	mean $\pm$ SD	RSD	mean $\pm$ SD	RSD
1	G2	5.88 $\pm$ 0.230	3.98	LOD	-	5.79 $\pm$ 0.013	0.22
	G1	20.65 $\pm$ 0.360	1.76	0.32 $\pm$ 0.006	1.86	18.70 $\pm$ 0.082	0.44
	B2	6.37 $\pm$ 0.120	1.85	0.36 $\pm$ 0.005	1.44	6.17 $\pm$ 0.050	0.81
	B1	18.61 $\pm$ 0.588	3.18	0.60 $\pm$ 0.006	1.05	15.49 $\pm$ 0.181	1.17
2	G2	5.83 $\pm$ 0.423	7.28	LOD	-	5.58 $\pm$ 0.020	0.44
	G1	35.32 $\pm$ 0.579	1.66	0.11 $\pm$ 0.019	17.20	33.69 $\pm$ 0.130	0.40
	B2	3.32 $\pm$ 0.191	5.77	0.03 $\pm$ 0.001	3.58	3.29 $\pm$ 0.050	1.58
	B1	17.63 $\pm$ 0.362	2.03	0.20 $\pm$ 0.016	8.13	15.34 $\pm$ 0.441	2.88
3	G2	9.27 $\pm$ 0.251	2.73	0.32 $\pm$ 0.006	1.97	7.15 $\pm$ 0.106	1.47
	G1	28.07 $\pm$ 1.187	4.24	0.52 $\pm$ 0.003	0.61	23.33 $\pm$ 0.352	1.49
	B2	8.02 $\pm$ 0.341	4.29	0.47 $\pm$ 0.008	1.73	7.66 $\pm$ 0.154	2.00
	B1	21.31 $\pm$ 0.912	4.25	0.93 $\pm$ 0.012	1.30	17.20 $\pm$ 0.414	2.39
4	G2	7.13 $\pm$ 0.185	2.51	LOD	-	5.70 $\pm$ 1.189	20.95
	G1	42.23 $\pm$ 2.422	5.73	0.06 $\pm$ 0.008	12.14	39.71 $\pm$ 7.163	18.04
	B2	3.85 $\pm$ 0.021	0.51	0.02 $\pm$ 0.004	28.78	3.64 $\pm$ 0.680	18.72
	B1	20.46 $\pm$ 0.404	1.96	0.09 $\pm$ 0.002	2.52	19.63 $\pm$ 3.492	17.76
5	G2	6.06 $\pm$ 0.090	1.41	0.21 $\pm$ 0.061	29.10	4.20 $\pm$ 0.332	7.75
	G1	17.84 $\pm$ 0.342	1.87	0.34 $\pm$ 0.033	9.77	16.30 $\pm$ 1.771	10.88
	B2	5.59 $\pm$ 0.330	5.91	0.33 $\pm$ 0.024	7.36	4.94 $\pm$ 0.520	10.57
	B1	14.59 $\pm$ 0.871	5.98	0.51 $\pm$ 0.075	14.70	14.39 $\pm$ 1.512	10.50

Similar findings were observed in olive oil samples. Ferracane et al (2007), found the presence of AFB1 only 3 out of 30 samples were contaminated ranging from 0.54 to 2.50  $\mu\text{g kg}^{-1}$  in olive oil (Ferracane et al 2007). Additionally, Papachristou & Markaki (2004), who studied 50 samples and 60 ng  $\text{kg}^{-1}$  of AFB1 was found only one of them (Papachristou & Markaki 2004). Daramidos et al (2000) determined AFB1 in 2.8-15.7 ng  $\text{kg}^{-1}$  of the concentration range in 72% of 50 olive oil samples. However one sample was contaminated with 46.3 ng  $\text{kg}^{-1}$ . In 14 samples AFB1 was not detectable (Daramidos et al 2000). Cavalier et al (2007) found that thirty-five olive oil samples were analysed and aflatoxins were not detected (Cavalier et al 2007). As it is observed in these studies, Aflatoxin is observed in a small amount in the olive oil samples, and the highest value reported is 2.5  $\mu\text{g kg}^{-1}$ . Aflatoxin is not passed to the oil in olive oil samples, which is the case in hazelnut oil, and exists in very small amounts.

A study was conducted on peanut oil by Yang et al (2011), and the following values were reported for 15 of the 31 samples; AFB1 (0.15-2.72  $\mu\text{g kg}^{-1}$ ), and for 6 of them as AFB2 (0.15-0.36  $\mu\text{g kg}^{-1}$ ), and for 3 of them as AFG1 (0.01-0.02  $\mu\text{g kg}^{-1}$ ). They examined other oils in the same study and AFB1 contamination was recorded in 15 peanut oils, six blended oils and single animal oil (fish oil). However, AFB2 and AFG1 were found only in peanut oil and in no other type of oil (Yang et al 2011). This could indicate that peanut oils are more susceptible to aflatoxin contamination. When compared with our results, AFB1 is the toxin found with the highest level in peanut oil and in the other oils analyzed in this study, as in the case of hazelnut oil. AFG1 was the toxin found at the second highest level in our results. This stems from the fact that the AFG1 amount is high in the hazelnut samples we used in the analyses in our study. It is passed to oil with the lowest level when examined the percentages of passed to oils. AFB types are passed to oil more than other. We may conclude this from the results of the study conducted by Yang et al (2011).

Elzupir et al (2010), conducted a study on a total of 81 vegetable oil samples including peanuts (n= 21), sesame (n= 14), and sunflower (n= 19). The average concentrations in peanut oil were found to AFB1 as 16.3, AFB2 as 1.0, AFG1 as 12.9 and AFG2 as 11.6  $\mu\text{g kg}^{-1}$  (Elzupir et al 2010). These values are extremely high when compared with the results of our study and other studies. AFB1 is the toxin found with the highest level in this study. However, AFG1 and AFG2 values were also determined to be high. Average levels in sesame oil were found AFB1 as 43.6; AFB2 as 0.3; AFG1 as 47.5 and AFG2 as 102.7  $\mu\text{g kg}^{-1}$ . In sunflower oil was detected AFB1 as 24.6, AFB2 as 0.3; AFG1 as 24.5 and AFG2 as 14.8  $\mu\text{g kg}^{-1}$ . These values are very different from the results we found in hazelnut oil.

#### 4. Conclusions

The hazelnuts that are used in hazelnut oil production industry are the ones that are not sold in the market with low quality. For this reason, it is highly probable that aflatoxin exists much in these hazelnuts. However, the results obtained in the analyses show that aflatoxin is passed to the oil at a very low concentration. Aflatoxin is not passed to oil and is accumulated in the oil cake. This reveals another problem that has to be investigated, because the oil cake of the hazelnuts is used as feed in animal feed industry. For this reason, it has to be investigated for aflatoxin before used in feed industry.

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