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Determination of Homogenization Performance of Grinder Used for Aflatoxin Analyses of Turkish Hazelnuts

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Abstract

This research was conducted to determine the homogeneity performance of laboratory grinder used for aflatoxin (Aflatoxin B1: AFB1; Aflatoxin B2: AFB2; Aflatoxin G1: AFG1; Aflatoxin G2: AFG2) analysis of hazelnut, one of the strategic products from the Black Sea Region. For the research, naturally aflatoxins-contaminated hazelnut samples in their unshelled state (A) and non-contaminated unshelled hazelnut samples (B) were used. Hazelnut samples in which aflatoxin (AFs) couldn't be detected by the analytical procedure were treated with standard solutions of 10 µg mL^{-1} AFB1, 5 µg mL^{-1} AFB2, 10 µg mL^{-1} AFG1, 5 µg mL^{-1} AFG2 at the levels of 2 mL, 0.6 mL, 4 mL and 1 mL, respectively. Then, hazelnut samples treated with AFs were grounded in laboratory grinders after 30 min of resting. The Cochran test procedure was applied to duplicate results to determine sufficient homogeneity of grinder. Variations among samples in both hazelnut groups were smaller than critical value of the statistical procedure of the Cochran test.

From the data obtained, it is concluded that laboratory grinder fulfills the sufficient homogeneity of both hazelnut samples of AFs detection.

INTRODUCTION

The homogeneity of test materials is required for all laboratory studies. The results of experiments can be affected tremendously if materials are not well homogenized [1]. The homogeneity of the tested materials has to be proved. The international harmonized protocol prescribes a test for sufficient homogeneity [2]. Therefore, particular care should be taken in handling and preparation of samples before analyses. Having the carcinogenic, teratogenic, mutagenic and immunosuppressive effects, AFs are toxic metabolites produced by especially *Aspergillus flavus* and *Aspergillus parasiticus* [3-9]. AFs consumption can cause malabsorption syndrome and decreased bone strength [10]. AFs have been detected in many food commodities such as peanuts, hazelnuts, pistachios, almonds, various walnuts [11,12], herbs

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and plant extracts [13], rice, peanut products, spices, white pepper, red pepper, and paprika [14]. The main AFs produced by species of *Aspergilli* are the B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) and B_1 being the most toxic one [15,16]. Apart from their adverse effects on health, food contamination of AFs results in serious economic losses.

Recently, contamination of foods with AFs has received much attention in international food trade. The frequent occurrence of AFs has led to European countries, including Turkey, to establish limits for maximum levels of AFs. The USA and European Union (EU) regulate AFs levels in their foods to lower than 20 ppb [17]. However, guidelines and legal limits vary from country to country. Turkey is considered the main producer and exporter of hazelnuts, supplying 65% of the world's total production [18]. With its suitable climate conditions, The Black Sea Region of Turkey possesses high importance in cultivation of hazelnuts. Turkey encountered AFs contamination of food products including hazelnuts in 1967. Because of the AFs contamination some parts of our hazelnuts were rejected. However, data following subsequent years were satisfying and determined that levels of AFs in hazelnuts and their products have gradually remained in a tolerant degree. Since hazelnuts are highly susceptible to AFs contamination, it is necessary to monitor levels of AFs continuously and sensitively. As mentioned above hazelnuts are cultivated mainly in the Black Sea Region and all AFs analyses of export hazelnuts of this region are performed in Trabzon Provincial Control Laboratory. The main aim of our presented research was to determine the homogeneity performance of the grinder used for grinding of hazelnuts for AFs analyses in the Trabzon Provincial Control Laboratory against established criteria presented in international harmonized protocol.

MATERIALS AND METHODS

Sample Collection and Preparation

Both naturally-contaminated (A) and non-contaminated hazelnut samples (B) were obtained from Arslanturk Organic Agriculture Products Co. in Arakli/Trabzon. Hazelnut samples were collected based on the Turkish national sampling method [19]. Unshelled hazelnut samples of each group (approximately 15 kg for each) were taken into polyethylene bags and stored at 20 ± 5ºC in the laboratory prior to analysis. Each sample was separately grounded in the laboratory grinder (robout coupe R23 type). Considerable emphasis was put on grinding of non-contaminated hazelnut samples to prevent contamination of it from naturally-contaminated hazelnuts in grinder. For this purpose grinder was cleaned with methanol solution (50%). Both grounded hazelnut samples were pre-analysed for the detection of AFs with HPLC and all analyses were conducted in duplicate. Aflatoxin levels from each group are presented in Table 1.

10 kg of A samples were weighed and grounded in laboratory grinder for 2 min at 3600 rpm. Then, 10 kg of B samples were taken and subjected to pre-grinding for 30 sec at 1800 rpm. For homogeneity

Table 1. AFs levels of naturally-contaminated and non-contaminated hazelnut samples (µg/kg)*

	AFB1	AFB2	AFG1	AFG2	Total AFs
Non-contaminated	ND**	ND	0.51- ND	ND	ND
Naturally-contaminated	1.54–3.45	0.47–0.77	4.67–7.98	0.66–0.98	7.97–14.65

* AFs values are given with their duplicates
** ND :Non-detected

testing, B samples were spiked with standard solutions of 10 µg mL^{-1} AFB_1, 5 µg mL^{-1} AFB_2, 10 µg mL^{-1} AFG_1, 5 µg mL^{-1} AFG_2 at the levels of 0.6 mL, 1 mL, 2 mL and 4 mL, respectively prior to extraction. These samples were left for 30 min and then grounded for 2 min at 3600 rpm. 100 samples were prepared from both A and B hazelnuts (each 100 g) and 12 samples were taken randomly and analysed in duplicate.

Chemicals, Standard Solutions and HPLC Conditions

The mobile phase (all HPLC grade) was the mixture of deionised water, methanol and acetonitrile (6 + 3 + 2). 350 µL of 4 M HNO_3 and 0.12 g of KBr were added to each litre of the mobile phase, followed by filtration of the mixture. Methanol and acetonitrile were procured from (Sigma-Aldrich, Steinhem, Germany). All the other chemicals were analytical graded and purchased from Merck (Darmstadt, Germany). The immuneoaffinity column, which was used for samples cleaning up, was purchased from VICAM (Watertown, MA, USA). Standard solutions of AFB_1, AFB_2, AFG_1 and AFG_2 were supplied from Sigma Chemical aflatoxin mix kit-m 0.3-1 ug mL^{-1}. Standard solutions of AFs were prepared according to AOAC Official Method 999.07 [20]. Determination of AFs was carried out by HPLC (HP1100 Series, Agilent Technologies) equipped with auto sampler, quaternary pump, degasser using FLD detector.

Flow rate	: 1 mL min^{-1}
Injection volume	: 100 µl
Column temperature	: 22ºC
Column	: C18, 4.5 x 250 mm
Retention times: AFB1	: 12.159, AFB2: 10.054,
AFG1: 8.951, AFG2	: 7.556
Derivatization unit	: Kobra Cell

The determination of recovery was also performed on non-contaminated hazelnut samples spiked with the mentioned levels of AFs. The average recoveries were 84.7% for AFB1, 79.3% for AFB2, 86.0% for AFG1, 91.6 % for AFG2 and 85.4% for total AFs.

High-Performance Liquid Chromatography Analyses

25 g of the hazelnut samples were weighed into blender (Waring Commercial Blender) with 5 g NaCl and extracted with 125 mL of 70% methanol by blending for 2 min. Then the extract was filtered through Schleicher & schwell 5971/2 filter paper and fluted filter paper (24 cm, Vicam, Watertown, MA, USA). 5 mL of filtrate was taken in an 20 mL syringe containing 10 mL of deionized water equipped with immunoaffinity column for cleaning at a flow rate of 3 mL min^{-1} and then the column was washed with 20 mL distilled water at a flow rate of 5 mL. Then air was passed through the syringe 3-5 times. The column was put into 2.5 mL volumetric flask and AFs were slowly eluted from the column by passing 1 mL of HPLC grade methanol through the column allowing to drop by gravity and flask was brought to volume with deionized water. After mixing in a vortex, 2 mL was transferred into a vial and 100 µL was injected into HPLC [21] (AOAC Official Method 991.31 was modified by Trabzon Province Control Laboratory Directorate and accredited by Turkish Accreditation Agency).

Statistical Analysis

In this study the statistical procedure proposed in the revision of International Harmonized Protocol (IUPAC Technical Report) was used [2]. According to the harmonized protocol the Cochran test procedure is proposed for duplicate results. The largest $D^2_{max}/\Sigma D_i^2$ ratio of each naturally-contaminated and non-contaminated hazelnut samples were calculated where D_i differences of each pair of duplicates and D_{max} the largest difference. The calculated ratio was compared with critical values for the Cochran test statistics for duplicates. Outlying pairs were detected at 95%

level of confidence. Analytical variance (s_{an}^2) and sampling variance (s_{sam}^2) were estimated by ANOVA. Allowable sampling variance (σ_{all}) was calculated as $\sigma_{all} = (0,3 \times \sigma_p)$ where σ_p is the target standard deviation. Then critical value for the test (c) is calculated and if the calculated s_{sam}^2 value is less than critical value (c) the test for homogeneity has been passed. To analyse concentrations less than 120 ppb target standard deviation was calculated at σ_p = 0,22c/mr by using Horwitz equation where c is concentration and mr is the dimensionless mass ratio [22].

RESULTS AND DISCUSSION

Despite best efforts to ensure homogeneity of testing materials, materials prepared for laboratory studies are usually heterogeneous to some degree. When heterogeneous materials are divided for analyses in laboratories, the parts produced will differ in a small degree. For the most part the differentiation will be negligible but we have to be certain of this. It is necessary that the estimated sampling standard deviation should be less than 30% of the target standard deviation [23]. According to the Harmonized Protocol, we obtain sufficient homogeneity once this condition is fulfilled [2]. It should be regarded that homogeneity tests are essential but not foolproof [23]. In contrary to total aflatoxin levels, AFB_1 levels of naturally-contaminated hazelnuts were in the limits of Turkish Food Codex. Aflatoxin levels of non-contaminated hazelnuts were below the limit of quantification for AFB_1: 0.16, for AFB_2: 0.14, for AFG_1: 0.19 and for AFG_2: 0.19. Twelve test materials of naturally-contaminated and non-contaminated hazelnut samples were analysed in duplicate for AFB_1, AFB_2, AFG_1, AFG_2 and total aflatoxins. The result of statistical evaluation is presented in Table 2 and Table 3 respectively. The largest $D^2_{max}/\Sigma D_i^2$ ratios of naturally-contaminated hazelnut samples for AFB_1, AFB_2, AFG_1, AFG_2 and total aflatoxins were 0.52, 0.48, 0.41, 0.21 and 0.24, and those of non-contaminated hazelnut samples treated with aflatoxins were 0.26, 0.36, 0.24, 0.27 and 0.31, respectively. These data were compared with critical value of 0.541 in Table Critical values from the Cochran test for variance outliers [24] at 95% confidence level. Results of comparison showed that there was no outlying pair to be excluded. According to variations of analyses results; calculated s_{sam}^2 values of both A and B samples were less than critical value (c). Harmonized protocol for proficiency testing (IUPAC) conveyed that if $s_{sam}^2 < c$ the test for homogeneity has been passed. The results of both A and B samples were fit in with that of IUPAC and confirmed that grounded hazelnuts can be considered as homogenous.

It can be concluded that grinder used for grinding of hazelnuts for AFs analyses in Trabzon Provincial Control Laboratory ensured sufficient homogeneity of samples.

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Table 2. Homogeneity data belong to naturally-contaminated hazelnut samples (A)

No	AFB1 µg/kg		AFB2 µg/kg		AFG1 µg/kg		AFG2 µg/kg		Total AFs	
	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2
1	3.08	2.55	0.27	0.37	6.06	5.11	0.59	0.42	9.35	8.53
2	3.03	2.97	0.37	0.33	4.80	4.66	0.53	0.51	8.72	8.46
3	2.90	2.92	0.37	0.34	4.62	4.84	0.46	0.46	8.34	8.54
4	2.81	2.89	0.29	0.29	4.79	4.42	0.50	0.42	8.38	8.01
5	3.08	3.30	0.35	0.29	5.27	4.54	0.43	0.41	9.10	8.41
6	3.03	3.08	0.35	0.35	4.45	5.01	0.45	0.56	9.78	9.09
7	3.00	2.83	0.27	0.28	4.80	4.65	0.40	0.35	8.82	8.10
8	2.89	2.93	0.28	0.28	4.65	4.45	0.34	0.31	7.93	7.95
9	3.02	3.07	0.32	0.29	5.12	4.91	0.51	0.65	9.03	8.67
10	3.16	2.93	0.38	0.33	5.48	5.21	0.44	0.48	8.46	8.92
11	3.02	2.83	0.33	0.31	4.92	5.01	0.71	0.48	8.96	8.60
12	3.03	3.02	0.32	0.29	4.66	4.89	0.33	0.44	8.59	8.62
Mean	3.03	2.94	0.33	0.31	4.97	4.81	0.47	0.46	8.79	8.49
origin of target sd (σp)	Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb	
abs. target sd (σp)&as RSD %san2	0.657	24	0.070	24	1.075	24	0.102	24	1.901	24
ssam2	0.02261		0.00087		0.09143		0.00574		0.11915	
σall 2	0.003		0.000		0.048		0.004		0.083	
critical value	0.0388		0.0004		0.1041		0.0009		0.3252	
ssam2< critical value	0.0889		0.002		0.2650		0.0066		0.6845	
	0.003 < 0.089		0.000 < 0.002		0.048 < 0.265		0.004 < 0.007		0.083 < 0.685	
	ACCEPT		ACCEPT		ACCEPT		ACCEPT		ACCEPT	

Table 3. Homogeneity data belong to non-contaminated hazelnut samples (B) Supplied with AFS

No	AFB1 µg/kg		AFB2 µg/kg		AFG1 µg/kg		AFG2 µg/kg		Total AFs	
	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2	Replicate1	Replicate 2
1	1.64	2.08	0.33	0.25	3.86	4.58	0.64	0.45	6.87	5.64
2	1.83	1.70	0.29	0.30	3.57	4.21	0.35	0.51	6.05	6.42
3	1.97	1.58	0.29	0.28	4.51	3.82	0.43	0.34	6.48	5.91
4	1.61	1.49	0.24	0.20	3.92	3.68	0.43	0.32	5.91	5.42
5	1.64	1.60	0.21	0.23	4.34	3.97	0.37	0.37	6.25	6.19
6	1.77	1.63	0.24	0.19	4.17	3.83	0.34	0.29	6.22	5.67
7	1.82	1.58	0.26	0.29	4.16	4.44	0.48	0.51	6.25	7.00
8	1.79	1.81	0.23	0.21	3.93	4.01	0.26	0.37	5.93	5.97
9	1.71	1.87	0.25	0.21	4.41	4.34	0.37	0.22	6.79	6.59
10	1.76	1.62	0.25	0.26	4.54	3.95	0.47	0.50	6.70	6.05
11	1.63	1.90	0.28	0.28	4.02	3.86	0.38	0.47	5.87	6.87
12	1.65	2.05	0.23	0.29	4.21	4.20	0.24	0.31	6.02	6.53
Mean	1.74	1.74	0.26	0.25	4.14	4.07	0.40	0.39	6.28	6.19
origin of target sd (σp)	Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb		Horwitz <120 ppb	
abs. target sd (σp)& as RSD %	0,383		0.056	24	0.903	24	0.086	24	1.371	24
san2	0,0306		0.0007		0.0907		0.0056		0.2003	
ssam2	-0,007		0.0006		-0.013		0.0046		-0.0191	
σall 2	0,0132		0.0003		0.0734		0.0010		0.1692	
critical value	0,0499		0.001		0.2095		0.0060		0.4752	
ssam2<critical value	-0.007 < 0.05		0.0006 < 0.001		-0.013 < 0.2095		0.0046 < 0.006		-0.0191 < 0.4752	
	ACCEPT		ACCEPT		ACCEPT		ACCEPT		ACCEPT	

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