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Determination of aflatoxin levels in nuts and their products consumed in South Korea

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Abstract

A total of 85 nuts and their products marketed in South Korea were assessed for aflatoxins using a monitoring scheme consisting of enzyme-linked immunosorbent assay (ELISA) for rapid screening, high performance liquid chromatography (HPLC) for quantification and LC–mass spectrometry (MS) for confirmation. Thirty-one out of 85 samples gave ELISA readings above 0.06 and were screened as possible positive samples. Aflatoxin contents of possible positive samples were determined using HPLC with a detection limit of 0.08–1.25 μg/kg and a quantification limit of 0.15–2.50 μg/kg. Nine samples including 1 raw peanut, 4 roasted peanuts, 2 peanut butters, 1 pistachio and 1 seasoned assorted nut were contaminated with aflatoxins (10.6% of incidence), ranging in various levels up to 28.2 μg/kg. LC–MS analysis on contaminated samples revealed that peaks eluting at 4.4, 5.2, 9.1 and 11.9 min were confirmed as aflatoxin G_1, aflatoxin B_1, aflatoxin G_2 and aflatoxin B_2, respectively.
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1. Introduction

Aflatoxins are a group of structurally related toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasticus* (Eaton & Groopman, 1994). Among the major aflatoxin of concern, aflatoxin B_1 (AFB_1) is the most frequent metabolite present in contaminated samples and is classified as a human carcinogen. Aflatoxin B_2(AFB_2), G_1(AFG_1) and G_2(AFG_2) are generally not reported in the absence of AFB_1 and are classified as possible carcinogens to humans (IARC, 1993; JECFA, 1998). Based on epidemiological studies, aflatoxins might be associated with human liver cancer and acute hepatitis (Li, Yoshizawa, Kawamura, Luo, & Li, 2001; Park, Kim, & Kim, 2004).

Because of potential health hazards to humans, regulatory levels have recently been documented. The range of worldwide regulations for $AFB_1$was from 0 to 30 μg/kg with a total from 0 to 50 μg/kg (Creppy, 2002; FAO, 1997). In the European Union, the total aflatoxins and AFB_1 level in human commodities are regulated with maximum residue levels (MRLs) that cannot be greater than 2 and 4 μg/kg, respectively (EEC, 1998). Recently, the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program adopted a limit of 15 μg/kg for total aflatoxins (Codex, 2001). In Korea, a residue limit of 10 μg/kg AFB_1 for foodstuffs has been established since 1989 (KFDA, 2000).

A review of monitoring studies on the occurrence of aflatoxins in food products has demonstrated that aflatoxins are still being found frequently in food products at levels that are of significant concern for consumer protection (Scott & Lawrence, 1997; Stroka & Anklam, 2002). Recently, the daily exposure of Koreans to AFB_1 through food consumption revealed that the calculated probable daily intake (PDI) of AFB_1 for Koreans ranged from

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1.19 to 5.79 ng/kg bw/day (Park et al., 2004). This exceeds the estimated provisional maximum tolerable daily intakes, 0.4 ng/kg bw/day for adults with hepatitis B or 1.0 ng/kg bw/day for adults and children without hepatitis B (Kuiper-Goodman, 1998). However, the PDI of AFB_1 for Koreans was estimated from the AFB_1 levels in 30 barley, 32 barley-based foods, 38 corn, 47 corn-based foods, 60 fermented soybean products and 108 rice. Levels of aflatoxins in nuts and nut products were not reflected though nuts and their products are the most important dietary sources of aflatoxins. Therefore, surveys concerning nuts and their products intended for human consumption are necessary in determining the risk assessment of aflatoxins for Koreans.

In this study, levels of aflatoxins in nuts and nut products marketed in South Korea were rapidly screened by enzyme-linked immunosorbent assay (ELISA), quantified by high performance liquid chromatography (HPLC) and confirmed by LC–mass spectrometry (MS).

2. Materials and methods

2.1. Sample collection

A total of 85 nuts and nut products were collected during the period from June to July 2004, according to stratified random sampling design (Yoon et al., 2003). Four cities including Seoul, Daejeon, Gwangju and Busan were chosen considering demographic and regional viewpoints (Fig. 1). Nuts and their products were selected to reflect (i) import and production (KERI, 2003), and (ii) the major dietary component of human consumption in South Korea, based on data available from the Ministry of Health and Welfare (KMOHW, 2002). In each city, 5 peanut samples, 3 walnut samples, 3 pine nut samples, 2 almond samples (Seoul; 3 samples), 3 peanut butter samples, 3 seasoned assorted nut samples and 2 pistachio samples were purchased from supermarkets, local stores and markets. With exception of 8 peanuts and 3 pine nuts, all other samples were imported. Samples ranging from 3 to 5 kg were delivered to the laboratory within 24 h of collection and immediately dispensed into plastic bags. The samples were stored at −4 °C until analyzed.

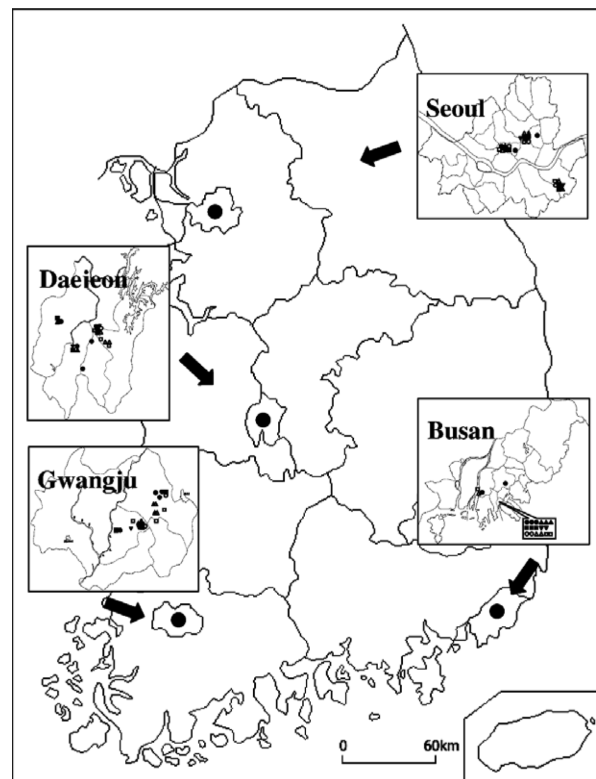

Fig. 1. Sampling of nuts and nut products to determine aflatoxin levels consumed in South Korea. Nuts and nut products were purchased at markets in Seoul, Daejeon, Gwangju and Busan as representative cities in South Korea considering balances in regions and populations. In each city, 5 raw peanut samples, 3 walnut samples, 3 pine nut samples, 2 almond samples (Seoul; 3 samples), 3 peanut butter samples, 3 seasoned assorted nut samples and 2 pistachio samples were purchased in markets. Sampling points were indicated using different symbols: closed circle; peanut, open circle; pistachio, closed triangle; walnut, open triangle; peanut butter, closed square; pine nut, open square; mixed nuts, closed reverse triangle; almond.

2.2. Chemical and reagents

Acetonitrile, acetone, ethanol, diethyl ether, hexane, methanol and chloroform were supplied by Merck (Darmstadt, Germany). HPLC-grade water was obtained from Burdick and Jackson (Muskegon, MI). The AFB_1, AFB_2, AFG_1, AFG_2, AFB_1-horseradish peroxidase conjugate, hydrogen peroxide and 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Sigma (St. Louis, MO). Anti-AFB_1 monoclonal antibody was produced as described (Yang et al., 2004) and cross-reacted with AFB_2(20.6%), AFG_1(64.6%) and AFG_2(25.3%). Stock standard solutions of aflatoxins with concentrations of 100 μg/ml were prepared in benzene–acetonitrile (98:2, v/v), wrapped in aluminum foil to prevent gradual break down of aflatoxins under UV light and kept under protected conditions at −20 °C. All other inorganic chemicals and organic solvents were of reagent grade or higher.

2.3. Sample preparation

Among collected nuts and their samples, hulled peanuts and pistachios were dehulled prior to sample preparation. Samples except peanut butter were pulverized using a food processor (Hanil FM-909T, Seoul, Korea). According to their lipid and moisture contents, the pulverized samples were classified as powder-type (raw and roasted peanuts, pistachios, almonds) and butter-type (peanut butters, walnuts, pine nuts, seasoned assorted nuts). The powdered samples were passed through a 20-mesh sieve and the butter-type samples were thoroughly pulverized until particles of nuts or nut products could no longer be detected. The prepared samples were stored at −4 °C until analyzed.

2.4. Enzyme-linked immunosorbent assay (ELISA) analysis

For ELISA analysis of AFB_1, the powder-type samples were extracted as follows: a 20 g sample was extracted with 100 ml of 60% methanol and 5 g of NaCl for 20 min with shaking. After extraction, the sample was centrifuged at 3000 rpm for 5 min and the supernatant was filtered with filter paper (Whatman No. 1) and glass microfibre filter pretreated by 1 ml of 100% methanol. For the butter-type samples, a 10 g sample was extracted with 50 ml of 50% acetonitrile for 3 min with shaking. After extraction, the sample was centrifuged at 3000 rpm for 15 min and the supernatant was filtered with filter paper (Whatman No. 4). An aliquot of sample was diluted with PBST and analyzed for AFB_1 (Yang et al., 2004). Direct competitive (DC)-ELISA procedure was performed as described by Kang et al. (Kang, Kang, & Chung, 2001). Briefly, wells of polystyrene microtiter plates were coated with anti-AFB_1 antibody. Standard AFB_1 or extracts were mixed with equal volumes of AFB_1-horseradish peroxidase conjugate, and 100 μl of this mixture was incubated over the antibody solid phase for 1 h at 37 °C. The plate was washed, and ABTS as a substrate was added to each well of the plate. Following incubation of the plate for 20 min at room temperature, the absorbance for each well at 405 nm was read with an ELISA reader (model 550, Bio-Rad, Hercules, CA).

2.5. HPLC determination of aflatoxins

Total aflatoxins in the samples were determined by HPLC subsequent to extraction, partitioning, and derivatization based on the Korean Food Code (KFDA, 2000) and the AOAC method 990.33 (AOAC, 2000). Briefly, 50 g of sub-samples were homogenized in 200 ml of methanol for 5 min and defatted twice with 100 ml of 1% NaCl and *n*-hexane in a separatory funnel. This was clarified with anhydrous ammonium sulphate followed by partitioning to 50 ml of chloroform twice. The chloroform was then evaporated to ca. 20 ml in rotary evaporator. This concentrate was introduced into a 22 × 300 mm glass chromatographic column packed with 50 g of silica gel. Then, 150 ml hexane followed by 150 ml of diethyl ether was passed through and discarded. Subsequently, aflatoxins were eluted with 200 ml of chloroform–ethanol (97:3), re-evaporated to dryness and derivatized with 0.1 ml of trifluoroacetic acid for 1 min. Following the reconstitution with 10 ml of acetonitrile–water (1:1, v/v), the resulting solution was used for HPLC analysis.

For HPLC analysis of aflatoxins, a Jasco HPLC system (Tokyo, Japan) equipped with two PU-980 intelligent pumps, an 851-AS intelligent autosampler, a FP-920 fluorescence detector and an 807-IT integrator was used. The chromatographic separation was performed on a μ Bondapak C_{18} column (3.9 × 300 mm i.d., Waters, Ireland) using a water–acetonitrile (3:1, v/v) mobile phase at a flow-rate of 1.0 ml/min. Detection of aflatoxin was carried out using 365 and 418 nm as wavelengths for excitation and emission, respectively.

Linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) were determined to test the validity of the HPLC procedure used for aflatoxins. Linearity was estimated by injecting triplicate aflatoxin standards of 1–100 ng of each aflatoxin. Accuracy was examined by the determination of the recoveries of the aflatoxins. The recoveries were ascertained by the addition of 20 ng/ml of AFB_1 and AFG_1, and 10 ng/ml of AFB_2 and AFG_2 to the clean peanut butter and walnut samples. Precision was established by performing multiple analysis of a spiked sample and was expressed by the mean ± relative standard deviation (RSD). LOD and LOQ were determined by spiked samples based on signal to noise ratio of 3:1 for LOD and 5:1 for LOQ.

2.6. LC–MS confirmation

For confirmation of aflatoxins, LC–MS analysis was carried out using a Nanospace SI-2 (Shiseido, Tokyo, Japan) liquid chromatographic system coupled with LCQ Deca XP Thermo Finnigan ion trap instrument with atmospheric pressure chemical ionization source, APCI (Thermo Finnigan, San Jose, CA). Separation was completed by reverse phase elution with a Luna C_{18}(2) column (1 × 150 mm i.d.; 5 μm particle size). The mobile phase consisted of 0.1% formic acid in 25% acetonitrile at a flow-rate of 0.2 ml/min. The MS detector was set as follows: vaporizer temperature 450 °C; a sheath gas flow-rate 25 arbitrary unit; auxiliary gas flow-rate 9 arbitrary unit; discharge current 3.5 μA; tube lens offset 38; capillary voltage 21 V. APCI-mass spectra ranging from m/z 100 to 500 were taken in positive-ion mode. The ions monitored in single ion recording were the protonated molecule $[M+H]^+$ at m/z 331 for AFB_{2a} (corresponding to AFB_1), 347 for AFG_{2a} (corresponding to AFG_1), 315 for AFB_2 and 331 for AFG_2.

3. Results and discussion

The collected 85 samples of nuts and nut products included 20 peanut, 12 walnut, 12 pine nut, 9 almond, 8 pistachio, 12 peanut butter and 12 seasoned assorted nut samples. Aflatoxin levels in the nuts and nut products were analyzed by ELISA as the first stage to screen aflatoxin contamination. To select the best solvent for aflatoxin extraction, four kinds of solvent systems, i.e. 50% acetonitrile and *n*-hexane, 80% methanol, 60% methanol, and 60% methanol and NaCl were tested. Considering an extraction yield of aflatoxin and matrix effects of samples, 60% methanol and NaCl were selected as the extraction solvent for powder-type samples. For butter-type samples, 50% acetonitrile was adopted as the extraction. When AFB_1 was spiked to powder-type samples at 1 and 5 ng/ml, 0.85 and 6.1 ng/ml of AFB_1 were detected in spiked samples and the accuracy expressed by recovery of AFB_1 in

powder-type samples were 85% and 122%, respectively. In the case of butter-type samples, the spiked AFB_1 at 5 and 10 ng/ml were as 6.5 and 9.8 ng/ml, with 130% and 98% of recovery, respectively.

ELISA analysis is convenient for simultaneous determination of contaminants in a large number of samples with relatively low cost and short time. However, it is not suitable for quantification of contaminants since it can be influenced by matrix effect of samples and has the possibility to overestimate the contaminants at very low concentration. Therefore, monitoring scheme, which consists of ELISA for screening of possible contaminated samples, HPLC for quantification of contaminated levels and LC–MS for confirmation of contaminated samples was applied to assess the aflatoxin levels in nuts and their products. As shown in Fig. 2, nuts and nut products were grouped according to ELISA readings to screen the possible contaminated samples. When ELISA readings were 0.05, 0.06, 0.10, 0.11, 0.20 and 0.21, AFB_1 contents calculated from standard curves were 0, 0, 6.1, 6.7, 10.4 and 10.8 μg/kg for powder-type samples, and 10.1, 11, 13.8, 14.4, 18.3 and 18.6 μg/kg for butter-type samples, respectively. Based on these results, 31 samples showing ELISA readings of more than 0.06 were selected as possible positive samples.

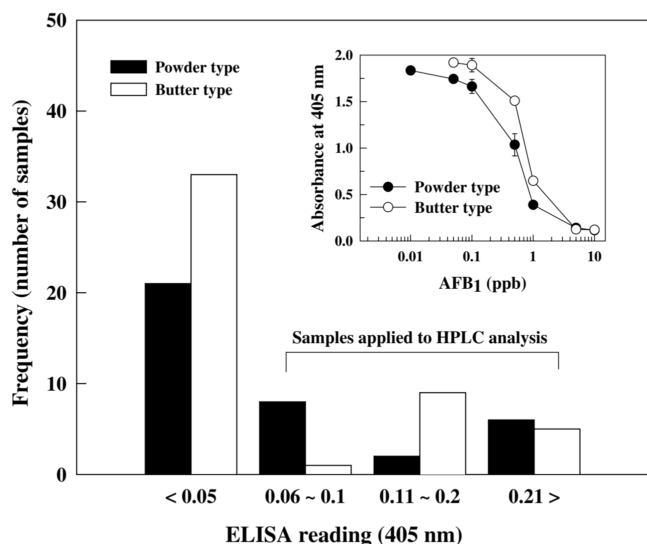

Fig. 2. Screening of aflatoxin B_1-contaminated nuts and nut products by ELISA analysis. Considering matrix effect, nuts and their products were divided into powder-type group and butter-type group according to their lipid contents and appearances. Different methods of sample treatment and standard curves for each group of samples were applied in the analysis of aflatoxin B_1 contamination. From ELISA results, samples showing ELISA readings of more than 0.06 were selected as possible contaminated samples and applied to HPLC analysis to determine aflatoxin levels. The inlet figure showed standard curves for aflatoxin B_1 by ELISA analysis. When ELISA readings were 0.05, 0.06, 0.10, 0.11, 0.20 and 0.21, aflatoxin B_1 contents calculated from standard curves were 0, 0, 6.1, 6.7, 10.4 and 10.8 μg/kg for powder-type samples, and 10.1, 11, 13.8, 14.4, 18.3 and 18.6 μg/kg for butter-type samples, respectively.

The levels of total and four individual aflatoxins of selected samples from ELISA were quantified by HPLC. Validation results including LOD, LOQ, linearity, recovery and precision on HPLC method are shown in Table 1. For AFB_1, AFB_2, AFG1 and AFG2, LOD were 0.08, 0.13, 0.40 and 1.25 μg/kg and LOQ were 0.15, 0.40, 1.30 and 5.00 μg/kg, respectively. Linearity of the measurements was checked for a standard solution containing aflatoxins in a range from the LOD up to a concentration of 100 ng/ml. Correlation coefficients, R^2, were in the range from 0.998 to 1.000. Accuracy was examined by the determination of the recoveries of the aflatoxins. When AFB_1 and AFG_2 were added at 20 ng/ml and AFB_2 and AFG_2 were added at 10 ng/ml, the recoveries were ranged from 83.4 to 102.1%. The precision of AFB_1, AFB_2, AFG_1 and AFG_2 expressed as the mean ± relative standard deviation (RSD) were 20.4 ± 7.11, 8.5 ± 22.59, 20.4 ± 5.42 and 8.3 ± 27.75, respectively. These data indicated that HPLC method adopted in this study was acceptable.

Levels of total aflatoxin and individual aflatoxins in 31 samples selected as possible positives in AFB_1 from ELISA analysis were determined by HPLC. As shown in Table 2, the contamination varied from below the LOQ to 28.2 μg/kg in total aflatoxins. Among 31 samples, 9 were contaminated with aflatoxins (10.6% incidence), ranging in levels up to 28.2 μg/kg. The levels of individual aflatoxin B_1, B_2, G_1 and G_2 were detected in the range of <0.2–18.0, <0.4–2.6, <1.3–4.1 and <2.5–3.5 μg/kg, respectively. Five peanut samples out of 20 raw and roasted peanut samples (25% incidence) and 2 peanut butter samples out of 12 peanut butter samples (17% incidence) were contaminated with aflatoxin, indicating that peanut and peanut products were the most common commodity contaminated with aflatoxin. Our result was supported by previous reports that the peanut is one of the most susceptible foodstuffs to contaminate by toxicogenic fungi producing aflatoxins (Escobar & Regueiro, 2002; Mphande, Siame, & Taylor, 2004). Particularly, the total aflatoxin and AFB_1 levels of roasted peanuts ranged from 2.0 to 28.2 μg/kg (mean; 10.7 ± 12.30) and from 1.9 to 18.0 μg/kg (mean; 8.0 ± 7.75), respectively. Moreover, 2 roasted peanut samples contained over 10 μg/kg of aflatoxin B_1, which is the legal tolerance limit for AFB_1 in South Korea. In a previous report, the probable daily intake (PDI) of Koreans to AFB_1 through food consumption was estimated as 1.19– 6.79 ng/kg bw/day. In data used for this estimation of PDI, barley-based food had the highest levels of AFB_1, which ranged from levels below the LOD (0.05–1 μg/kg) to 35 μg/kg among several food commodities (Park et al., 2004), but aflatoxin levels in nuts and nut products were not counted. Considering the high incidence of contamination (25%) and their contamination range up to 28.2 μg/kg of aflatoxin in nuts and nut products, PDI of aflatoxin in Korea could be affected by their consumption. However, the daily intake of peanut by the average Korean was estimated as 0.4 g (KMOHW, 2002), and was regarded as the minor contributor to the dietary intake of aflatoxin. Nevertheless, there

Table 1
Validation of aflatoxin determination by HPLC analysis

Aflatoxin	LOD[a] (μg/kg)	LOQ[b] (μg/kg)	Calibration curve	R^2	Recovery (%)[c]	Mean (μg/kg) ± RSD (%)[d]
AFB_1	0.08	0.15	$Y = 1.0198E{-}05x - 0.01$	0.9996	102.0	20.4 ± 7.11
AFB_2	0.13	0.40	$Y = 6.55E{-}05x - 0.06$	1.0000	84.8	8.5 ± 22.59
AFG_1	0.40	1.30	$Y = 1.31E{-}05x - 0.81$	0.9994	102.1	20.4 ± 5.42
AFG_2	1.25	2.50	$Y = 3.98E{-}05x - 0.47$	0.9978	83.4	8.3 ± 27.75

[a] Limit of detection.
[b] Limit of quantification.
[c] Accuracy was examined by the determination of the recoveries of the aflatoxins. Recoveries were determined by spiking 20 ng/ml aflatoxin B_1 and G_1, and 10 ng/ml aflatoxin B_2 and G_2 to the samples (peanut butter and walnut).
[d] Precision expressed by the mean ± relative standard deviation (RSD) was determined by a multiple analysis of a spiked sample. The level spiked was 20 ng/ml for aflatoxin B_1 and G_1, and 10 ng/ml for aflatoxin B_2 and G_2.

Table 2
Presence of aflatoxins in different nuts and their products by HPLC analysis

Sample category	Analyzed sample	Positive sample	Aflatoxins (μg/kg)					
			Total aflatoxin[a]	Range	AFB_1	AFB_2	AFG_1	AFG_2
Peanut (raw)	4	1	0.20 ± 0.14[b]	0.20	0.20 ± 0.14	nd[c]	nd	nd
Peanut (roasted)	8	4	10.67 ± 12.30	2.00–28.24	7.97 ± 7.75 (1.85–18.04)[d]	0.77 ± 1.25 (nd ~ 2.63)	1.04 ± 2.02 (nd ~ 4.07)	0.87 ± 1.75 (nd ~ 3.50)
Pistachio	4	1	3.41 ± 0.17	3.41	3.36 ± 0.14	nd	nd	nd
Peanut butter	2	2	7.36 ± 0.46	7.03–7.68	6.13 ± 0.44 (5.82–6.44)	1.07 ± 0.12 (nd ~ 1.15)	nd	nd
Assorted nuts (seasoned)	1	1	7.89 ± 0.73	7.89	6.68 ± 0.60	1.21 ± 0.18	nd	nd
Walnut	12	0	nd	–	nd	nd	nd	nd
Total	31	9						

[a] Total aflatoxin was represented by the summation of aflatoxin B_1, B_2, G_1 and G_2 levels.
[b] Total aflatoxin and individual aflatoxin levels were expressed by the mean ± standard deviation.
[c] Not detected (below the quantification limit); the mean of individual aflatoxin was calculated by assuming that the level of each aflatoxin in samples below the detection limit was equal to zero.
[d] The values in parenthesis represent the range of individual aflatoxin detected.

are groups with a much higher intake of nuts than the ordinary Korean population, such as certain age groups, heavy alcohol drinker and vegetarians, which would be important to address. In addition, there is still a lack of the intake data for imported tree nuts and their processed products.

Mass spectrometric detection was optimized by injecting standard aflatoxins in the positive ionization mode with same cone voltage (20 V) and analyzed for samples contaminated with aflatoxins. The representative MS chromatogram of samples contaminated with aflatoxins and their mass spectra are indicated in Fig. 3. Peaks eluting at 4.44, 5.23, 9.14 and 11.88 min were identified as aflatoxin AFG_1, AFB_1, AFG_2 and AFB_2, respectively, from retention time and the characteristic protonated molecules. The abundant fragments are protonated molecules at m/z 347.3 for AFG_{2a} (corresponding to AFG_1), 331.3 for AFB_{2a} (corresponding to AFB_1), 331.4 for AFG_2 and 315.5 for AFB_2. AFG_1 and AFB_1 are converted to the fluorescent hemiacetals, AFG_{2a} and AFB_{2a} by treatment with trifluoroacetic acid (Scott & Lawrence, 1997).

In this study, aflatoxin levels were monitored on 85 nuts and their products using a monitoring scheme consisting of ELISA for rapid screening, HPLC for quantification and LC–MS for confirmation. As a result, 9 samples among 85 nuts and their products (10.6% of the analyzed samples) were contaminated with aflatoxins and 2 peanut samples contained over 10 μg/kg of AFB_1, which is the legal tolerance limit for AFB_1 in South Korea. The high levels of aflatoxin in nut samples emphasize the need for regular monitoring and improved control of aflatoxin level. Therefore, this monitoring scheme could be applied to determine the aflatoxin contamination in a large number of samples in a cost and time effective manner. The data obtained from this monitoring can be used as a basis for risk analysis of aflatoxin, thereby maintaining the aflatoxin at the lowest possible levels.

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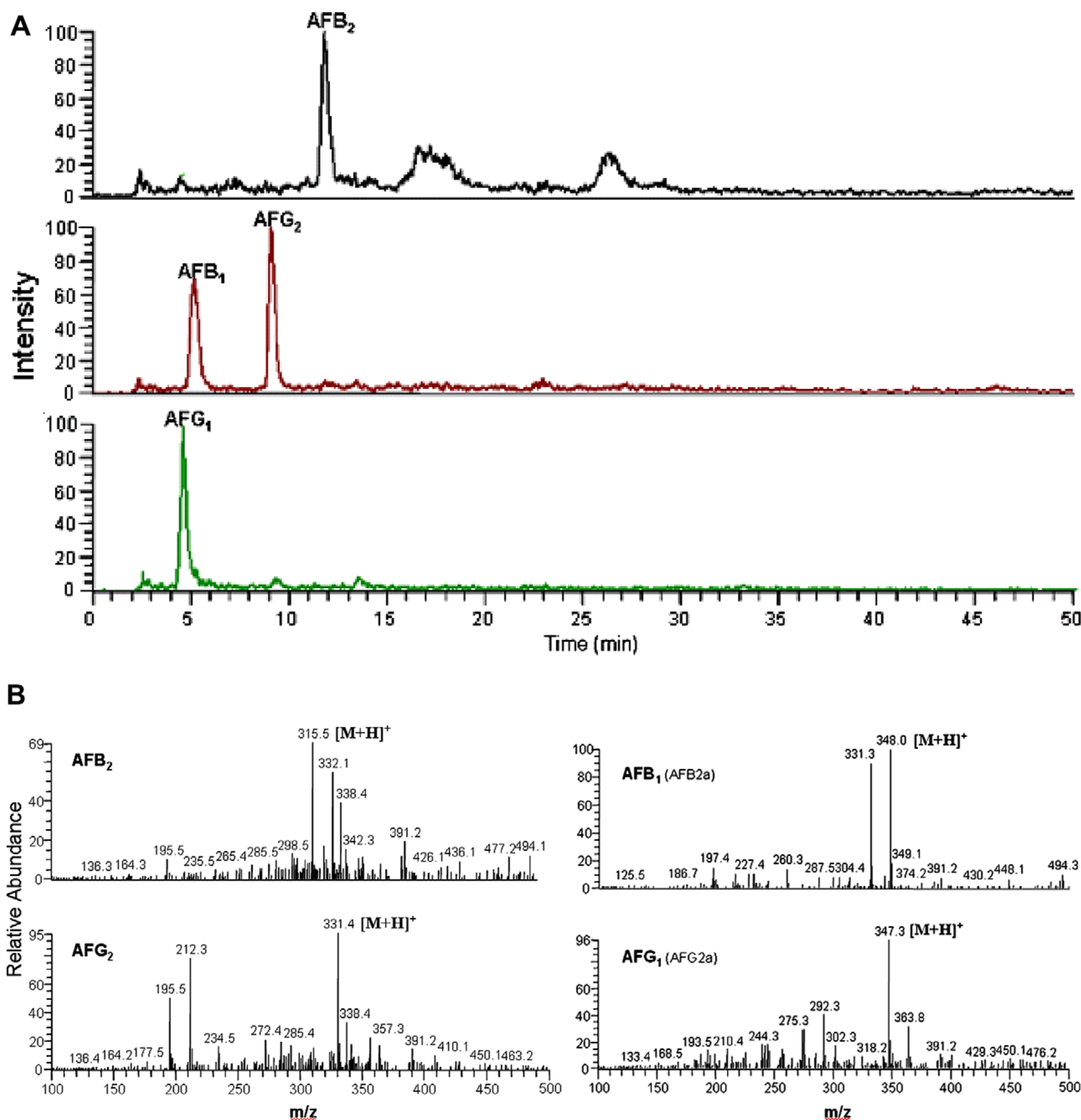

Fig. 3. Representative mass chromatogram (A) of samples contaminated with aflatoxins and their mass spectra (B). Mass chromatograms were obtained by monitoring the protonated molecule $[M+H]^+$ at m/z 331 for AFB_{2a} (corresponding to AFB_1), 347 for AFG_{2a} (corresponding to AFG_1), 315 for AFB_2 and 331 for AFG_2. Mass spectra were obtained from peaks eluting at 4.44, 5.23, 9.14 and 11.88 min and abundant fragments were protonated molecules at m/z 347.3 for AFG_1, 331.3 for AFB_1, 331.4 for AFG_2 and 315.5 for AFB_2.

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