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Analytical Methods

Aflatoxins contamination in spices and processed spice products commercialized in Korea

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Abstract

A survey for total aflatoxins (aflatoxins B_1, B_2, G_1, and G_2) was conducted on 88 spices and processed spice products commercialized in Korea. The presence of aflatoxins was determined by high-performance liquid chromatography (HPLC) with fluorescence detector using immunoaffinity column clean-up. Total aflatoxins (AFs) are detected in 12 samples (13.6% of incidence) including seven red pepper powder, two red pepper pastes (*Kochujang*), two curry and one ginger product. The contamination levels are 0.08–4.45 μg/kg as aflatoxin B_1 and 0.08–4.66 μg/kg as AFs. The liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis on contaminated samples was conducted for the confirmation of detected aflatoxins. The 12 samples which showed aflatoxins by HPLC/FLD were confirmed as aflatoxins by LC–MS/MS.

Keywords: Aflatoxins; Spices; Immunoaffinity column; HPLC–FLD; LC–MS/MS

1. Introduction

Aflatoxins are a group of toxic metabolites produced by species of *Aspergillus*, specifically *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, which were found worldwide in air and soil (Rustom, 1997). They are a significant threat to both human and animal health, because they are potent carcinogens, mutagens and teratogens (Blesa, Soriano, Moltó, & Mañes, 2004). Aflatoxins are also classified as Group 1 carcinogens by the International Agency of Research on Cancer (IARC), primarily affecting liver (IARC, 2002). Aflatoxins commonly found are aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2). AFB_1 is the most potent of all aflatoxins known to date and is generally found in the highest concentration in food and animal feeds (Lee, Wang, Allan, & Kennedy, 2004).

Aflatoxins in various agricultural products can be contaminated when drying of agricultural commodities is delayed or moisture level exceeds critical values for the mold growth during storage of the crops. Especially, spices are usually produced in countries with tropical climates that have high temperature, humidity and rainfall (Martins, Martins, & Bernardo, 2001). These climatic conditions are favorable to aflatoxin contamination. In recent years, the natural occurrence of aflatoxins in spices has been studied by several researchers (Zinedine et al., 2006; Romagnoli, Menna, Gruppioni, & Bergamini, 2007; Fazekas, Tar, & Kovacs, 2005; Martins et al., 2001; Aydin, Erkan, Baskaya, & Ciftcioglu, 2007).

In Korea, many kinds of spices are commonly used to cook various foods. Especially, Koreans prefer red pepper powder that made of red peppers which are generally dried in the sun and open air to that made of mechanical drying process. So red pepper powder made of natural dried red pepper is more likely to be contaminated with aflatoxins because of insufficient drying process. Red pepper paste

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(*Gochujang*) is manufactured from red pepper powder, so it is likely to be contaminated with aflatoxins.

Aflatoxin analysis on spice is not simple because of interference of high colored materials that are co-extracted with aflatoxins. Current analytical methods for the determination of aflatoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA) (Hu, Zheng, Zhang, & He, 2006). Although several of these methods achieve low levels of detection, in practice they consume large amount of time and solvent, and require one or more clean-up steps involving liquid–liquid partition or solid-phase extraction (Visconti & Pascale, 1998). As a fast screening method, ELISA processes good specificity, sensitivity, and simplicity. However, it has the possibility of false positives because of cross-reaction and interference in the complex matrixes (Hu et al., 2006; Nilüfer & Boyacioğlu, 2002). Several surveys of natural occurrence of aflatoxins were carried out using a monitoring scheme consisting of ELISA for rapid screening and HPLC for quantification (Chun, Kim, Ok, Hwang, & Chung, 2007; Kim et al., 2001; Park, Kim, Shon, & Kim, 2002). However, these researches did not include the kinds of spice samples. Sample with high pigment and lipid content makes the aflatoxins analysis difficult because of serious matrix interference. Therefore, a more selective treatment followed by specific purification is required before the analysis. The immunoaffinity columns (IAC), which contain antibodies specific to aflatoxins, are efficient for the purification of aflatoxins. These provide a quick and simple solution to sample clean-up and solve problems of interference that can be happened in adopting other methods.

In this study, we applied the IAC method to analyze AFs in several spices using HPLC with fluorescence detection coupled with immunoaffinity column clean-up step. We investigated the contamination of AFs in mainly consumed spices commercialized in Korea, and some samples which were contaminated with AFs were confirmed by LC–MS/MS.

2. Materials and method

2.1. Sample collection

A total of 88 spices and processed spice products samples were collected during the period from May to September 2006. The collected samples are made up of six different kinds of spices [red pepper powder (41 samples), red pepper paste (*Gochujang*) (15), curry (20), ginger products (7), black pepper (2), cinnamon powder (3)] commercialized in Korea. These samples were randomly purchased from different markets in ten cities including Gangneung, Wonju, Seoul, Anyang, Daejeon, Cheongju, Gwangju, Sunchang, Daegu, Busan. All samples were ground to pine powder and then until the beginning of the analyses, the ground samples were stored in a freezer (−20 °C).

2.2. Chemicals and materials

The standard stock solutions of AFB_1, AFB_2, AFG_1 and AFG_2 with concentration of 3 mg/kg were purchased from Supelco Chemical Co. Immunoaffinity column (AflaTest P) was supplied by VICAM (Watertown, MA, USA). Acetonitrile and methanol were HPLC grade and purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) and formic acid were obtained from Sigma Chemical Co. All other inorganic chemicals and organic solvents were of reagent grade or higher.

2.3. Sample preparation and immunoaffinity column clean-up

Samples were analyzed with HPLC–fluorescence detector (FLD) according to the annual report of Korea Food and Drug Administration (Lee et al., 2005), briefly described as follows. Each sample was ground into powder then for each powdered sample, 25 g of each sample was extracted with 100 ml methanol: water (70:30, v/v) containing 1% of sodium chloride by mechanical shaker for 20 min. The extract was filtered with filter paper (Whatman No. 1) and 10 ml of filtrate was diluted into 30 ml with deionized water and mixed vigorously. This mixture solution (15 ml) was then passed through an immunoaffinity column at a flow rate of about 3 ml/min (1 drop/s), washed with water (10 ml) at the same flow rate and flushed with air to remove remaining water. Final elution was accomplished by adding acetonitrile (3 ml) onto the column at the same flow rate and flushed with air. The eluate was evaporated to dryness by centrifugal concentrator at 40 °C. The dry residues were derivatized by adding 200 μl of TFA (trifluoroacetic acid), allowed to stand for 15 min at the place of protected from direct UV light, and then diluted with 800 μl of acetonitrile: water (20:80, v/v). This derivatized sample was filtrated with 0.45 μm membrane filter then transferred into HPLC vials for auto injection. Since aflatoxins are subject to light degradation, it must needs to protect analytical work adequately from daylight. Therefore, all the procedures were carried out in subdued light and protected from direct UV light.

2.4. Analysis of aflatoxins by HPLC

Determination of AFB_1, AFB_2, AFG_1 and AFG_2 levels in the derivatized samples was carried out by HPLC equipped with an auto sampler using a fluorescence detector. The HPLC equipment was a Shiseido (SI-2) system with 3023 pump, 3023 autoinjector and fluorescence detector set at 360 nm excitation and 460 nm emission. A Capcellpak C_{18} column (4.6 × 250 mm, 5 μm particle size, Shiseido, Japan) was used. The mobile phase was distilled water: acetonitrile (90:10) and the flow rate was 1 ml/min, injection volume was 20 μl.

2.5. Confirmation of aflatoxins by LC–MS/MS

For confirmation of aflatoxins, LC–MS/MS analysis was carried out using a ultra-performance liquid chromatography (UPLC) ACQUITY™/Micromass Quattro Premier XE API triple-quadrupole mass spectrometer (Waters, Micromass, Manchester, UK). The samples were separated by the ACQUITY UPLC™ BEH C_{18} column (Waters, 2.1 × 50 mm, 1.7 μm particle size), using the mobile phase with aqueous 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile, at a flow rate of 0.3 ml/min. The volume of each sample injected was 10 μl. The electrospray positive ionization (ESI+) source had the following settings: capillary voltage of 3.2 kV, cone voltage of 33–45 V, source temperature of 120 °C, desolvation temperature of 350 °C, cone gas flow rate of 50 l/h, desolvation gas flow rate of 700 l/h with nitrogen. Aflatoxins were determined by multiple reaction monitoring (MRM).

3. Result and discussion

The AFs were analyzed in 88 spices and processed spice products including 41 red pepper powder, 15 red pepper paste (*Gochujang*), 20 curry, seven ginger products, three cinnamon powder and two black pepper. When AFB_1, AFB_2, AFG_1 and AFG_2 were added to red pepper powder, black pepper and curry at the level of 3 μg/kg, the recovery ranges of 74.7–95.3% for AFB_1, 84.9–98.7% for AFB_2, 81.7–103.9% for AFG_1, and 68.1–88.5% for AFG_2 were obtained, respectively. Table 1 summarizes the linearity, limit of detection (LOD) and limit of quantification (LOQ) of the AFs measurements. The linearity was checked for standard solution containing total aflatoxins in range from 0.1 to 10 μg/kg. The squire of the correlation coefficient (R^2) were greater than 0.999. LOD was 0.01 μg/kg for AFB_1 and AFB_2, 0.15 μg/kg for AFG_1 and 0.02 μg/kg for AFG_2, respectively. LOQ was 0.03 μg/kg for AFB_1 and AFB_2, and 0.45 μg/kg for $AFG_1$and 0.06 μg/kg for AFG_2, respectively.

Table 1
Validation of aflatoxin determination by HPLC analysis

Aflatoxins	LOD (μg/kg)[a]	LOQ (μg/kg)[b]	Calibration curve[c]	R^2
Aflatoxin B_1	0.01	0.03	$Y = 466294X - 1391.7$	0.9999
Aflatoxin B_2	0.01	0.03	$Y = 716148X - 3900.2$	0.9998
Aflatoxin G_1	0.15	0.45	$Y = 96242X - 3571.2$	0.9992
Aflatoxin G_2	0.02	0.06	$Y = 266332X - 3193.7$	0.9997

[a] Limit of detection (LOD).
[b] Limit of quantification (LOQ).
[c] X = concentration of aflatoxins (μg/kg) and Y = intensity.

Table 2
Incidence and range of total aflatoxins level in spices and processed spice products

Sample category	Analyzed sample	Positive sample	Range of total aflatoxins[a] (μg/kg)
Red pepper flour	41	7	0.08–4.66
Red pepper paste (Gochujang)	15	2	0.21, 0.55
Curry	20	2	0.13, 0.46
Ginger product	7	1	0.18
Black pepper	2	0	ND
Cinnamon powder	3	0	ND
Total	88	12	0.08–4.66

[a] Total aflatoxins was represented by the summation of aflatoxin B_1, B_2, G_1 and G_2 levels.

Table 3
Mean of total aflatoxin levels and individual aflatoxin levels in positive samples of spices and their products

Category	Mean AFs (μg/kg)	Aflatoxins (μg/kg)				
		AFs	AFB_1	AFB_2	AFG_1	AFG_2
Red pepper flour	0.87 ± 1.68	0.08	0.08	ND	ND	ND
		0.09	0.09	ND	ND	ND
		0.11	0.11	ND	ND	ND
		0.19	0.19	ND	ND	ND
		0.34	0.34	ND	ND	ND
		0.59	0.59	ND	ND	ND
		4.66	4.45	0.21	ND	ND
Red pepper paste (Gochujang)	0.38 ± 0.24	0.21	0.21	ND	ND	ND
		0.55	0.18	ND	0.37	ND
Curry	0.30 ± 0.24	0.13	0.13	ND	ND	ND
		0.46	0.46	ND	ND	ND
Ginger product	0.18	0.18	0.18	ND	ND	ND

The levels of aflatoxins detected in different spices and spice products selected for this study are summarized in Tables 2 and 3. In this study, 12 samples were found contaminated with AFs in the range of 0.08–4.66 μg/kg. AFs contamination was detected in samples of red pepper powder, red pepper paste, curry and ginger products. Seven red pepper powder samples out of 41 red pepper powder samples (17.1% incidence) and two red pepper paste samples out of 15 red pepper paste samples (13.3% incidence) were contaminated with aflatoxins. Black pepper and cinnamon powder were detected below the limit of detection. AFB_1 were detected in all the contaminated samples. These results seem to suggest that AFB_1 is the most frequent one among aflatoxins.

Many researchers reported the levels of aflatoxins in red pepper powder or chilli powder. In Turkey, Aydin et al.

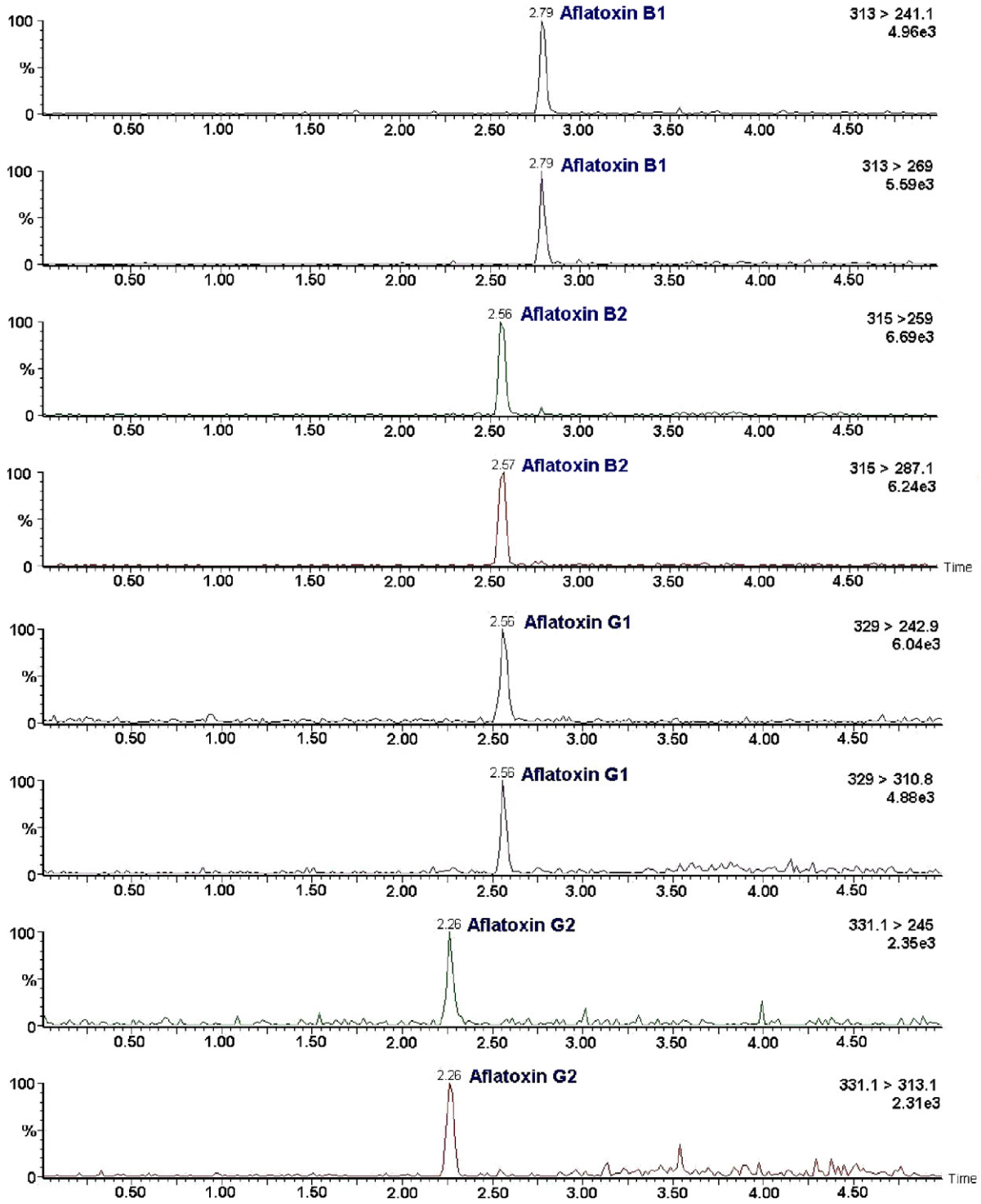

Fig. 1. LC–ESI-MS/MS chromatograms obtained in positive ion mode of aflatoxins standard solution at 1.5 μg/kg.

(2007) reported high levels of AFB_1 contamination in red pepper powder with levels of contamination up to 40.9 μg/kg. In Qatar, Abdulkadar, Al-Ali, Al-Kildi, and Al-Jedah (2004) reported that four samples out of six chilli powder were contaminated with AFs in the range of 5.60–69.28 μg/kg. Romagnoli et al. (2007) found AFs contamination in hot pepper (45.7% incidence) in the range of 0.57–30.7 μg/kg in Italy. In curry powder and ginger products, Martins et al. (2001) reported that curry powders were contaminated with AFB_1 (40% incidence) in the range of 1–5 μg/kg in Portugal. In Morocco, Zinedine et al. (2006) reported that AFB_1 was found in ginger and the average contamination level was 0.63 μg/kg. As a result, the incidence and levels of aflatoxins found in this study were relatively low as compared to the levels quoted in this literature. The incidence of aflatoxins in food is relatively high

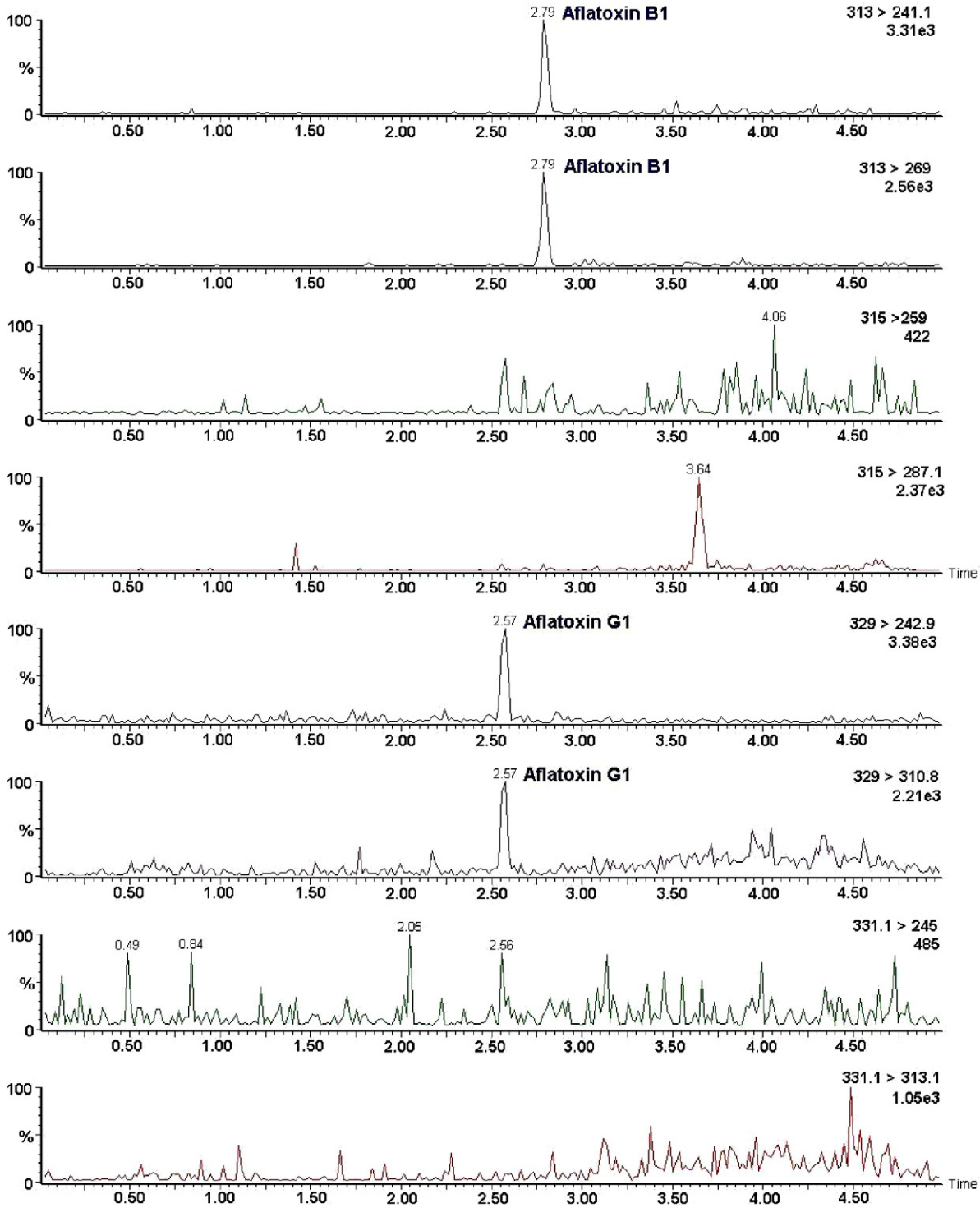

Fig. 2. LC–ESI-MS/MS chromatograms obtained in positive ion mode of red pepper powder sample contaminated with AFB_1 and AFG_1.

in tropical and subtropical regions, where the warm and humid weather provides optimal conditions for the growth of the molds (Rustom, 1997). As mentioned above, most of countries which were reported high levels of aflatoxin contamination in spices have tropical and subtropical climate. On the other hand, Korea has the temperate climate which has lower temperature and humidity than these of tropical climate. Therefore, the fact that incidence and levels of aflatoxins found in this study were relatively low is probably due to the climate of Korea which is less sensitive to AFs contamination than that of other countries reported high levels of aflatoxin contamination.

For confirmation of aflatoxins, LC–MS/MS analysis was carried out. For each aflatoxin, two characteristic fragmentations of the protonated molecular ion $[M + H]^+$ were monitored. For AFB_1, the parent ion is m/z 313, the product ions were m/z 241 and m/z 269, and for AFB_2, the parent ion is m/z 315, the product ions were m/z 259 and m/z 287. For AFG_1, the parent ion is m/z 329, the product ions were m/z 242 and m/z 310, and for AFG_2, the parent ion is m/z 331, the product ions were m/z 245 and m/z 313. The first and most abundant one was used for quantification, while the second one was used as a qualifier. In all MRM transitions, the dwell time was 0.1 s. Fig. 1 shows chromatogram of AFs standard solution level of 1.5 μg/kg. The representative mass chromatogram of red pepper powder sample contaminated with AFB_1 and AFG_1 is shown in Fig. 2.

The residue limit of aflatoxin levels in spices is not established in Korea, but in the European Union, aflatoxin levels in several spices are regulated with maximum residue levels that cannot be greater than 5 μg/kg for AFB_1 and 10 μg/kg for AFs (Commission Regulation (EC) No. 472/2002). In the result of this study 12 samples out of 88 spices and spice products were contaminated with AFs in the range of 0.08–4.66 μg/kg. One red pepper powder contained maximum level of 4.66 μg/kg for AFs and 4.45 μg/kg for AFB_1, these levels are lower than maximum residue levels in European Union. Therefore, it can be concluded that the contaminated levels of the samples such as red pepper powder, red pepper paste, curry and ginger product may not have any risk on public health. However, both red pepper products and other spices are likely to be contaminated with aflatoxins during processing and storage stages. Especially, red pepper powder and red pepper paste are one of the favorite spices and consumed continuously in Korea. AFs contamination in spices could be a serious problem even at low levels. Therefore, the regular monitoring of these spices will be needed continuously in Korea.

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