Aflatoxin M_1 levels in dairy products from South Korea determined by high performance liquid chromatography with fluorescence detection

BO RYUN YOON – SUNG-YONG HONG – SUNG MIN CHO – KYU RI LEE – MYUNGHEE KIM – SOO HYUN CHUNG

Summary

Sensitive methods for determination of aflatoxin M_1 (AFM_1) in milk, yoghurt and cheese were developed and evaluated. The analytical methods use high performance liquid chromatography-fluorescence detection after immunoaffinity column purification. The methods showed high linearity ($r^2 \geq 0.999$) for AFM_1 in the range of 0.05–10.00 $\mu g \cdot l^{-1}$. The limit of quantification for AFM_1 was 0.003 $\mu g \cdot kg^{-1}$, 0.07 $\mu g \cdot kg^{-1}$ and 0.05 $\mu g \cdot kg^{-1}$ in milk, yoghurt and cheese, respectively. The recovery rate of AFM_1 in the dairy products was 83–108% along with 2.1–12.8% repeatability and 0.0–13.1% reproducibility. Levels of AFM_1 were determined in 224 samples of milk, yoghurt and cheese collected from South Korea. The dairy samples were contaminated with AFM_1 in the range of 0.001–0.100 $\mu g \cdot kg^{-1}$ for milk and 0.015–0.136 $\mu g \cdot kg^{-1}$ for yoghurt and cheese. The levels of AFM_1 in the dairy products did not exceed the maximum limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by Korea Food and Drug Administration.

Keywords

aflatoxin M_1; milk; yoghurt; cheese; high performance liquid chromatography; fluorescence detection

Aflatoxins (AFs) are highly toxic secondary metabolites produced by *Aspergillus* spp. such as *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. These fungi can infect agricultural crops including maize, rice, wheat, barley, peanuts and cotton seeds to produce AFs during pre-harvest period and storage [1]. There are four naturally occurring AFs: AFB_1, B_2, G_1, and G_2. Of these, AFB_1 is the most common and toxic carcinogen [2]. AFB_1 is bioactivated to AFB_1-8,9-epoxide by cytochrome P450 (CYP) in liver. This highly reactive metabolite binds to DNA, forming adducts at N_7-guanine residues. This leads to mutations, resulting in hepatotoxicity, teratogenecity and carcinogenicity in humans and animals [3]. Hence, International Agency for Research on Cancer (IARC) of World Health Organization (WHO) categorized AFB_1 as Group 1 human carcinogen.

AFM_1, a 4-hydroxy derivative of AFB_1, is formed from AFB_1 by CYP, such as CYP1A and CYP3A, in liver and is excreted to milk of animals that have been fed with AFB_1-contaminated feeds and feedstuff [4–6]. Approximately 0.3–6.2% of AFB_1 ingested by livestock is biotransformed to AFM_1 depending on the level of AFB_1 contamination in feeds [7, 8]. AFM_1 can be detected in milk within 12 h after the first ingestion of AFB_1. The concentration of AFM_1 in milk decreases to an undetectable level within 72 h after the intake is stopped [9, 10].

Previously some researchers demonstrated that AFM_1 is less mutagenic and carcinogenic than AFB_1 based on lower mutagenic and carcinogenic potencies of AFM_1 in duckling [11], male Fisher rats [12, 13] and trout [14]. However, the cytotoxicity, genotoxicity and carcinogenicity of AFM_1

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are well established by recent studies [15]. Therefore, IARC re-categorized it to Group 1 human carcinogen from Group 2B [16].

AFM_1 shows resistance to thermal processes such as pasteurization and ultra-high-temperature (UHT) treatment, and to mild acidic conditions used in production of milk and various dairy products such as yoghurt, cheese, cream and butter [17–19]. The presence of AFM_1 in milk and dairy products is of great concern in humans, especially in susceptible population such as infants and young children [20].

Potential human exposure to AFM_1 through commercial milk and dairy products has been reported worldwide [9, 21–25]. Thus, many countries have set legal regulations for levels of AFM_1 in milk and milk products. European Commission (EC) has established a maximum allowable level of 0.05 $\mu g \cdot kg^{-1}$ for AFM_1 in liquid milk and dried or processed milk products [26], while US Food and Drug Administration (US FDA) has set the action level of 0.5 $\mu g \cdot kg^{-1}$ for AFM_1 in whole, low fat and skim milk [8, 27]. Korea Food and Drug Administration (KFDA) has set the legal limit of 0.5 $\mu g \cdot kg^{-1}$ for AFM_1 in milk [28].

Several analytical methods including thin layer chromatography (TLC), high performance liquid chromatography (HPLC), enzyme linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS-MS) have been used to determine concentrations of AFs in food [24, 29, 30]. Immunoaffinity column chromatography (IAC) and solid-phase extraction are used as clean-up procedures combined with the analytical methods [31, 32]. Although analytical methods for determination of AFM_1 in milk are established, no convenient and reliable methods have been validated for determination of AFM_1 levels in cheese and yoghurt products [33]. In addition, relatively much data on occurrence of AFM_1 in milk were published in the literature, but very few reports on that of AFM_1 in cheese and yoghurt products have been published worldwide. Moreover, no reports on levels of AFM_1 in cheese have been published in South Korea and the only previous study on levels of AFM_1 in yoghurt samples collected from South Korea reported the levels in samples based on the extraction method using less specific solid-phase cartridges for AFM_1 clean-up [34].

Therefore, this study was aimed to develop and validate precise, sensitive and convenient methods for quantification of AFM_1 by using IAC clean-up and HPLC combined with fluorescence detection (HPLC-FLD) along with LC-MS-MS as a confirmatory method. Further aim was to provide information on levels of AFM_1 in commercial milk, cheese and yoghurt products marketed in South Korea for the public by determination of its levels in dairy products to evaluate safety of the products.

MATERIALS AND METHODS

Chemicals and reagents

The AFM_1 standard was obtained from Supelco (Bellefonte, Pennsylvania, USA). HPLC-grade solvents were purchased from J. T. Baker (Center Valley, Pennsylvania, USA). Immunoaffinity columns (Afla M_1 Test) were purchased from VICAM (Milford, Massachusetts, USA). Ultra-pure water was prepared by Direct-Q system (Millipore; Billerica, Massachusetts, USA).

Samples for method development

An organic milk sample produced by Sul Farm (Pyungchang, Kangwon, South Korea) was used for development of analytical methods. Two-hundred grams of a milk sample was used for the method development. Eighty-five grams of a yoghurt sample produced by Denmark milk (Soowon, Gyunggi, South Korea) was used for development of methods. Also, eighteen grams of a cheese sample produced by Maeil (Gochang, Jeanbook, South Korea) was used for development of analytical methods.

Commercial samples for monitoring levels of AFM_1

One-hundred-eight milk samples, 55 yoghurt samples and 61 cheese samples were collected from supermarkets in South Korea from October 2013 to March 2014. All of the samples were stored at 4 °C until analysis.

Standard solutions

AFM_1 stock solution was prepared at a concentration of 10 $mg \cdot l^{-1}$ in acetonitrile (J. T. Baker). A series of AFM_1 standard solutions (0.05, 0.1, 0.25, 0.5, 1, 2, 5 and 10 $\mu g \cdot l^{-1}$) were prepared freshly using the stock solution diluted with acetonitrile-water (10 : 90, v/v). The determination coefficient (r^2) was determined by regression-correlation analysis using Sigmaplot 8.0 software (Systat Software, San Jose, California, USA). The standard solutions were used to calculate the fluorescence detector response and recovery rates of AFM_1 from the dairy samples.

AFM_1 extraction procedure

Briefly, 500 g of milk samples were homogenized with a blender (Heidolph DIAX 600;

Janke and Kunkel, Staufen, Germany) at 13000 ×*g* for 5 min. Then, the milk samples were filtered through glass microfiber (GF/A) filter paper (Whatman, Maidstone, United Kingdom) and collected in conical tubes. Fifty millilitres of the filtrate were loaded onto Afla M_1 Test IAC.

Twenty-five grams of yoghurt samples were weighed and placed in 100 ml of Erlenmeyer flasks, and 25 ml of the mixture acetonitile-water (60:40, v/v) were added to it. After shaking for 60 min using a Wrist Action Shaker (Burrell Scientific, Pittsburgh, Pennsylvania, USA), the extract was centrifuged at 2500 ×*g* for 20 min at 4 °C. Then, 25 ml of the supernatant were diluted with 75 ml of phosphate buffered saline (PBS, pH 8.4) and the solution was filtered through GF/A filter paper. The filtrate was gathered in a conical tube and 40 ml of the filtrate was loaded onto an Afla M_1 Test IAC.

Five grams of cheese samples were weighed and placed in 100 ml Erlenmeyer flasks. Then, 25 ml of the mixture acetonitile-water (60:40, v/v) was added to it. After sonication for 3 min using a Branson 8510 sonicator (Branson Ultrasonics, Danbury, Connecticut, USA), the extract was centrifuged at 2500 ×*g* for 5 min at 4 °C. Then, 10 ml of the supernatant were diluted with 30 ml of PBS (pH 7.4) and the solution was filtered through GF/A filter paper. The filtrate was collected in a conical tube and 30 ml of the filtrate were loaded onto Afla M_1 Test IAC.

Immunoaffinity column clean-up

Final filtrates were passed through Afla M_1 Test IAC. The column was washed with 20 ml of pure water (except for cheese, for which 10 ml of water were used) until 2–3 ml of air passed through it. AFM_1 was finally eluted with 3 ml of acetonitile-methanol (3:2, v/v). The eluates were evaporated to dryness under a stream of N_2 at 50 °C, and the residues were re-dissolved in 0.5 ml of acetonitrile-water (10:90, v/v). The solution was vortexed for 30 s and filtered through a syringe filter (pore size 0.22 μm; Pall, Port Washington, New York, USA).

Assessment of precision and sensitivity of the analytical method

In order to determine the recovery rate of AFM_1, dairy products were spiked with known concentrations of AFM_1 standard solutions previously diluted in 10% (v/v) acetonitrile to give contents of 0.025, 0.05, 0.1 and 0.5 $\mu g \cdot kg^{-1}$ for milk and 0.1, 0.25, and 0.5 $\mu g \cdot kg^{-1}$ for yoghurt and cheese. Extraction and clean-up of AFM_1 from the spiked samples were performed by the procedures described above. After AFM_1 contents in the samples were analysed by HPLC-FLD, they were expressed as mean ± standard deviation (*SD*).

The precision of the methods was assessed by repeatability (within-day precision) and reproducibility (between-day precision). AFM_1 contents were calculated for the samples spiked with AFM_1 solutions on the same day and on different days, respectively. The repeatability was determined by three consecutive injections of AFM_1 solutions extracted from the spiked samples within a day, and the reproducibility was determined by three injections per day during three days. The within-day and between-day precision were expressed as relative standard deviation (*RSD*) of AFM_1 levels obtained in triplicate.

The accuracy of the methods was evaluated by the recovery rate of AFM_1 obtained from samples fortified with known concentrations of AFM_1 standard solution. The recovery rate (*R*) was calculated by the following equation:

$$R = \frac{c_1}{c_2} \times 100 \quad (1)$$

where c_1 is concentration of AFM_1 obtained from the spiked sample and c_2 is AFM_1 concentration used for spiking the sample

The sensitivity of the methods using HPLC-FLD was determined by limit of detection (*LOD*) and limit of quantification (*LOQ*) for milk, yoghurt and cheese samples. These were calculated as a signal-to-noise (S/N) ratio of 3 and 10, respectively, by using Chromeleon 6.8 Chromatography Data System (Thermo Scientific, Sunnyvale, California, USA).

Analytical apparatus and HPLC conditions

HPLC (Dionex Ultramate 3000; Sunnyvale, California, USA) equipped with a fluorescence detector (FP-1520; Jasco, Easton, Maryland, USA) was used to detect AFM_1. The separation was carried out on a Hypersil GOLD column (4.6 mm × 150 mm, 5 μm particle size; Thermo Scientific) and column temperature was kept at 40 °C. The mobile phase, acetonitrile-methanol-water (17:15:68, v/v/v), was pumped at a flow rate of 0.5 $ml \cdot min^{-1}$, giving a total run time of 15 min. The injection volume of the samples was 50 μl. Determination of AFM_1 was performed at excitation wavelength of 360 nm and emission wavelength of 440 nm.

LC-MS-MS conditions

LC-MS-MS analyses were performed using an Agilent Technologies HPLC 1260 series apparatus (Agilent, Santa Clara, California, USA)

connected to MS-MS detector. Chromatographic separation of AFM_1 was achieved using a Poroshell 120 EC-C18 column (2.1 mm × 100 mm, 2.7 μm particle size; Agilent) with a mobile phase at a flow rate of 0.25 ml·min^{-1}. The mobile phase (A solution) consisted of water containing 0.1% formic acid while another mobile phase (B solution) consisted of acetonitrile containing 0.1% formic acid. A gradient elution program was applied as follows: after B solution linearly increased from 30% at 1.0 min to 65% at 2.0 min, it was held on 65% from 2.0 min to 5.0 min and linearly decreased from 65% at 5.0 min to 30% at 5.2 min. Subsequently, 30% of B solution was held on for 4.8 min for re-equilibration of the column before injection of the next sample, giving a total run time of 10 min. The injection volume was 10 μl. The column and sample temperatures were maintained at 40 °C.

MS-MS was performed using AB Sciex QTRAP mass spectrometer (Applied Biosystems, Foster City, California, USA). Analysis software (Analyst Software, version 1.5; Sciex, Framingham, Massachusetts, USA) was used to control the LC-MS-MS system and to acquire and process data. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) at unit resolution. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 0.34 MPa; auxiliary gas (GS2), 344.7 kPa; curtain gas, 344.2 kPa; capillary temperature, 550 °C; ion spray voltage (IS), 4,500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas.

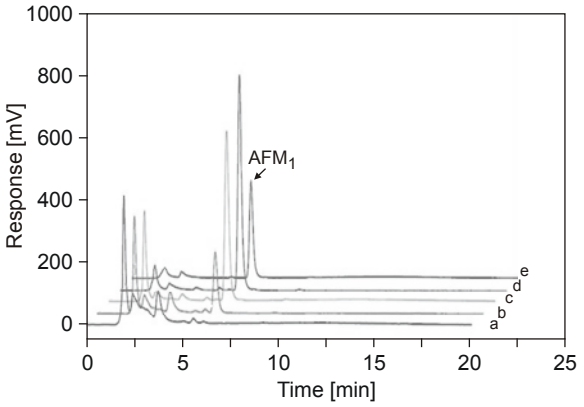

Fig. 1. Chromatogram of AFM_1 in milk spiked with AFM_1 standard solutions using HPLC-FLD.

The retention time of the AFM_1 peak was 6.7 min. A peak of 2.5 μg·l^{-1} of AFM_1 standard is shown as a positive control in the chromatogram.
Concentrations of AFM_1 standard: a – 0 μg·l^{-1}, b – 0.05 μg·l^{-1}, c – 0.1 μg·l^{-1}, d – 0.5 μg·l^{-1}, e – 20-fold dilution of 2.5 μg·l^{-1}.

The precursor-to-product ion transitions were mass/charge (m/z) 329/273 and m/z 329/259 for AFM_1, and collision energy was 29 eV for 329/273 and 329/259.

RESULTS AND DISCUSSION

Method validation

The analytical method using HPLC-FLD was validated for analytical parameters including linearity, sensitivity, accuracy and precision. Preliminary experiments using LC-MS-MS generated unsatisfactory results in measuring low levels of AFM_1 in milk. LC-MS-MS was able to quantify only levels above 0.05 μg·kg^{-1} of AFM_1 (*LOD* of LC-MS-MS, a legal limit of AFM_1 in milk in European Union). Thus, HPLC-FLD was used for determination of levels of AFM_1 in samples.

The choice of a mobile phase in HPLC is very important for a high separation efficiency of AFM_1. The acetonitrile-methanol-water system for the mobile phase is commonly used to separate AFs at a high efficiency [35]. Different ratios of acetonitrile-methanol-water and acetonitrile-water were compared for good separation of AFM_1. The mixture of acetonitrile-methanol-water (17 : 15 : 68, v/v/v) was selected for separation of AFM_1 by HPLC at a flow rate of 0.5 ml·min^{-1} (Fig. 1).

The linearity of determination of a series of AFM_1 concentrations in the analytical method was assessed by a standard curve using eight levels of AFM_1 (0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 5.0 and 10 μg·l^{-1}) dissolved in 10% (v/v) acetonitrile. Each standard solution was injected into HPLC-FLD in triplicate. The calibration curve was constructed by plotting the peak areas (y axis) versus AFM_1 concentrations (x axis) in the HPLC analysis. The linearity was determined by linear regression analysis and expressed as coefficient of determination (r^2). The curve showed r^2 value of 0.9996 (data not shown). Therefore, we concluded that the calibration curve was linear in the range of 0.05–10 μg·l^{-1} of AFM_1 dissolved in 10% acetonitrile (v/v). This range was equivalent to 0.0005–0.1 μg·kg^{-1} of AFM_1 in milk samples, 0.005–1.0 μg·kg^{-1} of AFM_1 in yoghurt and 0.02–4.0 μg·kg^{-1} of AFM_1 in cheese samples.

The extraction procedures of analytes of interest from samples influence the recoveries of the compounds from the specific matrix. Of particular importance is the selection of best solvent mixture for extraction so as to achieve the true values. Therefore, extraction conditions of AFM_1 from milk, cheese and yoghurt were optimized in

Tab. 1. Recovery rates and within- and between-day precision of AFM_1 in milk, cheese and yoghurt.

Sample	Spiking level [$\mu g \cdot kg^{-1}$]	Within-day precision (n = 3)		Between-day precision (n = 3)	
		Recovery rate [%]	*RSD* from repeatability [%]	Recovery rate [%]	*RSD* from reproducibility [%]
Milk	0.025	101.7 ± 0.1	0.1	106.4 ± 10.5	9.9
	0.05	99.2 ± 0.0	0.0	91.2 ± 8.6	9.5
	0.1	108.4 ± 0.4	0.4	96.8 ± 11.3	11.7
	0.5	96.6 ± 3.6	3.7	91.4 ± 3.8	4.1
Cheese	0.1	83.2 ± 10.0	12.0	90.3 ± 12.0	12.8
	0.25	83.7 ± 7.6	9.1	96.9 ± 2.0	2.1
	0.5	98.7 ± 13.0	13.1	89.4 ± 11.0	12.6
Yoghurt	0.1	91.3 ± 5.6	6.2	95.1 ± 4.4	4.6
	0.25	102.2 ± 1.2	1.2	95.7 ± 4.7	4.9
	0.5	106.9 ± 0.6	0.6	98.3 ± 8.2	8.4

Recovery rates values are expressed as mean ± standard deviation, *RSD* – relative standard deviation.

this study. A high percentage of organic solvents, such as acetonitrile, are widely used for extraction of AFs including AFM_1 from samples [36]. For milk samples, they were directly applied onto IAC (Afla M_1 Test) for extraction of AFM_1 from the samples. For cheese and yoghurt samples, a mixture of acetonitrile-water (60 : 40, v/v) was chosen as a solvent for extraction of AFM_1 from the samples since it provided the highest recoveries of AFM_1 from the samples. After AFM_1 extraction, its purification was performed with the same Afla M_1 Test IAC as used for milk.

The accuracy of the methods was evaluated by the recovery rate of AFM_1 recovered from samples fortified with known concentrations of AFM_1 standard solutions and the recovery rate (R) was calculated by the equation described in Materials and methods section Eq. 1. The recovery rates of AFM_1 in milk, cheese and yoghurt are shown in Tab. 1. The values in milk ranged from 91–108% at 0.025, 0.05, 0.1 and 0.5 $\mu g \cdot kg^{-1}$ of the fortified AFM_1 contents. The recovery rates in cheese were in the range of 83–99%, while those in yoghurt were in the range of 91–107% at 0.1, 0.25 and 0.5 $\mu g \cdot kg^{-1}$ of the fortified AFM_1 contents. Overall, the recovery rates in all of the samples were higher than 83%, which is in agreement with the recovery rates (70–110%) recommended by Joint Expert Committee on Food Additives in 2001 [37]. Thus, it was concluded that the analytical methods showed good recoveries from matrices of milk, cheese and yoghurt.

The precision of the methods was assessed by repeatability and reproducibility, as described in Materials and methods section. *RSD* obtained for repeatability and reproducibility tests in milk were in the range of 0.0–3.7% and 4.1–11.7%, respectively. The former and latter parameters in cheese were in the range of 9.1–13.1% and 2.1–12.8%, respectively, while those in yoghurt were in the range of 0.6–6.2% and 4.6–8.4%, respectively. Those values were below the reference levels recommended by European Commision [26]. Hence, we concluded that the analytical methods exhibited good precision in determination of AFM_1 in the samples.

The sensitivity of the methods using HPLC-FLD was determined by *LOD* and *LOQ* as described above. *LOD* and *LOQ* for AFM_1 in milk, yoghurt and cheese are shown in Tab. 2. *LOD* and *LOQ* values for AFM_1 in the samples were as low as those for detection of trace amounts of AFM_1,

Tab. 2. Limit of detection and limit of quantification for AFM1 in milk, yoghurt and cheese.

Sample	Linear equation	r^2	Range [$\mu g \cdot kg^{-1}$]	*LOD* [$\mu g \cdot kg^{-1}$]	*LOQ* [$\mu g \cdot kg^{-1}$]
Milk	$y = 24.7311475954x - 1.2168391376$	0.9994	0.0005–0.1	0.001	0.003
Yoghurt	$y = 29.1586865672x + 1.7402089552$	0.9996	0.005–1.0	0.020	0.070
Cheese	$y = 25.8142620232x - 1.5058043118$	0.9997	0.02–4.0	0.015	0.050

r^2 – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification.

indicating that the method was highly sensitive in determination of AFM_1 in the dairy samples.

Monitoring levels of AFM_1 in samples

The analytical methods validated above were used for determination of AFM_1 in 108 milk, 55 yoghurt and 61 cheese samples collected from local markets in South Korea. Eighty-eight out of 108 milk samples were contaminated with AFM_1 in the range of 0.001–0.100 $\mu g \cdot kg^{-1}$ with 0.023 $\mu g \cdot kg^{-1}$ of the mean content (Tab. 3). In particular, the levels of AFM_1 in organic milk samples were lower than those in general and low-fat milk samples, suggesting that cows grazing on grass produced milk contaminated with lower levels of AFM_1 than those fed with feedstuff. AFM_1 was detected above *LOD* (0.001 $\mu g \cdot kg^{-1}$) in 88 (77%) out of 108 samples in total. Of 88 AFM_1-positive samples, levels of AFM_1 only in nine samples exceeded the legal limit (0.05 $\mu g \cdot kg^{-1}$) set by European Commission, but were below 0.1 $\mu g \cdot kg^{-1}$. In addition, the levels of AFM_1 in all types of milk samples did not exceed 0.1 $\mu g \cdot kg^{-1}$, which were much lower than the maximum limit (0.5 $\mu g \cdot kg^{-1}$) set by KFDA.

Several studies reported on levels of AFM_1 in milk and dairy products. Kamkar reported the levels of AFM_1 to range between 0.015 $\mu g \cdot l^{-1}$ and 0.28 $\mu g \cdot l^{-1}$ in raw milk produced in Iran [29]. A survey from Portugal reported that the levels of AFM_1 in raw and UHT milk were in the range of 0.005–0.061 $\mu g \cdot l^{-1}$ [38]. Lee and co-workers reported that approximately half of raw milk collected from three different regions in South Korea was contaminated with low levels of AFM_1 in the range of 0.002–0.08 $\mu g \cdot l^{-1}$ with the mean level of 0.026 $\mu g \cdot l^{-1}$ [39]. In another study in South Korea, 0.05–0.1 $\mu g \cdot l^{-1}$ of AFM_1 were detected in raw milk [40]. These contamination levels of AFM_1 in raw milk are similar to those in commercial milk products analysed in this study. It indicates that heat treatment, such as UHT, did not reduce levels of AFM_1 in raw milk [21, 35]. Furthermore, commercial milk products collected from markets in other countries including South Korea also contained levels of AFM_1 similar to those in commercial milk products analysed in current study [34, 38, 41–43]. Thus, the analytical methods developed in this study are very sensitive and reliable for determination of AFM_1 in milk.

Fifteen out of 55 yoghurt samples were contaminated with AFM_1 in the range of 0.020–0.15 $\mu g \cdot kg^{-1}$ with the mean level of 0.020 $\mu g \cdot kg^{-1}$ (Tab. 4). AFM_1 was detected above *LOD* (0.02 $\mu g \cdot kg^{-1}$) in 15 (27%) out of 55 samples. The mean content of AFM_1 in positive samples was 0.051 $\mu g \cdot kg^{-1}$. In addition, the levels of AFM_1 in plain yoghurt were similar to those in liquid yoghurt samples, indicating that there was no significant difference between the two different types of yoghurt samples.

Several studies reported on levels of AFM_1 in yoghurt products. Galvano et al. reported that the levels of AFM_1 ranged between 0.001 $\mu g \cdot l^{-1}$ and 0.496 $\mu g \cdot l^{-1}$ in commercial yoghurt produced in Italy, while researchers from Portugal detected 0.045 $\mu g \cdot l^{-1}$ of AFM_1 in yoghurt products [22, 44, 45]. Kim et al. showed that approximately half of yoghurt products collected in South Korea were contaminated with AFM_1 in the range of 0.017–0.124 $\mu g \cdot l^{-1}$ [34]. These contamination levels of AFM_1 in yoghurt produced in South Korea are similar to those in commercial yoghurt products analysed in this study. In the previous study from South Korea, less selective cartridges were used to isolate AFM_1 from yoghurt samples. However, we used very specific immunoaffinity columns containing very specific antibodies against AFM_1 in this study. Although we used more selective immunoaffinity columns to isolate AFM_1 from yoghurt samples, approximately 1/3 of yoghurt samples were contaminated with AFM_1,

Tab. 3. Levels of AFM_1 in milk.

Milk	*n*	AFM_1 [$\mu g \cdot kg^{-1}$]	Number of samples in the range				
			< 0.001 $\mu g \cdot kg^{-1}$ (< *LOD*)	0.001–0.003 $\mu g \cdot kg^{-1}$ (*LOD–LOQ*)	0.003–0.05 $\mu g \cdot kg^{-1}$ (< L_{EC})	0.05–0.1 $\mu g \cdot kg^{-1}$ (> L_{EC})	0.1–0.5 $\mu g \cdot kg^{-1}$ (< L_{KFDA})
Fat-free	6	0.042	0	0	4	2	0
Low-fat	22	0.023	4	0	15	3	0
Organic	13	0.005	2	6	5	0	0
General	67	0.021	14	0	49	4	0
Total	108	0.023	20	6	73	9	0

Levels of AFM_1 represent the mean value of total number of samples.
n – number of samples, *LOD* – limit of detection (0.001 $\mu g \cdot kg^{-1}$), *LOQ* – limit of quantification (0.003 $\mu g \cdot kg^{-1}$), L_{EC} – limit set by European Commission (0.05 $\mu g \cdot kg^{-1}$) [26], L_{KFDA} – limit set by Korea Food and Drug Administration (0.5 $\mu g \cdot kg^{-1}$) [28].

Tab. 4. Levels of AFM_1 in yoghurt.

Yoghurt	*n*	AFM_1 [$\mu g \cdot kg^{-1}$]	Number of samples in the range			Mean of positive samples [$\mu g \cdot kg^{-1}$]
			< 0.02 $\mu g \cdot kg^{-1}$ (< *LOD*)	0.02–0.07 $\mu g \cdot kg^{-1}$ (*LOD–LOQ*)	0.07–0.15 $\mu g \cdot kg^{-1}$ (> *LOQ*)	
Liquid	27	0.021	18	8	1	0.039
Plain	28	0.019	22	4	2	0.062
Total	55	0.020	40	12	3	0.051

Levels of AFM_1 represent the mean value of total number of samples.
n – number of samples, *LOD* – (0.02 $\mu g \cdot kg^{-1}$), *LOQ* – limit of quantification (0.07 $\mu g \cdot kg^{-1}$).

Tab. 5. Levels of AFM_1 in cheese.

Cheese	*n*	AFM_1 [$\mu g \cdot kg^{-1}$]	Number of samples in the range			Mean of positive samples [$\mu g \cdot kg^{-1}$]
			< 0.015 $\mu g \cdot kg^{-1}$ (< *LOD*)	0.015–0.05 $\mu g \cdot kg^{-1}$ (*LOD–LOQ*)	0.05–0.15 $\mu g \cdot kg^{-1}$ (> *LOQ*)	
Local	8	0.042	1	5	2	0.047
Imported	53	0.007	44	8	1	0.026
Total	61	0.025	45	13	3	0.037

Levels of AFM_1 represent the mean value of total number of samples.
n – number of samples, *LOD* – limit of detection (0.015 $\mu g \cdot kg^{-1}$), *LOQ* – limit of quantification (0.05 $\mu g \cdot kg^{-1}$).

which was against our expectation. It may have resulted from more strengthened current safety concern of Korean dairy industries than 15 years ago. In addition, the levels of AFM_1 detected in yoghurt in current study were much lower than those in commercial yoghurts produced in Italy. These results could be explained by higher levels of AFs in cows' feeds used in Italy than those in South Korea. Furthermore, the levels of AFM_1 in all types of yoghurt samples in this study did not exceed 0.15 $\mu g \cdot kg^{-1}$. Although the legal limit is not set for AFM_1 in yoghurt, the levels of AFM_1 in yoghurt were below the maximum limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by KFDA.

Sixteen out of 61 cheese samples were contaminated with AFM_1 in the range of 0.015–0.15 $\mu g \cdot kg^{-1}$ with the mean value of 0.025 $\mu g \cdot kg^{-1}$ (Tab. 5). The mean content of AFM_1 in positive samples was 0.037 $\mu g \cdot kg^{-1}$. In addition, the levels of AFM_1 in local cheese produced in South Korea were much higher than those in imported cheese samples, suggesting that cows' feeds used in South Korea were more likely to be contaminated with AFs than those in other countries. The levels of AFM_1 in all types of cheese samples did not exceed 0.15 $\mu g \cdot kg^{-1}$. Although the legal limit is not set for AFM_1 in cheese, the levels of AFM_1 in cheese were far below the maximum allowable limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by KFDA.

Several studies showed that the contents of AFM_1 in cheese and yoghurt were higher than those in milk because of the association of AFM_1 with casein along with elimination of whey in cheese and yoghurt production [46]. This is consistent with our results, which showed higher levels of AFM_1 in yoghurt and cheese than those in milk. Although the levels of AFM_1 in commercial cheese and yoghurt products were below the maximum allowable limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by KFDA in South Korea, they should be much lowered because an exposure of humans to even low dietary AFM_1 levels could pose a carcinogenic risk to the public. Therefore, it is needed to continue to monitor the levels of AFM_1 in dairy products since susceptible population, such as infants, children and elderly persons, are more sensitive to AFM_1 when they often consume dairy products.

Confirmation of AFM_1 in products by LC-MS-MS

AFM_1 in milk, yoghurt and cheese samples, in which higher levels of AFM_1 than *LOQ* were detected, was further confirmed by liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS). Retention time and fragment ions of AFM_1 (m/z 329/273, m/z 329/259) from LC-MS-MS analysis were used to confirm AFM_1 in positive samples. The ion ratio of m/z 273 and m/z 259 was 2.4 ± 0.28, 2.5 ± 0.35, 2.4 ± 0.15 in milk, yoghurt and cheese, respectively. Also, LC-MS-MS analysis showed that there were no false-positive samples, and that no chromatographic peaks that interfered with that of AFM_1 within 2.1 min of retention time were produced in 88 samples. It indicated that the analytical methods developed in this study were able to

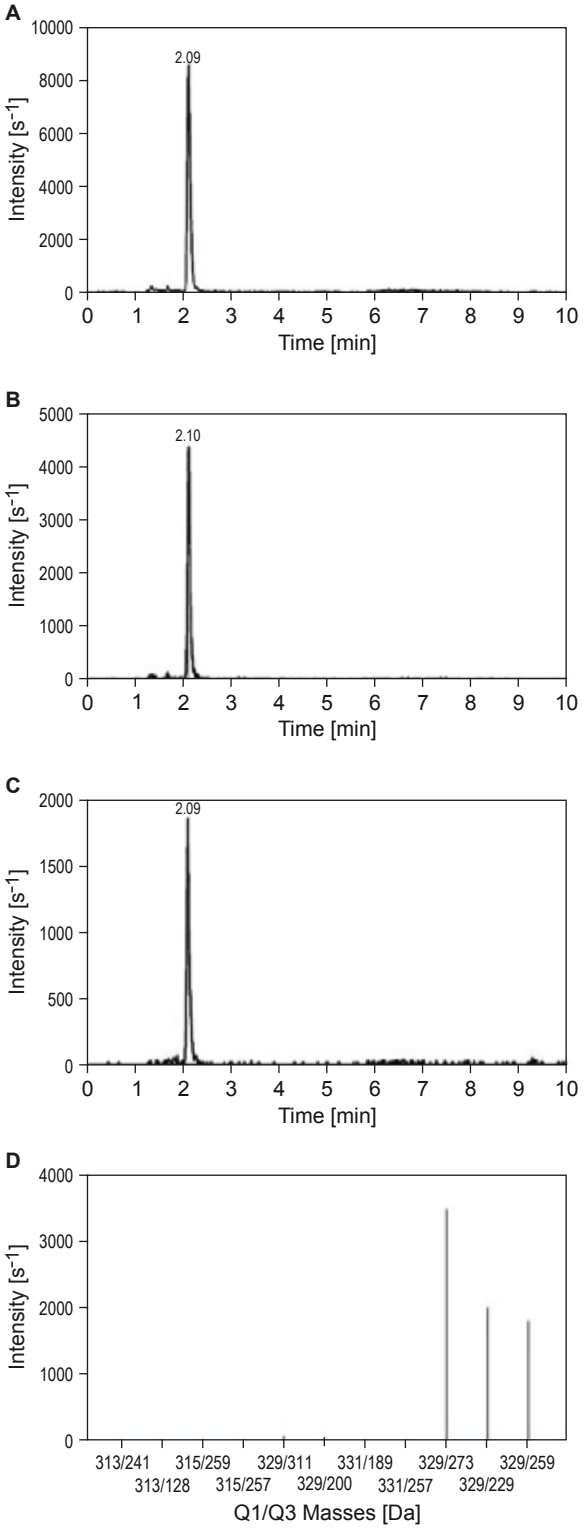

Fig. 2. Typical chromatograms of AFM_1 in milk using LC-MS-MS.

The retention time and fragment ions of AFM_1 (2.1 min; m/z 329/273, m/z 329/259) from LC-MS-MS analysis were used to confirm AFM_1 in the positive milk samples.
A – Total ion chromatogram (TIC), B – Extracted ion chromatogram (XIC) for primary ion (329 > 273), C – Extracted ion chromatogram (XIC) for secondary ion (329 > 259), D – Multiple reactions monitoring (MRM).

minimize interference effects by matrices in the samples, having appropriate sensitivity and specificity for determination of AFM_1 in this type of dairy samples. Typical multiple reactions monitoring (MRM) chromatograms of AFM_1 in a positive milk sample are shown in Fig. 2.

CONCLUSIONS

Highly reliable and sensitive HPLC–FLD methods combined with IAC clean-up were developed in this study for the determination of AFM_1 in milk and milk-based yoghurt and cheese products. The fluorescence detection method using IAC clean-up showed good results in parameters such as specificity, sensitivity, accuracy and precision of the analytical methods for determination of AFM_1 in milk, yoghurt and cheese. The methods were validated for three different matrices at levels as low as those of AFM_1 in milk regulated by European legislation. AFM_1, which was determined in the dairy samples at levels higher than *LOD* by HPLC-FLD, was confirmed by LC-MS-MS.

The legal limit of the level of AFM_1 in milk is set at 0.5 $\mu g \cdot kg^{-1}$ in South Korea and at 0.05 $\mu g \cdot kg^{-1}$ in European Union, but no legal limits on levels of AFM_1 in cheese and yoghurt products are set worldwide yet. The methods developed in this study were successfully applied to determination of AFM_1 in commercial milk, yoghurt and cheese products. The levels of AFM_1 in all types of milk samples did not exceed the maximum limit (0.5 $\mu g \cdot kg^{-1}$) set by KFDA. In addition, the levels of AFM_1 in yoghurt and cheese were also below the maximum allowable limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by Korean legislation.

In conclusion, although levels of AFM_1 in commercial milk, cheese and yoghurt collected from markets in South Korea in our study did not exceed the legal limit (0.5 $\mu g \cdot kg^{-1}$) set for milk by KFDA, more extensive and active research is required for continuous monitoring the levels of AFM_1 in dairy products as well as for the establishment of its legal limits for cheese and yoghurt marketed in South Korea.

Acknowledgements

This work was supported by Milltech, Seoul, South Korea.

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Received 3 February 2016; 1st revised 17 March 2016; accepted 11 April 2016; published online 21 May 2016.