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Determination of Aflatoxin M_1 in Milk and Milk Powder Using High-Flow Solid-Phase Extraction and Liquid Chromatography−Tandem Mass Spectrometry

CHIA-YANG CHEN,* WEN-JIUN LI, AND KAI-YAO PENG

Institute of Environmental Health, College of Public Health, National Taiwan University, 19 Hsu-Cho Road, Taipei City (10055), Taiwan

Animal feeds occasionally have some degree of contamination by *Aspergillus* spp. Even pasteurized milk at times contains the toxic liver carcinogen aflatoxin M_1 (AFM_1). Confirmation of its presence is now done with solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC)−fluorescence, using a small enough sample that SPE time is reasonable. In this study 200 mL of milk was extracted using a C18 disk at a flow rate of ∼100 mL/min and AFM_1 quantified by HPLC−tandem mass spectrometry with negative electrospray ionization. The effectiveness and cleanup efficacy of immunoaffinity columns (IAC) was compared with that of Mycosep multifunctional cleanup columns (MFC). Average recovery and detection limits of whole milk and low-fat milk cleaned up by IAC were significantly superior to those obtained with the MFC (78−87% and 0.59−0.66 ng/L, respectively). The new procedure improves extraction speed, sensitivity, and specificity.

KEYWORDS: Immunoaffinity column; multifunctional cleanup column; certified reference material; LC-MS/MS; aflatoxin M_1; solid-phase extraction

INTRODUCTION

Aflatoxins are typically found as secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* (*1*). Aflatoxins frequently contaminate cereal crops, such as corn, beans, peanuts, and dried fruit (*2*). Aflatoxin B_1 (AFB_1) has the highest toxicity. Epidemiological studies have shown that with prolonged exposure to AFB_1 liver cancer may develop, especially in persons with hepatitis B antigens (*3*, *4*). Consequently, the World Health Organization (WHO) classifies AFB_1 as a human carcinogen and proposes no safe dose (*5*). The major metabolite of AFB_1 is aflatoxin M_1 (AFM_1), which is detectable in the urine, blood, milk, and internal organs of animals ingesting AFB_1-containing feed (*6*, *7*). Concentrations of AFM_1 that show up in milk are ∼0.5−5% of ingested AFB_1 (*8*). Therefore, cows could excrete milk with up to 0.05 μg/L of AFM_1 if their daily intake of AFB_1 reaches 70 μg (*2*).

Although AFM_1 is less carcinogenic than AFB_1 (2−10% of potency), it is also a health danger. It has comparable liver toxicity, can reduce the immunity of infants, and is considered to be a possible human carcinogen (2B) by the International Agency for Research on Cancer (IARC) (*2*, *3*, *9*−*14*). The molecular structures of AFB_1 and AFM_1 are presented in **Figure 1**.

Because AFM_1 can survive pasteurization, there is a concern about exposure to it through milk or dairy products, especially for infants fed with breast milk or milk formula, as they are typically more susceptible to chemicals (*12*, *15*−*17*). In Taiwan and the United States regulations keep AFM_1 levels below 0.5 μg/L, and in Taiwan it should be nondetectable in baby food using standard methods. Since 1999 the European Union (EU), however, tolerates maximum levels of only 0.05 μg/L and 0.025 μg/kg in infant formulas and special dietary foods for medical purposes. They are considering further general reduction to 0.01 μg/kg (*18*−*20*).

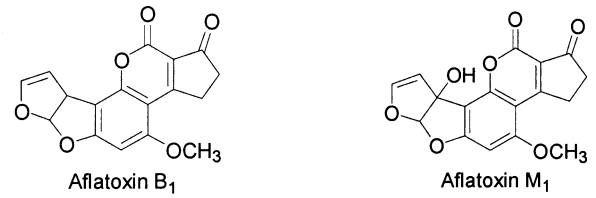

Figure 1. Molecular structures of aflatoxins B_1 and M_1.

Currently, milk samples are screened to detect the presence of AFM_1 using enzyme-linked immunosorbent assay (ELISA) with sensitivities as low as 0.01 μg/L (*21*, *22*). ELISA is not fully reliable due to cross-reaction interferences, especially at concentrations of below 0.05 μg/L (*23*). Recently, Magliulo and co-workers reported a more specific chemiluminescent assay reaching 0.001 μg/L (*24*). Another new screening assay using a headspace sensor array obtains results comparable to those of ELISA (*25*). For legal purposes, positive results from screening assays require unequivocal confirmation of the AFM_1. These are intensive, in both time and labor. Milk samples are defatted and extracted using chloroform or adsorbents, including C18 or AFM_1 immunoaffinity columns (IAC). They are then

* Corresponding author (telephone +886 2 2351 6478, ext. 47; fax +886 2 2351 9557; e-mail dbms@ntu.edu.tw).

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cleaned up by silica, IAC, or C18, and the analyte is separated using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC). It is then quantified with a fluorescence detector (FLD) (*17*, *22*, *26*−*30*). These methods require derivatization of AFM_1 with trifluoroacetic acid to enhance its fluorescence, and detection limits (LOD) (0.01−0.3 μg/L) are close to or higher than the regulating level of the EU. Recently, using a FLD, Lin and colleagues (*31*) obtained detection limits of 0.002 μg/L in milk and 0.02 μg/kg in milk powder without derivatization; Manetta et al. (*32*) announced a postcolumn derivatization with pyridinium hydrobromide perbromide and lowered the detection limit to 0.001 μg/L in milk.

For convenience, existing methods usually use 20−50 mL of sample. This avoids excessive use of chlorinated solvent in liquid−liquid extraction and saves time passing milk through cartridges in solid-phase extraction (SPE). Multifunctional cleanup columns (MFC) have also been successfully applied to the cleanup of aflatoxins B_1, B_2, G_1, and G_2 when analyzed by LC-FLD. MFC entrap matrix materials of cereal extracts but let the analytes pass through. This is simpler, quicker, and more stable than IAC (*33*, *34*).

AFM_1 and the other four aflatoxins have structural similarities. Although it is possible to apply MFC for AFM_1 cleanup, there have been no reports on this. Furthermore, MFC are also not set up for the combination of LC with mass spectrometry (MS), which is characterized as highly selective. The first analysis of AFM_1 using MS was by Plattner et al. (*35*), who compared the AFM_1 sensitivity of three ionization modes by direct injection of an AFM_1 standard onto a triple-quadrupole MS. There appear to be only two studies using tandem MS (MS/MS) for detecting AFM_1 in dairy products. Sørensen's (*36*) and Kokkonen's (*37*) groups extracted AFM_1 from milk and cheese, respectively, using the mixture of hexane and acetonitrile; Sørensen and Elbæk (*36*) further cleaned the extracts by Oasis HLB SPE cartridges. The detection limits of AFM_1 in milk and cheese were 0.01 μg/L and 0.3 μg/kg, respectively (*36*, *37*). However, by their sample preparation procedures these two methods did not eliminate matrix effects and must use matrix standards for calibration. Blank matrixes free of the analyte are not easy to obtain, and their storage time is short. As a result, there would be considerable practical benefit from further work on sample preparation and methods for separation prior to MS.

We here report an extremely sensitive LC-MS/MS method for detecting AFM_1 in milk and milk powder and contrasted the cleanup efficiencies of IAC and MFC. Our method was validated using certified reference materials BCR-282, ERM-BD283, ERM-BD284, and BCR-282 spiked samples.

MATERIALS AND METHODS

Materials. Standard solutions of aflatoxin M_1 (10 μg/mL in acetonitrile) and dimethyldichlorosilane (DMDCS) were purchased from Supelco (Belletonte, PA), aflatoxin B_1 powder was from Sigma (St. Louis, MO), and 4-methylmorpholine (>99.5%) was from Aldrich (St. Louis, MO). We imported BCR-282 (AFM_1 certified <0.05 μg/kg, probable range of 0.01−0.02 μg/kg), ERM-BD283 (certified at 0.111 ± 0.018 μg/kg), and ERM-BD284 (certified at 0.44 ± 0.06 μg/kg) certified reference whole milk powder from Fluka (Buchs, Switzerland). Formic acid (88%) and Bakerbond Speedisk cartridges (50 mg) and disks (50 mm i.d.) (including C18 and PolarPlus C18 with ≈4% higher carbon content and no end-cap) were purchased from J. T. Baker (Phillipsburg, NJ). Immunoaffinity columns (IAC) Afla M_1 (capacity ≈ 150 ng; stored at 4 °C until use) and Mycosep 226 multifunctional cleanup columns (MFC) (stored at room temperature) were acquired from Vicam (Watertown, MA) and Romer Labs (Union, MO), respectively. A Betabasic C18 silica column (150 × 2.1 mm i.d., 3 μm) with a guard column in the same packing material (10 × 2.1 mm i.d., 3 μm) was from Thermo Hypersil-Keystone (Reno, NV). Whole milk (K brand, produced in Taiwan), low-fat milk (T brand, produced in Taiwan), and milk powder (F brand, imported from New Zealand) for development of the methodology were purchased from supermarkets in Taipei City (detected AFM_1 2.8−3.5 ng/L in milk and 47 ng/kg in milk powder). All solvents were of HPLC grade (J. T. Baker). The AFM_1 working standards were diluted from the purchased stock solution in acetonitrile.

Sample Extraction. We used either 20 g of milk powder dissolved in 200 mL of deionized water (40 °C for instantly dissolved milk and 60 °C for the BCR 282) or 200 mL of liquid milk. When protocol called for it, we spiked the milk with AFM_1 standard, stirring for 20 min before centrifuging (4000 rpm, 15 min). Floating lipids and condensed solids at the bottom were discarded. The milk was heated to 70 °C and was extracted with activated 50-mm Speedisk C18 (washed sequentially before use with 10 mL of acetonitrile, methanol, and deionized water) at a flow rate of ∼100 mL/min on a Diskmate II rotary extraction station (J. T. Baker), able to extract six samples simultaneously. After washing with 10 mL of deionized water, the disks were moved to a vacuum manifold (J. T. Baker) and dried (5 min, 20-in. mercury vacuum) and then eluted with two portions of 5 mL of acetonitrile.

Sample Cleanup. The eluate was cleaned up using either IAC or MFC. With IAC, disk eluate was concentrated to 0.5−1.0 mL at 45 °C with a Thermo Savant SpeedVac SPD 1010 (Holbrook, NY), and then deionized water was added to bring the final volume to 15 mL. The acetonitrile fraction was then smaller than 7%, the upper limit that IAC can tolerate (*38*). The solution was gravity fed through the column, and an additional 2 mL of deionized water was used to wash the column. After forcing extra water out of the column with a syringe, the IAC was eluted twice with 2 mL of acetonitrile/methanol (3:2, v/v) and the eluate was concentrated to 300 μL using the SpeedVac.

With MFC, the eluate from the disk was concentrated to 8.6 mL, and deionized water was added to bring the final volume to 10 mL. This brought the acetonitrile/water mixture (86:14, v/v) to the ratio recommended by the manufacturer. The solution was then transferred to a test tube, and the MFC was pushed into the tube to force the extract upward through the column. The supernatant (∼7 mL) was filtered with a syringe PTFE filter (pore size = 0.2 μm) and was concentrated to 300 μL using the SpeedVac.

Liquid Chromatography−Tandem Mass Spectrometry. Fifty microliters of the concentrate was analyzed using a Hitachi L-7100 pump (Tokyo, Japan) coupled to a Thermo Finnigan TSQ 7000 triple-quadrupole mass spectrometer (Finnigan Mat, San Jose, CA) with negative electrospray ionization (ESI) and selected reaction monitoring (SRM) mode. Separation was performed on a 100 × 2.1 mm i.d., 3 μm, PRP-1 poly(styrene-divinylbenzene, SDB) column with a 25 × 2.3 mm i.d., 12−20 μm, guard column of the same material (Hamilton, Reno, NV). Temperature was kept at 40 °C using an ECO-1 column oven (Analab, Taipei, Taiwan). The mobile phase was 10 mM 4-methylmorpholine aqueous solution (pH 9.7)/acetonitrile. Initially, the organic portion was 20%, linearly changing to 100% within 6 min, where it was kept for 6.5 min. The system was re-equilibrated for 14 min before subsequent use. The flow rate was kept at 0.2 mL/min with no split. The monitored precursor ion and the product ion were m/z 327 (i.e., $[M - H]^-$) and 312, respectively. The collision gas and energy conditions were argon and 22 V, respectively. Tube lens, capillary offset, lens 1-1, and collision energy were tuned to optimize the transition of the ions using 10 ng/μL AFM_1 solution in acetonitrile at 20 μL/min mixed with the same flow rate as the aqueous mobile phase. Nitrogen was used as the sheath and auxiliary gas at 70 and 20 psi, respectively. The retention time of AFM_1 was 9.3 min.

QA/QC, Quantitation, and Data Analysis. Glassware was soaked in 2 M sulfuric acid aqueous solution for a day before use to remove possible active adsorption sites for AFM_1. Used glassware that contacted the AFM_1, standards, or samples was soaked in aqueous NaOCl to destroy AFM_1 residue before cleaning and reuse. Plastics and vials were disposed of after use. To deactivate the surface, glass tubes and vials were silylated with 7% DMDCS in toluene (v/v) and were rinsed twice with toluene and methanol in sequence. A deionized water sample and

a blank milk or milk powder sample (from the same bottle used for spiking) were run with each batch of samples to check experimental contamination and existing residue levels. An external calibration curve was made at each analysis using seven points of AFM_1 standard solutions in acetonitrile/water (1:4, v/v) between 0.4 and 600 pg/μL. If detected, concentrations in milk blanks were deducted in the calculation of the recoveries of spiked samples. Concentrations in MFC-cleaned samples were adjusted by multiplying by 10 mL and dividing by the volume (in mL) of the supernatant. LOD and on-column detection limits were defined with the signal-to-noise (S/N) ratio at 3.

To determine the LODs in milk, 3 was divided by the S/N ratios of four replicate milk samples spiked at 25 ng/L (half of the EU limit) and then multiplied by the spiked plus detected blank concentration. The LOD in milk powder was calculated the same way with four replicates of nonspiked BCR-282 milk powder using the middle level of probable range in BCR-282 (0.015 μg/kg). The MDL was the LOD at 99% confidence level and was estimated using the following equation assuming that the LODs distributed as a normal distribution (*39*):

$$\mathrm{MDL} = \bar{X} + \frac{t_{n-1,0.99} \times \mathrm{SD}}{\sqrt{n}}$$

where $\bar{X}$ and SD are the mean and standard deviation of LODs of replicated samples, respectively; $t_{n-1,0.99}$ is the t value at the 99% of t-distribution, and n is the number of tested samples. Data were acquired and analyzed using Finnigan Xcalibur Home Page v 1.1 and Microsoft Excel 2002. Two-tailed Student's t test was used to compare the differences in recoveries and detection limits.

RESULTS AND DISCUSSION

Optimization of the Instrumental Analysis. In ESI, mobile phase pH is critical to signal intensity. We tested 10 mM formic acid (pH 2.9) and 10 mM 4-methylmorpholine aqueous solution (pH 9.7) for positive and negative ESI, respectively, by injecting 30 ng of AFM_1 onto the SDB column in the selected ion monitoring (SIM) mode. The $[M - H]^-$ (*m*/*z* 327) ion was better than that of the $[M + H]^+$ (*m*/*z* 329) ion not only in peak shape but also in the S/N ratio (≈6.7-fold higher). The higher S/N ratio of the negative ion could be explained by the weak acidity of AFM_1 (*40*). Aflatoxin signals from negative ions produced by chemical ionization can be ~100 times higher than those of protonated ions similarly produced or molecular ions created by electron ionization (*35*). We further searched a product ion of *m*/*z* 327 with stable and strong signals and its best collision energy, which was *m*/*z* 312 at 22 V. In contrast, Sørensen and Elbæk (*36*) chose the $[M + H]^+$ ion of AFM_1 for the precursor ion using a mobile phase of water/methanol mixture acidified with 0.02% acetic acid and a postcolumn reagent of 0.1 mM ammonium acetate in 80% methanol. Kokkonen et al. (*37*) also selected positive ESI using a water/acetonitrile gradient containing 0.1% acetic acid. However, they did not report negative ESI for comparison.

On the basis of the retention time, peak shape, and S/N ratio, the best chromatographic gradient for AFM_1 was 20% organic increased to 100% in 6 min and then kept for 6.5 min at a temperature of 40 °C. The AFM_1 retention on the SDB column was compared with that on a Betabasic C18 silica column using a 6-minute gradient from 20% organic phase to 100%. The silica column AFM_1 peak was much broader than that of the SDB column. Also on the SDB column, we investigated factors such as the initial organic percentage (15−30% at 5% interval), the gradient (20−100% organic within 6−12 min at 2-min intervals), and the chromatographic temperature (40 or 50 °C).

Sample Extraction. Extraction methods for AFM_1 were compared using Speedisk C18 and Speedisk PolarPlus C18. Both cartridges retained AFM_1 well at 99.3 ± 0.60 and 98.9 ± 0.50% (mean ± range, $n = 2$), respectively, when using 500 μL of 0.2 μg/mL AFM_1 in deionized water. PolarPlus C18 elution using acetonitrile was more difficult. Subsequent evaluation was done with Speedisk C18 alone. One liter of 0.1 μg/L AFM_1 in deionized water was passed through, and the acetonitrile elution volume was compared (10 vs 15 mL). The recoveries were 83.6 ± 4.2 and 84.4 ± 4.2%, correspondingly (mean ± range, $n = 2$). Acetonitrile elution at 10 mL was as effective as that at 15 mL.

Filtration was not critical to our procedure. Milk sample filtration before extraction is widely used, although filters frequently clog and must be replaced (*27*, *29*−*31*). Extracting milk at room temperature using the C18 disk was extremely slow even though we prefiltered samples with two layers of glass fiber filters (pore sizes = 20−25 and 1 μm, respectively). When heated to 70 °C, however, 250 mL of milk passed through the disk within 2 min regardless of filtration.

Defatting the milk improved retention and reduced breakthrough. Breakthrough of the adsorbent was checked with a whole milk sample spiked at 0.17 μg/L of AFM_1. Up to 500 mL of milk could pass through the C18 disk quickly without clogging. The fractions that were not retained were 4.1 ± 1.0, 19.7 ± 0.70, and 38.4 ± 4.8% in the first 200 mL, the second 200 mL, and the last 100 mL, individually (mean ± range, $n = 2$). Total recovery was 41.7 ± 1.7% after the IAC cleanup. To improve the retention, we defatted the milk, and the breakthrough of 200-mL samples was reduced to 1.71 ± 0.40% (mean ± range, $n = 2$). The Speedisk particle size was much smaller than a usual SPE adsorbent (10 vs 50−60 μm) and provided a much larger surface area to attain quick equilibrium of an analyte between the adsorbent and the matrix. Although the high temperature of 70 °C may reduce adsorption of AFM_1, breakthrough was negligible when sample volume was limited to 200 mL. Further studies might extract milk at lower temperature, which could increase the breakthrough volume without significantly decreasing the flow rate.

Sample Cleanup. Checking the elution efficacy following the IAC manufacturer's suggested protocol, we found that 4 mL of acetonitrile/methanol (3:2) was sufficient to elute most trapped AFM_1. After passing through IAC, it was eluted three times with 1.25 mL of the solution. The recoveries in each portion were 74.2 ± 0.49, 16.7 ± 0.65, and 0.89 ± 0.060%, respectively, for a total of 91.8 ± 0.27% (mean ± range, $n = 2$). We confirmed the combination of acetonitrile/water (86:14) for cleanup in MFC. We spiked 100 ng of AFM_1 into the solution and passed through MFC; the recoveries were 108 ± 7.9% (mean ± SD, $n = 3$).

A higher concentration factor enables a larger fraction of the extract to be analyzed and usually improves the sensitivity. We concentrated 1.25 mL of 16 ng/mL AFM_1 in acetonitrile/methanol (3:2) to almost dryness or to 300 μL using the SpeedVac, and the recoveries were 3.38 ± 0.22 and 92.7 ± 0.04%, respectively (mean ± range, $n = 2$). The final volume of residue was chosen at 300 μL, and 50 μL could be injected without influencing the chromatography.

The milk fat content did not influence recovery. Recoveries of spiked whole milk and low-fat milk (both at 0.025 μg/L) cleaned up by IAC were 78.2 ± 7.3 and 86.6 ± 4.1%, respectively (mean ± SD, $n = 4$; **Table 1**). Although recoveries of IAC extraction or cleanup may vary (usually 70−95%) with manufacturer or even between different batches (*41*−*43*), our recoveries were comparable with previous work using the same IAC product (83.3−89.5%) (*31*). Our RSDs from milk (4.7−9.3%) were equivalent to or better than the intralaboratory results

Table 1. Recoveries in Different Matrixes Cleaned up by Immunoaffinity Columns (IAC) or Multifunctional Cleanup Columns (MFC) [Mean ± SD (RSD), $n = 4$]

matrix	spiked (s) or certified (c) level	cleaned up by IAC (%)	cleaned up by MFC (%)
whole milk	0.025 μg/L (s)	78.2 ± 7.3 (9.3%)	6.51 ± 0.28 (4.3%)[a]
low-fat milk	0.025 μg/L (s)	86.6 ± 4.1 (4.7%)	15.8 ± 4.6 (29%)
BCR-282	<0.05 μg/kg (c)	0.0238 ± 0.0048 μg/kg	0.0532 ± 0.0173 μg/kg
ERM-BD283	0.111 ± 0.018 μg/kg (c)	0.109 ± 0.017 μg/kg 98.6 ± 15.4 (15.6%)[b]	
ERM-BD284	0.44 ± 0.06 μg/kg (c)	0.485 ± 0.040 μg/kg 110 ± 9.1 (8.3%)[b]	
BCR-282 milk powder	1.0 μg/kg (s)	72.4 ± 6.5 (9.0%)	33.5 ± 12.2 (36%)
BCR-282 milk powder	8.0 μg/kg (s)	62.7 ± 8.5 (14%)	31.0 ± 7.7 (25%)

[a] $n = 3$. [b] Recoveries were calculated on the basis of 0.111 and 0.44 μg/kg, respectively.

of two round-robin tests using AOAC 2000.08 and 986.16 standard methods (8−18% and average 28%, respectively) (*44*, *45*). Recoveries of the spiked whole milk and low-fat milk samples by MFC cleanup were 6.51 ± 0.28 and 15.8 ± 4.6%, individually (**Table 1**), which were much lower than those of IAC cleanup. The AFM_1 recovery was the same in the two forms of milk whether cleaned by IAC or MFC (*p* values were 0.11 and 0.074, respectively).

MFC recovery levels were much lower than IAC recovery levels, whether milk was spiked above or below the U.S. and Taiwanese regulation level (5 μg/kg). We used BCR-282 milk powder spiked at 1.0 or 8.0 μg/kg. The IAC recoveries were 72.4 ± 6.5 and 62.7 ± 8.5%, correspondingly (mean ± SD, $n = 4$), at these two levels. MFC recoveries were 33.5 ± 12.2 and 31.0 ± 7.7%, respectively (**Table 1**). When sample concentrations increase, recoveries are usually higher. This is because both the relative percentage of analyte loss during sample preparation is less and analytical variations are smaller. However, the average recovery and RSD of IAC cleanup samples at 8 μg/kg were 10% lower and 4.6% higher than those at 1 μg/kg, respectively, although statistically identical. The spiked amount at 8 μg/kg was 160 ng of AFM_1, which was slightly greater than IAC column capacity (150 ng). This explains the lower recovery and a larger RSD. Recoveries of AFM_1 using MFC cleanup were dramatically lower with high RSDs in real samples than those in deionized water. After cleanup, real sample solutions were white-yellow. Additional filtration of the solutions using a syringe filter of 0.2 μm pore size was needed to prevent the HPLC system from blockage. The background noise in the MS/MS was also higher than with IAC cleanup. Thus, AFM_1 passed MFC without difficulties, but MFC failed to entrap most of the milk components like it did for cereal extracts.

Evaluation of Matrix Effect. There was no significant matrix effect with IAC cleanup. ESI is subject to the matrix effect, which may suppress signals significantly (*46*, *47*). The deep drop in recoveries by MFC for real samples would result from signal suppression. To clarify the issue, we re-injected milk powder samples spiked at the 1.0 μg/kg level cleaned up by IAC or MFC at original condition, with 10× dilutions of the samples and using a shorter column (50 mm) with the same packing material and column i.d. Compared to recoveries at the original condition, MFC recoveries using a shorter column decreased 33.6 ± 7.3% (mean ± SD, $n = 3$), but those of the diluted MFC samples increased 3.04 ± 0.38 times. Consequently, there was significant ion suppression for MFC-cleanup samples. With IAC-cleanup samples, recoveries of the diluted samples or analyzed using a shorter column were 1.11 ± 0.17 and 0.94 ± 0.06 times (mean ± SD, $n = 3$) compared to those analyzed at the original condition.

Use of a stable-isotope-labeled analyte as an internal standard (IS) can overcome the matrix effect usually encountered in LC-MS(/MS). It can also correct the variations in sample preparation and instrumental analysis if added at the beginning of sample handling. Because there is no commercially available isotopically labeled AFM_1, we attempted to synthesize deuterium- or ^{18}O-labeled aflatoxins but did not succeed. Three methods based on previous reports were used for deuterium−hydrogen exchange (*48*−*50*). These reactions required basic conditions at 65−80 °C for 16−40 h. No matter which approaches were used, there was no aflatoxin detected in the reacted solutions, but several new peaks did appear on the liquid chromatograms. Because aflatoxins are subject to ammoniation (*51*), the six-membered ring of the lactone on the molecule could be opened rapidly in the basic reaction solutions and form other products. In contrast, we did replace one or two ^{16}O with ^{18}O on AFM_1 using the process of Leis et al. (*52*), but the reaction was not complete after 4 h. The relative signal intensity of unlabeled AFM_1 was 34% of $^{18}O_1$-AFM_1 and was 44% of $^{18}O_2$-AFM_1. The $^{18}O-^{16}O$ exchange did not go further even if the reaction time was extended to 8 h.

AFB_1 was unsuitable for an IS of AFM_1, and vice versa, at least for the milk matrix. The impact of matrix effects is clearly different between AFM_1 and AFB_1. Another IS trial used a fixed amount of AFB_1 (20 pg/μL) in calibration standards. The best precursor ion, product ion, and collision energy of AFB_1 by negative ESI were *m*/*z* 311, 296, and 20 V, respectively. The monitored product ions of both AFB_1 and AFM_1 were 15 amu less than their precursor ions and formed at low collision energy. This may result from loss of a methyl group. Nevertheless, the r^2 of calibration curves was only 0.970, not reaching external standards (≥0.995). Moreover, the signal intensity of AFB_1 did not show the same tendency in real samples as that of AFM_1, and quantitation precision deteriorated (data not shown). The retention time of AFB_1 was ∼2 min longer than with AFM_1 (**Figure 2**). Vahl and Jørgensen quantified four aflatoxins (including B_1) in food by LC-MS/MS with atmospheric pressure chemical ionization (APCI) using AFM_1 as the IS and reported a similar problem for spice samples (*53*).

Quantitation and Sensitivity. The LODs of AFM_1 for whole milk and low-fat milk using IAC cleanup were 0.59 ± 0.19 and 0.66 ± 0.12 ng/L, respectively (mean ± SD, $n = 4$) (**Table 2**); those cleaned up by MFC were 14 ± 2.6 and 9.2 ± 3.3 ng/L, respectively. The MDLs of AFM_1 using IAC steps were 25- and 18-fold lower than those using MFC for whole milk and low-fat milk samples, respectively. Obviously, using IAC

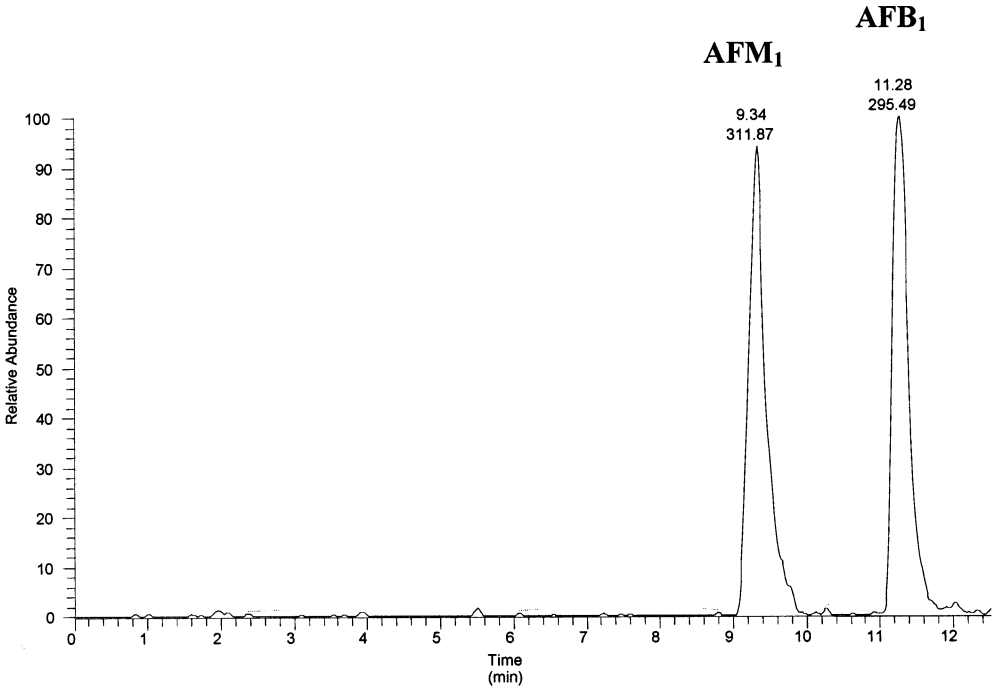

Figure 2. Chromatogram of 50 μL injected AFM_1 (10 pg/μL) and AFB_1 (20 pg/μL) standards at SRM mode.

Table 2. Limit of Detection (LOD) and LOD at 99% Confidence Level (MDL)[a] in Different Matrixes Cleaned up by Immunoaffinity Columns (IAC) or Multifunctional Cleanup Columns (MFC) (Mean ± SD, $n = 4$)

	cleaned up by IAC		cleaned up by MFC	
matrix	LOD	MDL	LOD	MDL
whole milk	0.59 ± 0.19 ng/L	1.0 ng/L	14 ± 2.6 ng/L[b]	25 ng/L
low-fat milk	0.66 ± 0.12 ng/L	0.94 ng/L	9.2 ± 3.3 ng/L	17 ng/L
BCR-282 milk powder	8.5 ± 0.50 ng/kg	9.6 ng/kg	8.2 ± 2.2 ng/kg	13 ng/kg

[a] Mean + $t_{n-1,0.99} \times SD/\sqrt{n}$. [b] $n = 3$.

cleanup was more sensitive than using MFC for milk samples. Regarding the LODs between the two matrixes, there were no significant differences either by IAC ($p = 0.55$) or by MFC ($p = 0.083$). The LODs of AFM_1 for nonspiked BCR-282 milk powder were 8.5 ± 0.50 and 8.2 ± 2.2 ng/kg, respectively, for IAC and MFC (mean ± SD, $n = 4$) (**Table 2**), and there were no statistical differences between these two procedures ($p = 0.83$). However, the variation in the MFC LODs was much higher (1.4 times) than that for IAC. In summary, the IAC cleanup provided 14−24 times lower detection limits than the MFC did for liquid milk samples and was more precise for milk powder.

The lowest level of detection was with IAC cleanup. The other study of MS/MS using another cleanup approach was less sensitive by a factor of 17, and other methods using FLD with or without derivatization were less sensitive by a factor of from 2 to 500. The MDL was ≤1.0 ng/L, which indicated that once AFM_1 in milk was above 1 ppt, this method could detect it with 99% probability regardless of variations in sample preparation and instrumental analysis (**Table 2**).

Method Validation. The method presented here was further assessed using certified reference materials of BCR-282 (very low level), ERM-BD283 (low level), and ERM-BD284 (high level). The detected concentrations in the samples were 0.0238 ± 0.0048, 0.109 ± 0.017, and 0.485 ± 0.040 μg/kg (mean ± SD, $n = 4$), respectively (**Table 1**), which were all close to the certified levels. The deviations from the spiked or certified levels were from −28 to +10% using IAC cleanup in the range of 0.11−1.0 μg/kg. This complied with Directive 96/23/EC of the European Union, giving an acceptable range between −50 and +20% for samples ≤1.0 μg/kg (*54*). Accordingly, the method is linear, at least for milk powder, and does not require the use of matrix standards for calibration.

The method accommodated a larger sample volume of milk than existing methods. By using a 4−10-fold volume, detection is more reliable. Even with this volume increase, extraction time decreased using a SPE disk allowing a flow rate faster (20×) than that of the standard SPE cartridge. This extraction technique may be applied to other liquid matrixes such as water, juice, and urine. The tandem MS provided better sensitivity and specificity than a fluorescence detector. The LOD in milk was 76−85 times more sensitive than the most demanding EU regulatory limits (50 ng/L). The LOD in milk powder was 3 times more sensitive than the EU maximum level of baby formulas (25 ng/kg) and 590 times more sensitive than the maximum regulated level for Taiwan and the United States (5 μg/kg). Although the recovery and precision with MFC cleanup were much less satisfactory than with the IAC cleanup for confirmatory analysis, MFC sensitivity was still comparable to that of the most sensitive existing methods. Further studies can develop a multifunctional column for milk matrix to save time and labor on sample cleanup. Also, research synthesizing an isotopically labeled AFM_1 or use of 2D-LC (e.g., with restricted access material) would alleviate the concern of matrix effect and increase the applications of the method.

SAFETY

Aflatoxin M_1 is toxic and a liver carcinogen. To minimize exposure, it should be handled only in a fume hood while wearing protection (e.g., gloves, lab coat, and eye protection). The chemical itself, and all contacted materials, should be disposed of in a legal and environmentally safe manner.

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