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ARTICLE

Development of a Novel Immunoaffinity Column for Aflatoxin Analysis Using an Organic Solvent-Tolerant Monoclonal Antibody

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An organic solvent-tolerant monoclonal antibody specific to aflatoxins B_1, B_2, G_1, G_2, and M_1 (AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1) was prepared. In an indirect competitive enzyme-linked immunosorbent assay, the half maximal inhibitory concentration (IC_{50}) values were 1.9, 2.1, 2.1, 2.4, and 2.8 ng/mL for AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1, respectively. Antibody reactivity was retained at 40% methanol concentration or at acetonitrile concentrations up to 40%. An immunoaffinity column (IAC) was prepared using agarose gel beads with bound antibody. The IAC retained the tested AFs that were 89, 90, 95, 90, and 89% for AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 at 20% acetonitrile concentrations or that were 81, 87, 79, and 83% for AFB_1, AFB_2, AFG_1, and AFG_2 at 60% methanol concentrations. Roasted peanuts and seven kinds of spices were spiked with 8.0, 1.0, 6.0, and 1.0 ng for AFB_1, AFB_2, AFG_1, and AFG_2 per 1 g sample and extracted with 90% acetonitrile. The roasted peanuts and cayenne pepper out of the spices were also extracted with 70% methanol. The extracts were diluted 5-fold with phosphate-buffered saline and applied to the IAC. The spiked aflatoxins were recovered with satisfactory rates: 78 (RSD, 2.1%) to 127% (RSD, 1.7%). The developed IAC was used for the analysis of aflatoxins in naturally contaminated samples of roasted peanuts and cayenne pepper. The newly developed IAC showed substantially organic solvent tolerance at the concentration that could not be used for existing IACs, and the column showed good ability to clean up samples for food analysis.

KEYWORDS: Mycotoxin; immunoassay; cleanup

INTRODUCTION

Aflatoxins (AFs) are secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, fungi that are distributed throughout the tropics. AFs are potent hepatotoxins, causing acute liver damage to humans and other animal species when taken up in large amounts. A lethal outbreak of aflatoxicosis due to AF contamination of maize in Kenya occurred in 2004 (*1*). AFs are also potent carcinogens; the International Agency for Research on Cancer categorized naturally occurring AFs as group 1 carcinogens (carcinogenic to humans) .

Levels of AF contamination in foods are regulated worldwide; the regulations specify AFB_1 concentrations, total AFs (sum of AFB_1, AFB_2, AFG_1, and AFG_2), and AFB_1 metabolite (AFM_1) concentrations (**Figure 1**). AFB_1 is of particular concern because of its toxicity and carcinogenicity. Some countries, including Japan, regulate AFB_1 levels, whereas other countries regulate total AFs. The Codex Alimentarius Commission adopted a maximum total AFs level for tree nuts in 2008 (*2*), and the number of countries that regulate total AFs will increase in the near future.

Total AFs in foods are generally extracted with aqueous solutions containing high concentrations of organic solvents such as acetonitrile or methanol. The extracts are purified by multifunctional column (MFC) chromatography (*3*) or by immunoaffinity column (IAC) chromatography (*4*, *5*), and the AFs are determined by high-performance liquid chromatography (HPLC) with fluorescence detection or by liquid chromatography with detection by mass spectrometry or tandem mass spectrometry. Because IAC has a higher AF purity than MFC, IAC has become a major tool for AF analysis (*6*−*9*). However, the currently available columns show low tolerance for organic solvents, which is problematic because sample extracts generally contain high concentrations of acetonitrile, methanol, or acetone (*10*). The extracts have to be diluted before application to the column, which not only increases the sample volume but also produces insoluble substances that affect AF recovery. Acetonitrile, in particular, is rarely used as an organic solvent for IAC

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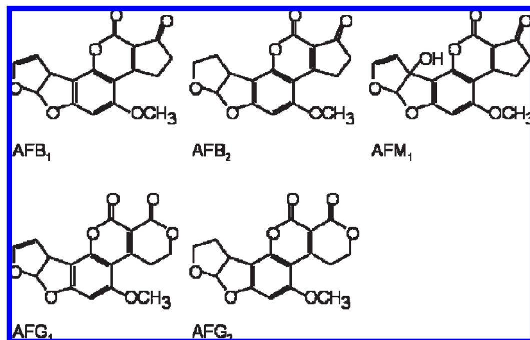

Figure 1. Structures of AFs.

because of this effect (*11*), although it is a good extraction solvent for polar low molecular weight compounds and is used for AF extraction for MFC cleanup (*3*).

It was expected that a high-performance IAC could be prepared with an organic solvent-tolerant antibody and that such a column could be used for cleanup of food extracts for which the use of the existing IACs is not appropriate. In this paper, the preparation and characteristics of a new monoclonal antibody (MoAb) and the performance of an IAC prepared from the MoAb were described.

EXPERIMENTAL PROCEDURES

Reagents and Apparatus. AFB_1 was purchased from Enzo Life Sciences (Lausen, Switzerland). AFM_1 was purchased from Biopure (Tulln, Austria). AFB_2, AFG_1, AFG_2, and keyhole limpet hemocyanin (KLH) were purchased from Wako Pure Chemical (Tokyo, Japan). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Freund's complete adjuvant and incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). Horseradish peroxidase (HRP)-labeled rabbit antimouse IgG antibody, 96-well microplates used for cell culture, and 96-well microtiterplates used for enzyme-linked immunosorbent assay (ELISA) were purchased from Thermo Fisher Scientific (Rockford, IL). Agarose gel activated with cyanogen bromide and the protein G column were purchased from GE Healthcare UK (Buckinghamshire, England). All other chemicals and reagents were of analytical grade and were purchased from Wako Pure Chemical or Nacalai Tesque (Kyoto, Japan). ELISA absorbance was measured with a microplate reader (MPR-01, Horiba, Kyoto, Japan).

AFB_2−Protein Conjugate Preparation. AFB_2 carboxymethyloxime (AFB_2 oxime) as a hapten was prepared according to the method of Chu and Ueno (*12*, *13*), except that AFB_2 was used instead of AFB_1. In brief, AFB_2 (26.1 μmol) was dissolved in 6.3 mL of a 1:4:1 (v/v) mixture of pyridine, methanol, and water, and the solution was mixed with aminooxyacetic acid hemihydrochloride (93.9 μmol) and heated at reflux for 2 h. The reaction mixture was concentrated and then purified by silica gel (1 g) column chromatography with a mobile phase of 9:1 chloroform:methanol by gravity flow. The fractions in which the product was contained were examined on thin-layer chromatography (TLC; silica gel 70F254 plate from Wako) with a developing solvent of 9:1 chloroform:methanol. After pooling of the fraction, the product was confirmed as a single spot on the TLC. The R_f value was 0.54 (as compared with 0.70 for AFB_2). After evaporation, AFB_2 oxime (23.5 μmol) was obtained as a white powder in 90% yield.

The AFB_2 oxime was covalently coupled with KLH or BSA by the activated ester method, as described previously (*14*). In brief, the AFB_2 oxime (20 μmol) in dried dimethyl sulfoxide (1.5 mL) was added dropwise to a mixture of *N*-hydroxysuccinimide (40 μmol) and 1-ethyl-3-(3-dimethylamiopropyl) carbodiimide hydrochloride (40 μmol) in dried dimethyl sulfoxide (0.5 mL). The solution was stirred at room temperature for 1.5 h. The stirred solution (220 μL) was added to KLH (20 mg) or BSA (20 mg) dissolved in 1 mL of borate-buffered saline (100 mM sodium borate, 150 mM NaCl, pH 8.0), and the mixture was gently stirred at room temperature for 1.5 h. After dialysis against 10 mM phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.2) for 4 days at 4 °C, the AFB_2−KLH conjugate prepared was used for mouse immunization, and the AFB_2−BSA conjugate was used as an ELISA antigen.

MoAb Preparation. MoAb preparation was briefly described in the applied patent (*15*). Seven week old female BALB/c mice from Nippon SLC (Shizuoka, Japan) were intraperitoneally immunized with 50 μL of the AFB_2−KLH conjugate (100 μg/mouse) after it had been emulsified with an equal volume of Freund's complete adjuvant. Booster injections (25 μg/mouse) were performed three times using the emulsion with Freund's incomplete adjuvant at intervals of 2 weeks. Three days after the last injection, spleen cells from the mouse (5×10^8 cells) were fused with P3-X63-AG8.653 myeloma cells (5.5×10^7 cells) by using polyethylene glycol (MW 1500). The fused cells were suspended at 2.5×10^6 cells/mL (spleen cells) in hypoxanthine aminopterin thymidine medium, transferred to the wells of a 96-well microplate, and incubated at 37 °C in 5% CO_2. Ten days after the start of incubation, the cultured fluids in which a hybridoma had formed a colony were screened by reactivity with the AFB_2−BSA conjugate in 40% methanol in a direct-bind ELISA (db-ELISA), and the fluids were subjected to secondary screening of their reactivity with AFB_1, AFB_2, AFG_1, and AFG_2 in an indirect competitive ELISA (ic-ELISA), as described below. Hybridoma grown in the positive well was cloned by the limiting dilution technique, and the representative cell clone was used for preparation of the MoAb.

For MoAb preparation, BALB/c mice were pretreated by intraperitoneal injection with 0.5 mL of pristane, and 1 week after the pretreatment, the mice were inoculated with 2×10^7 viable cells. Seven to 10 days after the inoculation, ascite fluids produced were collected from the mice, and the MoAb in the fluid was purified on a protein G column. The MoAb concentration was determined from the extinction coefficient (1.4 for 1 mg/mL of IgG).

db-ELISA and ic-ELISA. A db-ELISA was used to screen hybridomas producing methanol-tolerant MoAbs. An ic-ELISA was used to screen hybridomas producing anti-AF group-specific MoAbs and to determine the reactivity of the prepared MoAb, as described previously (*14*). In brief, 100 μL of AFB_2−BSA conjugate (1 μg/mL) in PBS was added to each well of 96-well microtiter plates and was physically coated by incubation overnight at 4 °C. After they were washed three times, the wells were blocked by the addition of 300 μL of 1% BSA in PBS.

In a db-ELISA, 50 μL of cultured fluids of the hybridomas diluted with an equal volume of 80% methanol containing PBS was added to the wells, and the plate was incubated for 1 h at 25 °C. After the plate had been washed three times, 100 μL of HRP-labeled rabbit antimouse IgG antibody (1 μg/mL) in PBS modified with 0.3% BSA was added to each well. The plate was incubated for 1 h at 25 °C and then washed three times with PBS. HRP substrate solution (100 μL; 2 mg/mL of 3,3′,5,5′-tetramethylbenzidine and 0.006% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5) was added to each well, and the plate was incubated for 10 min at 25 °C. Color development with the HRP reaction was stopped by the addition of 100 μL of 0.5 M sulfuric acid, and the absorbance at a wavelength of 450 nm was measured with a microplate reader.

In an ic-ELISA, each AF was dissolved at a concentration of 0.16−10 ng/mL in 2% acetonitrile solution or 0.064−1000 ng/mL (only AFB_1) in 2−100% acetonitrile solutions diluted with PBS, and cultured fluid of the hybridomas was diluted 2-fold with PBS modified with 0.3% BSA; the final concentration showed 50% of maximum absorbance in the above db-ELISA. The AF solution (50 μL) was added to the above blocked wells, and an equal volume of the diluted cultured fluid was immediately added to the wells. The plate was incubated for 1 h at 25 °C. Furthermore, HRP-labeled rabbit antimouse IgG antibody was added followed by incubation, washing, and substrate addition steps as described before for the db-ELISA.

Preparation of IAC. Agarose gel was covalently coupled with the MoAb according to the following procedure. MoAb (40 mL, 0.5 mg/mL) dissolved in PBS was mixed with 20 mL of activated agarose gel, and the mixture was gently agitated for 2 h at 25 °C. The gel coupled with the MoAb was separated from the liquid phase by filtration through filter paper. The coupled gel was added to 40 mL of a blocking buffer (1 M monoethanolamine modified with 0.5 M NaCl; pH 8.0), and the mixture was gently agitated for 2 h at 25 °C. The gel was alternately washed four times with the blocking buffer and 0.1 M sodium acetate modified with 0.5 M NaCl (pH 4.0). The gel was then washed with PBS and 2 M NaCl

and finally with PBS. Gel (0.2 mL) was packed in empty columns (ø 9 mm) with polyethylene frits. The prepared IACs were filled with PBS and used for the experiments described below.

Organic Solvent Tolerance of the IAC. Total AFs (0.5, 1.0, 2.5, 5.0, 10, or 20 ng/mL for each AF) were dissolved in 2% acetonitrile for determination of the adsorption ability of the IAC. Total AFs (5.0 ng/mL for each AF) were dissolved in 2−80% acetonitrile or 2−80% methanol to determine the organic solvent tolerance; AFM_1 (5.0 ng/mL) was dissolved only in 2−80% acetonitrile. Each solution (10 mL) was applied to an IAC at a flow rate of 1 drop/s. After all of the solution had flowed through, the column was washed twice with 3 mL of PBS and twice with 3 mL of distilled water. The adsorbed AFs were eluted with 3 mL of acetonitrile; the first 1 mL was retained in the column for 5 min, and the remaining 2 mL was flowed through. The eluent was dried under a N_2 stream, and the residue was dissolved in 1 mL of acetonitrile-water (1:9; v/v). Fluorescent derivatives of AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 were produced by UV radiation according to the method of Joshua (*16*), and the derivatives were analyzed by HPLC as described below.

Preparation and Analysis of Food Samples. Roasted peanuts and seven kinds of spices (cayenne pepper, paprika, white pepper, black pepper, cinnamon, turmeric, and coriander) were collected from markets. The absence of AFs in these samples was confirmed by preliminary examination for which these samples were cleaned up by the prepared IAC and analyzed by HPLC (data not shown). Roasted peanuts and cayenne pepper samples naturally contaminated with AFs were also collected. Ten kinds of the samples were ground, and 10 g aliquots were transferred to Erlenmeyer flasks (100 mL). For recovery tests, 100 μL of total AFs (equivalent to 16.0 ng total AFs per 1 g sample; spiking levels for AFB_1, AFB_2, AFG_1, and AFG_2 were 8.0, 1.0, 6.0, and 1.0 ng/g, respectively) in acetonitrile were added to the uncontaminated samples. After vigorous shaking, the samples were allowed to stand for 30 min.

A mixture acetonitrile:water 90:10, v/v, or methanol:water 70:30, v/v (20 mL for roasted peanuts, 80 mL for spices), was added to each flask, and the mixture was extracted with a blender for 5 min. After filtration through filter paper, the sample extracts were diluted to 18% acetonitrile or 14% methanol (5-fold dilution) with PBS. The milky turbidity produced by dilution of the samples was dispersed by the addition of Tween 20 at the following percentages: paprika, 1% Tween; cayenne pepper, 2%; white pepper, 4%; black pepper, 2%; and cinnamon, 10%. The diluted sample solutions (10 mL) were applied to the IAC, which was then washed twice with 3 mL of PBS and twice with 3 mL of water. Total AFs were eluted with 3 mL of acetonitrile; the first 1 mL was retained in the column for 5 min, and the remaining 2 mL was flowed through, and the eluate was collected in a vial and was evaporated to dryness under an N_2 stream. The residue was treated with 0.1 mL of trifluoroacetic acid and allowed to stand for 15 min in the dark based on the official method of Japan although this is inconvenient method as compared to that of Joshua; then, 0.9 mL of acetonitrile−water (1:9; v/v) was added to each vial and mixed. Each AF was determined by injection of the above solution into the HPLC under the conditions described below.

HPLC Conditions. To analyze the adsorption ability and organic solvent tolerance of the IAC, the 1100 series HPLC system equipped with a fluorescence detector (1200) (Agilent Technologies, Santa Clara, CA) was used. For food sample examination, an HPLC system consisting of an LC-10AD pump, a SIL-1-A autoinjector (100 μL loop), a CTO-10AC column oven, an RF-10AXL fluorescence detector, a DGU-3A degasser, a CBM-10A communication bus module, and a class LC-10 chromatography data system (Shimadzu, Kyoto, Japan) was used. The column was an Inertsil ODS-3 V (5 μm, 4.6 mm i.d. × 250 mm) (GL Sciences, Tokyo, Japan). The HPLC mobile phase was a mixture of 1:3:6 acetonitrile, methanol, and water. The flow rate was 1.0 mL/min, the column oven temperature was 40 °C, and the injection volume was 100 μL. The fluorescence detector was set at excitation and emission wavelengths of 360 and 450 nm, respectively.

RESULTS AND DISCUSSION

Preparation of Anti-AF MoAb. Previous reports have indicated that antibodies raised against AFB_1 are AFB_1 specific (*12*, *17*) and that antibodies raised against AFB_2 are AFB_2 specific (*18*). This is the normal immune response between a hapten and its corresponding antibody. However, a MoAb that was equally reactive to AFB_1, AFB_2, AFG_1, and AFG_2 and that was organic solvent-tolerant was needed for a novel IAC preparation (*15*).

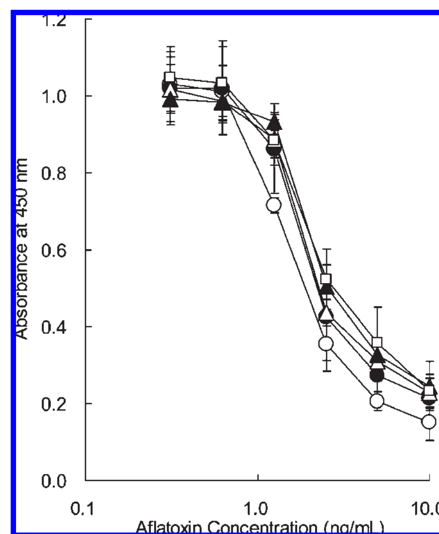

Figure 2. Reactivity of MoAb2-3 with AFs in ic-ELISA: AFB_1 (○), AFB_2 (●), AFG_1 (△), AFG_2 (▲), and AFM_1 (□). Each data point is the mean of three replicates; error bars indicate standard deviations.

AFs share a bisfuran and coumarin structure, but AFB_2 and AFG_2 have a single bond in the bisfuran moiety, whereas AFB_1 and AFG_1 have a double bond (**Figure 1**). Because the single bond structure is slightly larger than the double bond structure, the anti-AFB_2 antibody population raised against AFB_2 can be expected to contain molecules that can accommodate the smaller AFB_1 molecule. In fact, Pestka et al. and Groopman et al. prepared antibodies specific to both AFB_1 and AFB_2 by using an AFB_1−protein conjugate (*19*,*20*), and Gaur et al. did the same with an AFB_2−BSA conjugate (*21*). However, an antibody specific to the total AFs group has not yet been prepared. Because AFB_1 and AFB_2 have a cyclopentanone structure and AFG_1 and AFG_2 have a δ-lactone structure, it was expected that if the carbonyl group was attached to a carrier protein, an antibody population containing a few molecules that did not distinguish the difference between the two types of AFs could be prepared. An AFB_2 oxime was therefore chosen as a hapten to satisfy this requirement.

The AFB_2 oxime was conjugated with KLH, and BALB/c mice were immunized with the conjugate. The mice, in which the antibody titer against AFB_2 was raised, were used to make a spleen cell preparation for cell fusion. Ten days after cell fusion, wells in which hybridomas were grown were screened in a db-ELISA at a final methanol concentration of 40% to obtain an organic solvent-tolerant MoAb, and the positive wells were subjected to an ic-ELISA secondary screen. Six wells showed reactivity not only to AFB_2 but also to AFB_1, AFG_1, and AFG_2, as well as tolerance to 40% methanol. The cells in each positive well were cloned by the limiting dilution technique, and the representative cell clone secreting MoAb AFB2-3-7F3-3 (abbreviated as MoAb2-3) was selected. The subclass was IgG_1, and the light chain was λ.

The IC_{50} values of MoAb2-3 were 1.9, 2.1, 2.1, 2.4, and 2.8 ng/mL for AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1, respectively, in the ic-ELISA; their reaction curves almost completely overlapped with one another (**Figure 2**). The affinity of AFM_1 was similar without steric hindrance, even though it has a hydroxyl group in its bisfuran structure. MoAb2-3 seemed to recognize the common overall structure of the AFs. This result validated our working

hypothesis and indicated that the reactivity of MoAb2-3 was sufficient for all of the AFs that are important for food sanitation.

Acetonitrile Tolerance of MoAb2-3. AFs are generally extracted with aqueous solutions containing high concentrations of a water-miscible solvent, such as acetonitrile or methanol. However, acetonitrile cannot be used with commercially available IACs for AFs at concentrations $>7\%$ because it denatures antibodies (*10*). In fact, acetonitrile has rarely been used at concentrations above 5% because of the low recovery observed for foodstuffs such as peanut butter (*11*).

MoAb2-3 had a methanol tolerance of at least 40%, as indicated by the results of MoAb screening. To get more information about the acetonitrile tolerance of the reaction of MoAb2-3 with AFB_1, ic-ELISA was performed at acetonitrile concentrations ranging from 1 to 50% (**Figure 3**). The reactivity of MoAb2-3 with AFB_1 at 10% acetonitrile was the same as that at 1% acetonitrile. The reactivity was lower at 20 and 40% acetonitrile, and there was no reactivity at 50% acetonitrile. On the other hand, the reactivity with AFB_2−BSA was maintained even at 40% acetonitrile and 30% of the reactivity resided at 50% acetonitrile. The difference might have arisen from competitive reaction of the free AFB_1 and the coated AFB_2−BSA in the ic-ELISA.

These results indicate that MoAb2-3 tolerated moderate acetonitrile concentrations. If this tolerance extended to higher concentrations, the prepared IAC would not elute the adsorbed AFs with organic solvents. Therefore, the moderate tolerance was deemed adequate.

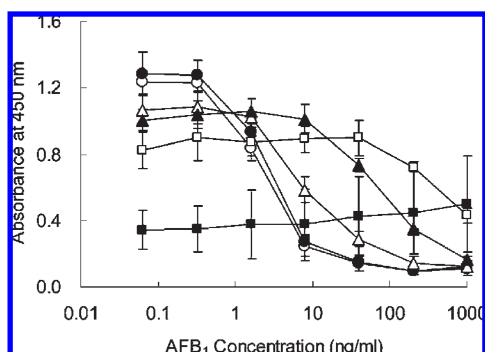

Figure 3. Acetonitrile tolerance of MoAb2-3 reactivity with AFB_1 in ic-ELISA: 1 (○), 10 (●), 20 (△), 30 (▲), 40 (□), and 50% acetonitrile (■). Acetonitrile percentages are the final acetonitrile concentrations in the competitive reaction mixture. Each data point is the mean of three replicates; error bars indicate standard deviations.

Adsorption Ability and Organic Solvent Tolerance of the Prepared IAC. An IAC was prepared with MoAb2-3. The nature of the support gel is important for binding of the MoAb (*22*). Agarose gel did not adsorb any matrices from the food extracts examined (data not shown); therefore, it was selected as the support gel for the IAC. Activated agarose gel was coupled with purified MoAb2-3 under saturated conditions, and the gel was packed in a disposable plastic column (0.2 mL/column). Acetonitrile solutions (2%, 10 mL) containing equal amounts of AFB_1, AFB_2, AFG_1, and AFG_2 (5.0, 10, 25, 50, 100, or 200 ng of each AF) were applied to the IAC, and the adsorbed AFs were eluted with acetonitrile. AF recovery was more than 85% up to 400 ng total AFs, but the recovery rate decreased at 800 ng total AFs. Thus, the binding capacity of the prepared IAC was at least 400 ng total AFs, and this capacity was deemed adequate for total AFs analysis of foods. These results also confirmed that the acetonitrile tolerance was not so strong that the adsorbed AFs could not be eluted from the column.

Acetonitrile and methanol were used to examine the applicable organic solvent concentrations. Ten milliliters of 2−80% acetonitrile or methanol solutions containing total AFs (5 ng/mL of each AF) was applied to the IAC, and the AF recoveries were determined. More than 90% of each AF was recovered at up to 20% acetonitrile, and AFB_1 and AFB_2 recoveries were high even at 40% acetonitrile (**Figure 4**A). This behavior was similar to that of AFB_2−BSA not AFB_1 in the above ic-ELISA. The reason is not clear, but AFB_2−BSA is rigidly coated on the wells as well as MoAb2-3 bound on support gels in the IAC. These rigid forms might be effective to the organic solvent tolerance. AFM_1 (5 ng/mL) was also applied separately, and the recovery of this AF was also more than 90% up to 20% acetonitrile. The IAC showed even higher tolerance for methanol than for acetonitrile (**Figure 4**B). More than 80% of the AFs were recovered at 60% methanol. This result implies that methanol extracts from food samples may be directly applied to the column.

The prepared IAC showed sufficient AF adsorption capacity at higher organic solvent concentrations than is the case for existing IACs, as described by Scott et al. (5−7% acetonitrile; 16−30% methanol) (*10*). Because of this organic solvent tolerance, this IAC is expected to be applicable for samples that are difficult to analyze with existing IACs.

Recovery Test. Spices are typically difficult to clean up because they contain large amounts of essential oils. After simultaneous

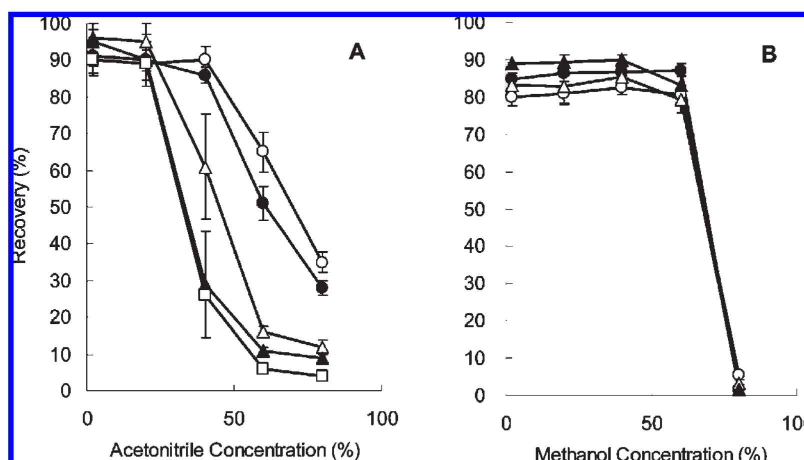

Figure 4. Acetonitrile (**A**) and methanol (**B**) tolerance of the prepared IAC: AFB_1 (○), AFB_2 (●), AFG_1 (△), AFG_2 (▲), and AFM_1 (□). Each data point is the mean of three replicates; error bars indicate standard deviations.

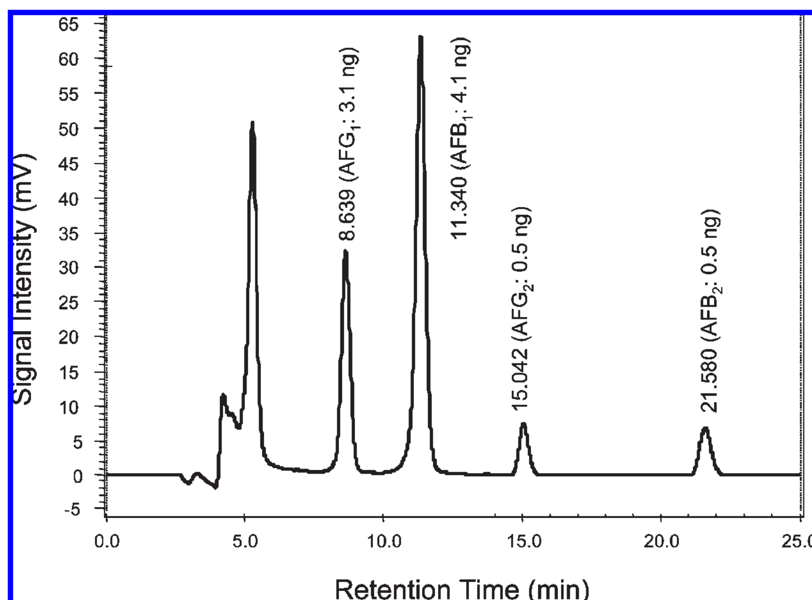

Figure 5. Typical chromatogram for paprika extracted with 90% acetonitrile.

Table 1. Mean Total AFs Recoveries Extracted with 90% Acetonitrile from Spiked Food Samples by Means of IAC Cleanup

	AFB_1		AFB_2		AFG_1		AFG_2	
food sample	recovery (%)	RSD[a] (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
roasted peanuts	85	1.8	79	1.7	92	2.1	81	2.2
	76[b]	2.7	78[b]	2.2	72[b]	2.8	82[b]	1.9
cayenne pepper	94	3.5	93	2.0	99	4.5	96	2.0
	76[b]	2.6	78[b]	2.7	72[b]	2.5	84[b]	2.6
paprika	91	4.9	89	4.4	99	6.2	99	5.4
white pepper	97	1.1	98	1.0	88	7.0	86	6.0
black pepper	127	1.7	97	1.7	81	10.6	82	9.7
cinnamon	78	2.1	82	0.4	87	1.6	89	0.5
turmeric	86	1.6	88	1.0	87	1.8	88	1.0
coriander	104	0.3	115	0.6	99	3.6	102	3.9

[a] RSD, relative standard deviation for three replicates. [b] Recoveries obtained by extraction with 70% methanol.

extraction of total AFs and the essential oils with an organic solvent, the oils are precipitated by dilution with an aqueous solution; however, the precipitates physically inhibit total AFs cleanup. It was expected that less dilution of the organic solvent extracts as compared to dilution rate for existing IACs, followed by dispersion of the turbidity with Tween 20, might effectively reduce inhibition on the total AFs cleanup. To test this procedure, total AFs-spiked samples of roasted peanuts and seven kinds of spices (cayenne pepper, paprika, white pepper, black pepper, cinnamon, turmeric, and coriander) were used. The samples were extracted with 90% acetonitrile (this concentration is based on the extraction conditions used for MFC) (*3*); the roasted peanuts and cayenne pepper were also extracted with 70% methanol, which is usually used for existing IACs (*6*, *9*). All of the sample extracts were diluted 5-fold with PBS (from 90 to 18% acetonitrile; from 70 to 14% methanol). Any resulting milky turbidity was dispersed by the addition of Tween 20 before application to the IAC.

The AF peaks in the HPLC chromatogram of paprika were sharp and clear, without noise, and the baseline was stable (**Figure 5**). Similar results were observed for the other food samples (data not shown). Recoveries for all of the food samples at 90% acetonitrile were satisfactory as 78 (RSD, 2.1%) to 127% (RSD, 1.7%) (**Table 1**). Two commercially available IACs showed extremely low recoveries under these conditions and thus could not be used for total AFs cleanup (data not shown). For the 70% methanol extractions, the recoveries were 72 (RSD, 2.8%) to 82% (RSD, 1.9%) for roasted peanuts and 72 (RSD, 2.5%) to 84% (RSD, 2.6%) for cayenne pepper. For the roasted peanuts and the cayenne pepper, the values were −1 to 20% and 12−27% lower than the values for 90% acetonitrile extraction. Stroka et al. showed that the use of acetonitrile−water as a solvent led to false positive analytical results because the acetonitrile solution easily loses water by adsorption into dry foods and the observed recovery values are consequently higher than the true values (*23*). Our extraction conditions differed from those used by Stroka et al.; nevertheless, the influence of water adsorption on the acetonitrile extraction had to be considered. For cayenne pepper, which is a dry material, recovery was higher than that for roasted peanuts, which contain water; for 70% methanol, the recoveries were the same for cayenne pepper and roasted peanuts. Again, recoveries observed by means of acetonitrile extraction may be higher than the true values, because of water adsorption. However, the influence of water adsorption was minor, and all of the recoveries, except the recovery of AFB_1 from black pepper (127%), were between 70 and 120%.

Stroka et al. have reported that antibody sensitivity to organic solvents is a critical factor in the IAC cleanup procedure (*23*). With our newly prepared IAC, total AFs were able to be obtained with high purity and high recovery from all of the tested food samples after 5-fold dilution of 90% acetonitrile extracts. This is the first IAC to show satisfactory acetonitrile tolerance and, of

Table 2. Mean Concentrations of AF Extracted from Naturally Contaminated Roasted Peanuts and Cayenne Pepper by Using 90% Acetonitrile and 70% Methanol

		AFB_1		AFB_2		AFG_1		AFG_2	
food sample	extraction solvent	concn (ng/g)	RSD[a] (%)	concn (ng/g)	RSD (%)	concn (ng/g)	RSD (%)	concn (ng/g)	RSD (%)
roasted peanuts	90% acetonitrile	22.5 (26.5)[b]	4.4	2.7 (3.4)	6.9	1.1 (1.2)	11.0	0.2 (0.2)	8.9
	70% methanol	19.1 (25.1)	1.4	2.3 (2.9)	3.9	1.0 (1.4)	34.7	0.3 (0.4)	20.4
cayenne pepper	90% acetonitrile	6.7 (7.1)	5.3	0.3 (0.3)	8.1	ND[c]		ND	
	70% methanol	5.3 (7.0)	1.1	0.2 (0.3)	1.4	ND		ND	

[a] RSD, relative standard deviation for three replicates. [b] Brackets showed values corrected by dividing the above results by the corresponding recovery test results/100. [c] ND, not determined.

course, methanol tolerance; therefore, this IAC extends the range of applicable foods because it enables the use of increased organic solvent concentrations for the extractions.

Analysis of Foods Naturally Contaminated with AFs. Extraction of AFs from naturally contaminated foods is known to be less efficient than extraction in recovery tests. The lower efficiency is due to the fact that fungi that produce AFs invade the tissue of the host plant by elongation at the tip of the hypha and by production of side branches; therefore, the AFs penetrate deep into the tissue. Therefore, the recovery results were compared for homogenized roasted peanuts and cayenne pepper naturally contaminated with AFs with the results of the recovery tests with 90% acetonitrile and 70% methanol (**Table 2**). The recoveries of the major AF contaminant, AFB_1, obtained in both samples with 90% acetonitrile, were higher than those obtained with 70% methanol, as was the case in the recovery tests. AFs concentrations were revised by using the results from the recovery tests, and the difference between the revised values was lower than that between the unrevised values. This result suggests that the values of AF contaminated foods would become truer by the revision as compared to the obtained values. Further experiments should be performed for a large number of natural samples to confirm this suggestion in future study.

In conclusion, our newly developed IAC containing the anti-aflatoxin MoAb2-3 showed dramatically improved organic solvent tolerance and good cleanup ability for food analysis as compared with existing IACs. This IAC permitted acetonitrile extraction, which cannot be used with existing IACs. In addition, the new IAC also allowed extraction at methanol concentrations up to 60%. This high organic solvent tolerance will be useful for cleanup of AFs. It is expected that this IAC will eliminate various AF cleanup difficulties derived from sample matrices, as well as restrictions on the applicable organic solvents.

Safety. This study was approved by the Biomanagement Committee at HORIBA, Ltd., and was carried out according to the guidelines of the committee.

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