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Article

FOOD CHEMICAL CONTAMINANTS

Determination of Aflatoxin M1 in Liquid Milk, Cheese, and Selected Milk Proteins by Automated Online Immunoaffinity Cleanup with Liquid Chromatography–Fluorescence Detection

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Abstract

Background: Aflatoxins are secondary metabolites produced by a number of species of *Aspergillus* fungi. Aflatoxin M1 (AFM1) is a hydroxylated metabolite of aflatoxin B1 and is found in the milk of cows fed with feed spoilt by *Aspergillus* species. AFM1 is carcinogenic, especially in the liver and kidneys, and mutagenic, and is also an immunosuppressant in humans.

Objective: A high-throughput method for the quantitative analysis of AFM1 that is applicable to liquid milk, cheese, milk protein concentrate (MPC), whey protein concentrate (WPC), whey protein isolate (WPI), and whey powder (WP) was developed and validated.

Method: AFM1 in cheese, milk, and protein products is extracted using 1% acetic acid in acetonitrile with citrate salts. The AFM1 in the resulting extract is concentrated using RIDA®CREST/IMMUNOPREP® ONLINE cartridges followed by quantification by HPLC–fluorescence.

Results: The method was shown to be accurate for WP, WPC, WPI, MPC, liquid milk, and cheese, with acceptable recovery (81–112%) from spiked samples. Acceptable precision for WP, WPC, WPI, MPC, liquid milk, and cheese was confirmed, with repeatabilities of 4–12% RSD and intermediate precisions of 5–13% RSD. Method detection limit and ruggedness experiments further demonstrated the suitability of this method for routine compliance testing. An international proficiency scheme (FAPAS) cheese sample showed that this method gave results that were comparable with those from other methods.

Conclusions: A method for high-throughput, routine testing of AFM1 is described. The method was subjected to single-laboratory validation and was found to be accurate, precise, and fit-for-purpose.

Highlights: An automated online immunoaffinity cleanup HPLC–fluorescence method for milk proteins, cheese, and milk was developed and single-laboratory validated. It allows for high-throughput analysis of AFM1 and can be used for the analysis of AFM1 in whey protein products.

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Figure 1. Structure of aflatoxin M1.

The most abundant aflatoxins found in plant-based foods are B1, B2, G1, and G2; they are secondary metabolites produced by species of *Aspergillus* fungi, particularly *A. flavus* and *A. parasiticus* (1). Cows that are fed with stored grain-based feed spoilt by *Aspergillus* fungi hepatically hydroxylate approximately 5% of the aflatoxin B1 to produce aflatoxin M1 (AFM1, Figure 1), the major aflatoxin metabolite expressed in milk (2–4). Because AFM1 is heat stable, it is potentially found in pasteurized milk and other dairy products derived from contaminated raw milk, and countries therefore require dairy products to be tested for and certified free of AFM1 as a requirement for international trade (5, 6). AFM1 is currently not found in New Zealand as the local climate does not favor *Aspergillus* growth (pre harvest) and dairy cows in New Zealand are predominantly fed on local pasture or pasture-based silage (7, 8).

AFM1 has been detected in milk and other dairy products produced in many areas around the world, including countries from Africa, Europe, the Middle East, Asia, and North and South America (9–13). This compound has been shown to cause hepatocellular carcinomas and renal damage, and is cytotoxic as well as mutagenic (14–17). Because of these adverse health effects, the level of AFM1 allowed in milk and other dairy products is regulated in many countries (6, 11, 13).

In the past, AFM1 was analyzed using HPTLC, but is now more commonly analyzed using chromatography with fluorescence detection or MS, or by immunoassay techniques such as enzyme-linked immunosorbent assay, thin film electrochemical biosensors, and optical biosensors (18–23). As AFM1 is usually regulated and present at very low concentrations (ng/L), extracts need to be concentrated using either immunoaffinity or C_{18} solid phase extraction cartridges before chromatographic analysis (19, 24, 25).

For the high-throughput analysis of aflatoxins, R-Biopharm has developed an automated immunoaffinity cartridge system (RIDA®CREST ICE) to replace the manual operation required with conventional immunoaffinity columns (26). This device allows for a higher sample throughput and improved precision, and is less prone to blockage compared with manual immunoaffinity columns.

The purpose of this study was to develop and validate a high-throughput method for the routine analysis of AFM1 in whey protein concentrate (WPC), whey protein isolate (WPI), whey powder (WP), milk protein concentrate (MPC), liquid milk, and cheese using RIDACREST ICE coupled to HPLC–fluorescence.

METHOD

Apparatus

(a) *Pipettes*.—Eppendorf Research® Plus 2–20, 20–200, 100–1000, 1000–10 000 microlitres (Eppendorf, Hauppauge, NY).
(b) *Centrifuge*.—Heraeus Multifuge X3 centrifuge (ThermoFisher, Waltham, MA).
(c) *Centrifuge tubes*.—Polypropylene 15 and 50 mL (ThermoFisher).
(d) *Vortex mixer*.—Genius 3 (IKA, Wilmington, NC).
(e) *Analytical balance*.—AE 260 analytical delta range (±0.1 mg) or equivalent (Mettler-Toledo, Columbus, OH), calibrated with National Institute of Standards and Technology (Gaithersburg, MD) traceable calibration weights.
(f) *HPLC column*.—Kinetex Phenyl-Hexyl 2.6 μm, 4.6 × 150 mm (Phenomenex, Torrance, CA).
(g) *HPLC system*.—Prominence HPLC system consisting of two LC-20AT pumps, an SIL-20AC autosampler, a CTO-20AC column oven, a CBM-20A control module, an RF-20AX fluorescence detector, a DGU-20A5R degasser unit, and data processing with Lab Solutions software version 5.73 (Shimadzu, Kyoto, Japan). RIDACREST ICE controlled by Clarity software version 8.2 (R-Biopharm, Darmstadt, Germany).
(h) *Immunoaffinity cartridges*.—IMMUNOPREP® ONLINE AFM1 cartridges (R-Biopharm Rhône, Glasgow, Scotland).
(i) *AFLAPREP® M WIDE immunoaffinity columns*.—(R-Biopharm Rhône).
(j) *Graduated cylinders*.—100, 250, and 1000 mL.
(k) *Volumetric flasks*.—500 and 1000 mL.
(l) *HPLC injection vials*.—Amber, 2 mL with Teflon-coated caps.
(m) *Conical flasks*.—150 mL.
(n) *Linear shaker*.—HS 501 digital (Ika-Werke, Staufen, Germany).
(o) *pH meter*.—S220 pH/Ion meter (Mettler Toledo).
(p) *Evaporator*.—Techne sample concentrator (Cole-Palmer, Vernon Hills, IL).
(q) *Glass test tubes*.—15 mL.
(r) *Nylon syringe filters*.—0.2 μm (Phenex, Phenomenex).
(s) *Disposable plastic syringes*.—6 mL (Electrolube, Brookvale, NSW, Australia).

Reagents

(a) *Acetic acid (CH_3COOH)*.—Reagent grade (Mallinckrodt, Staines, UK).
(b) *Ammonium acetate (NH_4CH_3COO)*.—Reagent grade (Sigma Aldrich, St Louis, MO).
(c) *Sodium hydroxide (NaOH)*.—Reagent grade (Merck, Kenilworth, NJ).
(d) *Methanol (CH_3OH)*.—HPLC grade (Mallinckrodt).
(e) *Water (H_2O)*.—HPLC grade (Genpure18.2 MΩ/cm Barnstead, Waltham, MA).
(f) *Formic acid (HCOOH)*.—HPLC grade (Fisher, Waltham, MA).
(g) *Acetonitrile (CH_3CN)*.—HPLC grade (Mallinckrodt).
(h) *Nitric acid (HNO_3)*.—Reagent grade (Mallinckrodt).
(i) *Triton X-100*.—Reagent grade (Mallinckrodt).
(j) *Supel™QuE citrate extraction tube salts*.—Reagent grade (Sigma Aldrich).
(k) *Tris(hydroxmethyl)aminoethane*.—Reagent grade (Sigma Aldrich).
(l) *AFM1 standard*.—0.5 μg/mL (R-Biopharm Rhône).
(m) *Isopropanol*.—HPLC grade (Fisher).

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Solutions

(n) *Extraction solution*.—5 mL acetic acid, 495 mL acetonitrile.
(o) *1 M NaOH solution*.—Dissolve 4 g NaOH pellets in 100 mL water.
(p) *Loading buffer*.—Dissolve 1.54 g ammonium acetate in 1 L water, and adjust the pH to 6.8–7.0 using *1 M NaOH solution*.
(q) *Reconstitution buffer*.—Mix 450 mL *Loading buffer* with 50 mL methanol.
(r) *Elution buffer*.—Dissolve 3.85 g ammonium acetate in 640 mL water. Then add 100 mL acetonitrile and 260 mL methanol and adjust the pH to 2.0 using concentrated nitric acid.
(s) *Cartridge wash buffer*.—Dissolve 1.54 g ammonium acetate and 3.02 g tris(hydroxymethyl)aminomethane in 875 mL water, add 125 mL methanol, and pH adjust to 8.0 with nitric acid.
(t) *Mobile phase A*.—980 mL water, 20 mL methanol, and 1 mL formic acid.
(u) *Mobile phase B*.—900 mL acetonitrile, 100 mL water, and 1 mL formic acid.
(v) *Autosampler wash*.—250 mL water and 250 mL acetonitrile.
(w) *Pump seal wash*.—800 mL, 200 mL isopropanol.

Standards

Prepare an intermediate standard solution (10 ng/mL) by diluting 20 μL of the 500 ng/mL stock standard with 0.980 mL acetonitrile. Prepare standards of 0.025, 0.050, 0.100, and 0.150 ng/mL to validate detector linearity.

Samples

A sample each of WPC, WPI, WP, and MPC known to be free from AFM1 was used to carry out the AFM1 spiking experiments. A permeate-free, full cream homogenized milk and grated Edam cheese purchased retail were also used to carry out spiking experiments, and an Food Analysis Performance Assessment Scheme (FAPAS) reference cheese sample was used (Fera Science Limited, UK).

Sample Preparation

Milk protein powders

Add milk protein powder (6 ± 0.05 g) to a 125 mL conical flask with 50 mL water. Place the flask on a hot plate stirrer at 50°C and mix with a stir bar for 30 min; then cool to room temperature. Weigh milk protein solution (10 ± 0.05 g) into a 50 mL centrifuge tube and spike at 50 and 100 ng/L.

Liquid milk

Add milk (10 ± 0.01 g) at ambient temperature directly to a 50 mL centrifuge tube and spike at 50 and 100 ng/L.

Cheese

Add cheese (5 ± 0.05 g) at ambient temperature to a 50 mL centrifuge tube. Add water (50°C, 10 mL), cool the sample to room temperature, and spike at 50 and 100 ng/L.

Sample Extraction

Add extraction solution (20 mL) and the salts in one extraction tube to the sample and vortex mix. Shake (200 rpm, 90 min) the sample tubes and centrifuge (3400 rcf, 10 min). Transfer the top acetonitrile layer (2 mL) to a test tube, add one drop of Triton X-100, and dry the sample under N_2 at 60°C until a small viscous residue remains. Add reconstitution buffer (2 mL) and vortex mix the test tube. Use a syringe filter if the sample extract is cloudy, prior to transfer of a minimum of 1.5 mL to an autosampler vial.

Table 1. HPLC gradient conditions used following automated cartridge cleanup

Time, min	Flow rate, mL/min	Mobile phase composition, %	
		A[a]	B[b]
0.0	1.0	100	0
5.0	1.0	100	0
5.3	0.1	100	0
8.0	0.1	100	0
8.3	0.8	60	40
15.7	0.8	60	40
16.0	0.8	0	100
28.0	0.8	0	100
28.3	1.0	100	0
29.0	1.0	100	0
30.0	0.8	100	0

[a] Mobile phase A = 2% methanol, 0.1% formic acid.
[b] Mobile phase B = 90% acetonitrile, 0.1% formic acid.

HPLC Conditions

(a) *Column temperature*.—40°C.
(b) *Injection volume*.—1000 μL.
(c) *Binary gradient*.—Settings in Table 1.
(d) *Fluorescence detector*.—Excitation: 355 nm, emission: 430 nm.
(e) *RIDACREST ICE conditions*—Settings in Table 2.

Carry out external standard calibration using a single-level 50 ng/L aqueous aflatoxin standard subjected to the entire procedure.

Results and Discussion

Method Optimization

The experimental method followed the protocol supplied by the manufacturer, apart from the following modifications: (1) addition of extraction salts before the 90 min shaking period followed by centrifugation; (2) the cartridge wash buffer pH = 8; (3) extension of the automated immunoaffinity cycle time to 24 min; (4) an increase in the chromatographic run time to 30 min, as seen in Figure 2.

Method Validation

Detector linearity was demonstrated through analysis of multilevel AFM1 standard solutions ($n = 4$) covering an analyte range from 25 to 150 ng/L and was performed by direct injection bypassing the RIDACREST ICE. Linearity was evaluated by least-squares regression analysis of peak area versus concentration, with acceptable values for r^2 of 0.997 being obtained, as shown in Figure 3.

Method recovery was determined by spiking samples of WP, WPC, WPI, MPC, liquid milk, and cheese with 50 and 100 ng/L AFM1, as shown in Table 3. The recovery results fit within the AOAC guidelines of between 70–125% recovery for analytes present at 10 μg/L or less (27).

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Table 2. RIDA®CREST ICE conditions[a]

Step	High pressure dispenser flow, mL/min	Solution	Volume, mL
Conditioning	5.0	Loading buffer	2.0
Sample extract	1.0	Loading buffer	1.0
Cartridge wash	2.0	Cartridge wash buffer	6.0
Elution	0.3	Elution buffer	0.6
Clamp wash	5.0	Loading buffer	2.0

[a] Run time = 24 min, configured to operate in single-cartridge mode only.

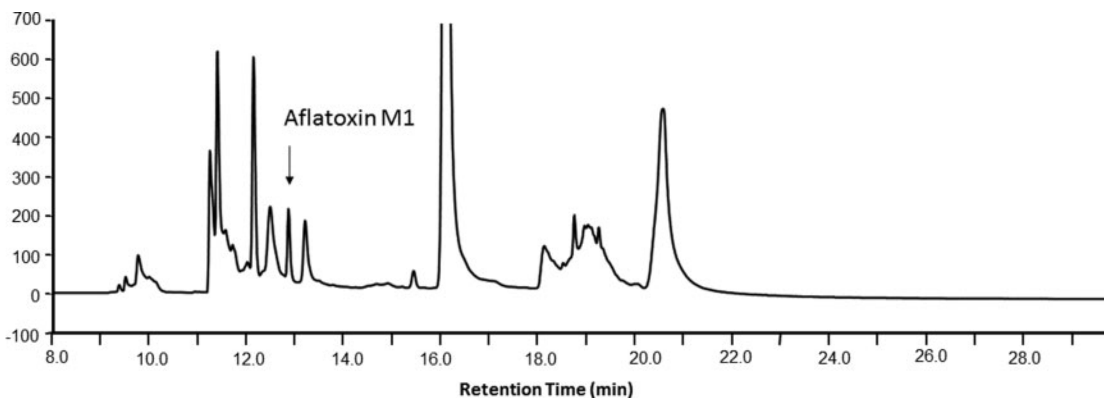

Figure 2. Chromatogram of aflatoxin M1 in whey powder.

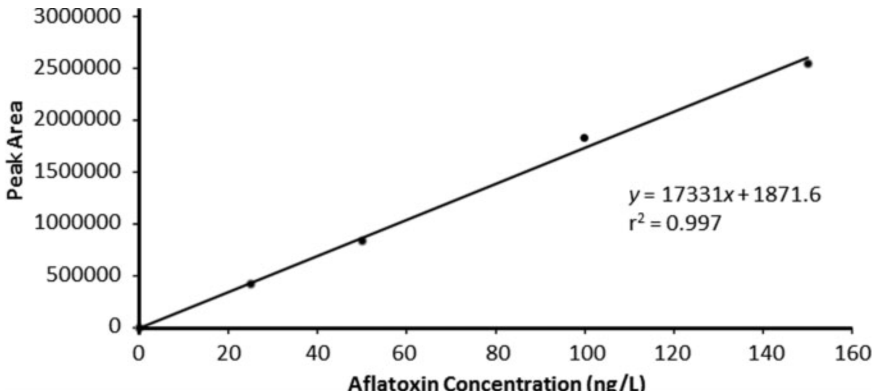

Figure 3. Calibration curve for aflatoxin M1 showing linear detector response.

Table 3. Recovery of aflatoxin M1 from a range of spiked matrices

	Average recovery, %	
Sample	50 ng/L	100 ng/L
Whey powder	90	102
Whey protein isolate	97	103
Whey protein concentrate	88	101
Milk protein concentrate	112	108
Milk	99	85
Cheese	80	81

Because of the absence of appropriate certified reference material, a cheese sample containing AFM1 from the FAPAS proficiency scheme with assigned mean and range values was analyzed. The FAPAS cheese sample had an assigned value of 2.005 ± 0.510 μg/kg (uncertainty at 95% confidence interval) with duplicate results obtained using this method of 1.705 and 2.005 μg/kg, yielding a z-score of −1.42, below the limit of |2| for outlying results. This method was also compared with duplicate 50 ng/L spiked WPI and WPC samples, which were prepared and analyzed using the manual immunoaffinity column method. The results of this study are shown in Table 4. The manual immunoaffinity and HPLC method was based on reference methods (AOAC Offiical Method **2000.08** and IDF 171, 28, 29) with minor modifications: (1) the milk samples were dissolved in 25 mL water; (2) aflatoxin was extracted from the cartridges using 7.5 mL acetonitrile with back flushing; (3) analytical chromatography conditions were isocratic elution with 30% acetonitrile at 0.7 mL/min on a 5 μm, 150 × 4.6 mm Prodigy column (Phenomenex).

Method precision was evaluated by the analysis of duplicates of WP, WPI, WPC, MPC, liquid milk, and cheese samples spiked with 50 ng/L AFM1. Acceptable precision was demonstrated, with a repeatability of between 3.7 and 11.7% RSD, an intermediate precision of between 4.5 and 12.8% RSD, and

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calculated HorRat values between 0.3 and 1.2 (30). The full precision results are shown in Table 5.

The instrumental LOD was estimated by serial dilution of the AFM1 until a signal-to-noise ratio of three was obtained, yielding a measured value for the LOD of 0.010 ng/mL in diluent.

Method robustness was investigated by performing a Plackett–Burman trial (31, 32), as previously described (33). The seven factors assessed were: extract volume (9 and 11 mL), volume of extraction solvent (18 and 22 mL), shaking time (105 and 75 min), centrifuge speed (3450 and 3350 × *g*), centrifuge time (12 and 8 min), Triton X-100 drops (2 drops and ½ normal drop), and a dummy factor (G, g). The method was found to be robust for the parameters evaluated, and the results obtained were normally distributed, with variances conforming to those expected by chance (Figure 4). As with similar external-standard-based methods, critical method parameters included accurate measurement of the sample weight, extract volume, and aliquot volume.

Table 4. Comparison of aflatoxin M1 results measured by automated online (cartridge) and manual (column) cleanup in whey protein concentrate and whey protein isolate spiked at 50 ng/L

Sample	RIDA®CREST ICE, ng/L	Manual column, ng/L
Whey protein concentrate	43–55 (48)[a]	46–48 (47)[b]
Whey protein isolate	49–64 (55)[c]	49–66 (58)[b]

[a] Range and mean of 12 replicates.
[b] Range and mean of 2 replicates.
[c] Range and mean of 11 replicates.

Table 5. Precision results from aflatoxin M1 samples spiked at 50 ng/L

Sample	Repeatability, RSD, % (HorRat)	Intermediate precision, RSD, %
Whey powder	9.6 (1.2)	12.6
Whey protein isolate	5.6 (1.1)	12.0
Whey protein concentrate	3.7 (0.7)	7.3
Milk protein concentrate	11.7 (0.9)	10.9
Milk	4.8 (0.3)	4.5
Cheese	9.8 (0.3)	12.8

The RIDACREST ICE offers a practical solution to the requirement for the high sample throughput (up to 42 samples in a single run per day) needed for measuring AFM1 for global compliance with maximum regulatory limits for AFM1 in milk and milk-based products (34, 35).

Conclusions

A method that is intended for use in high-throughput laboratories as part of routine product compliance release testing, to show that these dairy products contain less than the maximum regulatory levels of AFM1, was developed. This method was subjected to single-laboratory validation and was found to be accurate, precise, and fit-for-purpose.

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Conflict of interest

The authors declares that they have no conflict of interest.

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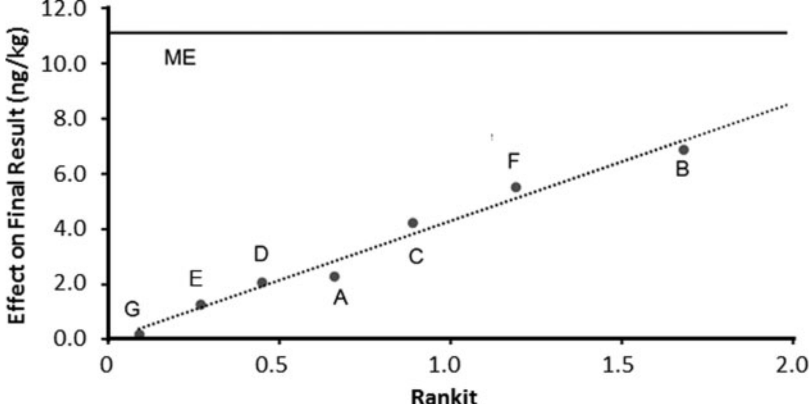

Figure 4. Half-normal plot of method ruggedness evaluation for aflatoxin M1 in whey protein isolate.

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