



FOOD CHEMICAL CONTAMINANTS

Enhanced Automated Online Immunoaffinity Liquid Chromatography–Fluorescence Method for the Determination of Aflatoxin M₁ in Dairy Products

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Abstract

Background: Aflatoxin M₁ (AFM₁) is found in the milk of cows exposed to feed spoiled by *Aspergillus* fungi species. These fungi may produce the secondary metabolite aflatoxin B₁, which is converted in the cow liver by hydroxylation to AFM₁ and is then expressed in milk. AFM₁ is regulated in milk and other dairy products because it can cause serious health issues, such as liver and kidney cancers, in humans and is an immunosuppressant.

Objective: To optimize the chromatographic protocol and to extend the matrix scope to include a wider range of dairy products: whey powder, whey protein concentrate, whey protein isolate, liquid milk, skim milk powder, whole milk powder, adult nutritional products, and yogurt.

Methods: AFM₁ is extracted using 1% acetic acid in acetonitrile incorporating ionic salts. The AFM₁ in the resulting extract is concentrated using an automated RIDA[®] CREST IMMUNOPREP[®] online cartridge coupled to quantification by HPLC–fluorescence.

Results: The method was shown to be accurate, with acceptable recovery (81.2–97.1%) from spiked samples. Acceptable precision was confirmed, with a relative standard deviation (RSD) for repeatability of 6.6–11.2% and an RSD for intermediate precision of 7.5–16.7%. Method LOD and robustness experiments further demonstrated the suitability of this method for routine compliance testing. Analysis of an international proficiency trial sample generated results that were comparable with the value assigned from alternative independent methods.

Conclusion: A method with improved chromatography for high-throughput, routine testing of AFM₁ in an extended range of dairy products is described. The method was subjected to single-laboratory validation and was found to be accurate, precise, and fit for purpose.

Highlights: Single-laboratory validation of an automated online immunoaffinity cleanup fluorescence HPLC method for AFM₁ in whey proteins, milk powders, nutritional products, liquid milk, and yogurt. Allows for high-throughput analysis of AFM₁ with enhanced chromatographic performance. Method applicable to the analysis of AFM₁ in an extended range of milk and milk-based products.

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Aspergillus fungi grow primarily on cereal grain, corn, soyabean concentrates, and other cow feeds in tropical and subtropical conditions (1, 2), with aflatoxin B₁ being a secondary metabolite (3). Aflatoxin M₁ (AFM₁) is a liver-hydroxylated metabolite of aflatoxin B₁ and may be found in the milk from cows that have ingested feed contaminated with *Aspergillus flavus* and *A. parasiticus* (4, 5). Because of the climatic conditions and the predominant pasture-feeding practice, the presence of AFM₁ in the milk of the New Zealand dairy herd is a rare event (6–8). AFM₁ is a highly regulated potential contaminant in dairy products because of the multiple serious and deleterious human health conditions that it can induce when consumed (9–11). The Codex regulatory limit for AFM₁ in milk is 0.5 µg/kg and the European regulatory limit is 0.05 µg/kg (12, 13). AFM₁ at or above regulatory limits in raw bovine milk has commonly been reported in several countries as part of global surveys (14, 15), and, because AFM₁ is not degraded by pasteurization, it will be found subsequently in processed dairy products (16–18).

A wide variety of quantitative chromatographic or semi-quantitative ELISA and biosensor methods to measure the AFM₁ content in foods have been developed (19–21). Irrespective of the quantitative end point technique used, most methods reported for the quantitation of AFM₁ in complex food matrices rely on highly manipulative and time-consuming manual solid-phase extraction or immunoaffinity cartridge cleanup, thereby limiting the overall sample throughput (19, 21). Recently, methods utilizing online solid-phase extraction or immunoaffinity cleanup with either LC-MS or HPLC-fluorescence detection have been published (22, 23). It has previously been reported that, because of the high specificity of the binding antibody, immunoaffinity cleanup facilitates superior performance and that fluorescence detection can be more sensitive than LC-MS for aflatoxins (19, 24).

ELISA has been widely used to screen milk and some dairy product samples for AFM₁ because it is rapid, less costly, has fewer cleanup steps, and uses small sample sizes (19, 21, 25). However, as ELISA can suffer both from cross-reactivity with similar compounds and from matrix interferences, confirmatory analysis is usually performed using HPLC with either MS or fluorescence detection (21, 26, 27). Screening methods for the analysis of AFM₁ using electrochemical and optical biosensors have also been reported; however, these are not commonly utilized by analytical food chemistry laboratories (28–30). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS has been used to screen for other aflatoxins, with a recent quantitative method coupling MALDI-TOF with triple quadrupole MS to quantify AFM₁ in milk (31, 32).

Only one method has reported a fully automated technique that couples both immunoaffinity cleanup and LC in an integrated system for the analysis of AFM₁ in selected dairy products (33). The present work reports the enhancement of that methodology through a more robust and selective chromatographic protocol and a wider scope of sample type. The chromatography for the previous method was achieved utilizing a phenyl-hexyl chemistry that allowed for the analysis of samples spiked with AFM₁ as it has a very characteristic peak; however, the chromatogram was relatively congested with unidentified peaks with retention times close to that of AFM₁. This may lead to confusion, particularly when AFM₁ is either absent or close to the LOD in the sample, with the risk of misidentification of AFM₁. The improved chromatography system reported in this study utilized octadecylsilane column chemistry, as used by AOAC INTERNATIONAL and International Dairy Federation (IDF) reference methods; however, it was

implemented with gradient rather than isocratic elution, thereby facilitating superior and unequivocal peak resolution (34, 35).

This manuscript describes the validation of an improved high-throughput method for the routine analysis of AFM₁ in a variety of dairy products including whey powder, whey protein concentrate, whey protein isolate, liquid milk, skim milk powder, whole milk powder, adult nutritional products (supplementary powders for expectant mothers, sport nutritionals, and special dietary powders), and yogurt. Cheese and milk protein concentrate contain higher concentrations of casein than other products and were excluded from this study as low recovery was observed, putatively because of the known binding affinity of casein for AFM₁ (36).

Experimental

Apparatus

- (a) *Pipettes*.—Research plus, 20 and 200 µL, and 1 and 10 mL (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, USA), calibrated with NIST (Gaithersburg, MD) traceable calibration weights.
- (f) *HPLC column*.—Prodigy octadecylsilane 5 µm, 4.6 mm × 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). RIDA[®]CREST ICE controlled by Clarity software version 8.2 (R-Biopharm, Darmstadt, Germany).
- (h) *Immunoaffinity cartridges*.—IMMUNOPREP[®] ONLINE AFM₁ cartridges (R-Biopharm Rhone, Glasgow, United Kingdom).
- (i) *Graduated cylinders*.—100, 250, and 1000 mL.
- (j) *Volumetric flasks*.—500 and 1000 mL.
- (k) *HPLC injection vials*.—Amber, 2 mL with Teflon-coated caps.
- (l) *Conical flasks*.—250 mL.
- (m) *Linear shaker*.—HS 501 digital (Ika-Werke, Staufen, Germany).
- (n) *pH Meter*.—S220 pH/Ion meter (Mettler Toledo).
- (o) *Evaporator*.—Techne sample concentrator (Cole-Palmer, Vernon Hills, IL).
- (p) *Glass test tubes*.—15 mL.
- (q) *Nylon syringe filters*.—0.2 µm, Phenex (Phenomenex).
- (r) *Disposable 6 mL plastic syringes*.—(Electrolube, Brookvale, NSW, Australia).
- (s) *HPLC sample vials*.—1.5 mL, screw-top, polypropylene (Machery Nagel, Düren, Germany).

Reagents

- (a) *Acetic acid (CH₃COOH)*.—Reagent grade (Mallinckrodt, Staines, United Kingdom).

- (b) Ammonium acetate ($\text{NH}_4\text{CH}_3\text{COO}$).—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).—Purified to $\geq 18.2 \text{ M}\Omega/\text{cm}$ with Genpure water system (ThermoFisher).
- (f) Acetonitrile (CH_3CN).—HPLC grade (Mallinckrodt).
- (g) Nitric acid (HNO_3).—Reagent grade (Mallinckrodt).
- (h) Triton X-100.—Reagent grade (Mallinckrodt).
- (i) SupelTMQuE citrate extraction tubes.—Reagent grade, each containing 4 g magnesium sulfate, 1 g sodium chloride, 1 g sodium citrate tribasic dihydrate, and 0.5 g sodium citrate dibasic sesquihydrate (Sigma Aldrich).
- (j) Tris(hydroxymethyl)aminoethane.—Reagent grade (Sigma Aldrich).
- (k) AFM_1 stock standard.—500 $\mu\text{g/L}$ (R-Biopharm Rhone, Glasgow, UK).
- (l) Isopropanol ($\text{CH}_3\text{CH}(\text{OH})\text{CH}_3$).—HPLC grade (ThermoFisher).

Solutions

- (a) Extraction solution.—5 mL acetic acid and 495 mL acetonitrile.
- (b) Sodium hydroxide solution (1 M).—4 g sodium hydroxide pellets were dissolved in 100 mL water.
- (c) Loading buffer.—1.54 g ammonium acetate was dissolved in 1 L water, with the pH adjusted to 6.8–7.0 using sodium hydroxide solution, (b).
- (d) Reconstitution buffer.—450 mL loading buffer, (c), was mixed with 50 mL methanol.
- (e) Elution buffer.—3.85 g ammonium acetate was dissolved in 640 mL water; 100 mL acetonitrile, and 260 mL methanol were added and the pH was adjusted to 2.0 using concentrated nitric acid.
- (f) Cartridge wash buffer.—1.54 g ammonium acetate and 3.02 g tris(hydroxymethyl)aminomethane were dissolved in 875 mL water, 125 mL methanol was added, and the pH was adjusted to 8.0 with nitric acid.
- (g) Mobile phase A.—Water.
- (h) Mobile phase B.—Acetonitrile.
- (i) Autosampler wash.—250 mL water and 250 mL acetonitrile.
- (j) Pump seal wash.—Isopropanol.

Standards

An intermediate AFM_1 standard solution (10 $\mu\text{g/L}$) was prepared by diluting 0.020 mL of the 500 $\mu\text{g/L}$ stock standard with 0.98 mL acetonitrile. Standards of 0.025, 0.05, 0.15, and 0.2 $\mu\text{g/L}$ were prepared by dilution of the intermediate standard and used to validate detector linearity.

The spike standard was prepared by diluting 100 μL of the intermediate standard with 9.9 mL reconstitution buffer.

Samples

Samples of whey powder (WP), whey protein concentrate (WPC), whey protein isolate (WPI), liquid milk, skim milk powder (SMP), whole milk powder (WMP), adult nutritional products (ANP), and yogurt that were known to be free of AFM_1 were used to carry out spiked recovery experiments. A WMP interlaboratory proficiency scheme sample with an assigned consensus value of 0.026 $\mu\text{g/kg}$ of AFM_1 (Fapas, York, United Kingdom) was used during the evaluation of method accuracy.

Sample Preparation

Whey protein solutions were made by dissolving WP, WPC, and WPI powders ($18 \pm 0.05 \text{ g}$) into separate 250 mL conical flasks with 150 mL water. The flask was placed on a hot plate stirrer at 50°C, mixed with a stir bar for 30 min, and then cooled to room temperature. The whey protein solutions ($10 \pm 0.05 \text{ g}$) were weighed into 50 mL centrifuge tubes, to which 50 and 100 μL spike standards (0.05 and 0.1 $\mu\text{g/L}$) were added.

Milk ($10 \pm 0.01 \text{ g}$) and whey protein solutions ($10 \pm 0.05 \text{ g}$) were accurately weighed in 50 mL centrifuge tubes, to which 50 and 100 μL spike standards (0.05 and 0.1 $\mu\text{g/L}$) were added.

Yogurt ($5 \pm 0.05 \text{ g}$), and SMP and WMP ($4 \pm 0.05 \text{ g}$) were accurately weighed in 50 mL centrifuge tubes, to which 50 and 100 μL spike standards (0.05 and 0.1 $\mu\text{g/L}$) were added. Water (50°C, 10 mL) was added, and the sample was cooled to room temperature.

ANP ($1.4 \pm 0.05 \text{ g}$) was accurately weighed in 50 mL centrifuge tubes, to which 50 and 100 μL spike standard (0.05 $\mu\text{g/L}$) was added. Water (50°C, 10 mL) was added, and the sample was cooled to room temperature.

Sample Extraction

Extraction solution (20 mL) and the citrate salts in one extraction tube were added to the prepared sample solutions and vortex mixed. The sample tubes were shaken (200 revolutions per min, 90 min) and centrifuged (3400 relative centrifugal force, 10 min). The top acetonitrile layer (2 mL) was transferred to a test tube, one drop of Triton X100 was added, and the sample was dried under nitrogen at 60°C until a small viscous residue remained. Reconstitution buffer (2 mL) was added, and the test tube was vortex mixed. A syringe filter was used if the sample extract was cloudy, prior to transfer of a minimum of 1.5 mL to an autosampler vial.

Online Immunoaffinity–HPLC Conditions

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

Quantitation

Routine quantitation of the AFM_1 content in samples was performed by interpolation of the calibration curve (forced through

Table 1. RIDACREST 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 |

^aRun time = 49 min, configured to operate in single-cartridge mode only, with each cartridge used in accordance with manufacturer's instructions, a maximum of 15 times.

zero) of a single-level external standard (0.05 µg/L) subjected to the entire procedure.

Final results were calculated as in Equation (1):

$$\text{AFM}_1 \text{ (ng/g)} = \frac{A}{L} \times \frac{2}{M} \quad (1)$$

where A = peak area of AFM₁ in sample; L = slope of single point calibration curve; 2 = volume of extracted sample (mL); and M = mass of sample (g).

Results and Discussion

Method Optimization

To facilitate extraction and to avoid potential matrix interferences during chromatographic detection, QuEChERS (Quick Easy Cheap Effective Rugged Safe) ionic salts mixtures are commonly used during the extraction of milk products prior to immunoaffinity cleanup (37). The efficacy of these salts in optimizing recovery during the solvent extraction of AFM₁ from milk has recently been demonstrated (38).

Table 2. HPLC gradient conditions used following automated cartridge cleanup

| Time, min | Flow rate, mL/min | Mobile phase composition | |
|-----------|-------------------|--------------------------|-------------------|
| | | A, % ^a | B, % ^b |
| 0.0 | 0.7 | 80 | 20 |
| 5.0 | 0.7 | 80 | 20 |
| 5.3 | 0.1 | 80 | 20 |
| 8.0 | 0.1 | 80 | 20 |
| 8.3 | 1.0 | 80 | 20 |
| 10.7 | 1.0 | 80 | 20 |
| 12.7 | 1.0 | 75 | 25 |
| 26.0 | 1.0 | 32 | 68 |
| 29.0 | 1.0 | 32 | 68 |
| 32.0 | 1.0 | 50 | 50 |
| 36.0 | 1.0 | 100 | 0 |
| 37.0 | 1.0 | 80 | 20 |
| 49.0 | 1.0 | 80 | 20 |

^aMobile phase A = water.

^bMobile phase B = acetonitrile.

The use of polypropylene vials was adopted to avoid the risk of AFM₁ binding to the vial surface, which can occur when non-silanized glass vials are used (34).

The modified chromatographic protocol implemented in this enhanced method was developed to overcome the partial co-elution of unknown compounds with AFM₁, which was occasionally observed in milk powder samples with the previous method (33). Initially, chromatography on the octadecylsilane column was evaluated isocratically with different percentages of the modified eluents as reported in IDF/ISO (International Standards Organisation) and AOAC methods; however, it was determined that gradient elution consistently yielded an AFM₁ peak that was fully resolved from other unidentified compound peaks, thereby achieving unequivocal identification and quantitation (Figure 1).

Method Validation

Method validation procedures were performed consistent with those described by the Stakeholder Program on Infant Formula and Adult Nutritionals (39). These procedures describe the estimation of parameters including recovery, LOD, and precision.

Linearity was demonstrated through the analysis of multilevel AFM₁ standard solutions (n = 4) covering an analyte range of 0.025–0.2 µg/L by direct injection, bypassing the RIDACREST ICE and yielding a linear regression with a correlation coefficient of 0.9982 (Figure 2).

Method recovery was determined by spiking samples of WP, WPC, WPI, SMP, WMP, ANP, liquid milk, and yogurt with 0.05 and 0.1 µg/L AFM₁ (Table 3). Average recoveries were estimated as 81–97% and are consistent with a prescribed expected recovery (50–120%) at <1 µg/kg concentration (40).

Method precision was evaluated by the analysis of independent duplicates of WP, WPI, WPC, WMP, SMP, ANP, liquid milk, and yogurt with 0.05 µg/L AFM₁. Acceptable precision was demonstrated, with a within-day repeatability of 6.6–11.2% RSD_r, and calculated HorRat values of 0.3–0.5. Intermediate precision was calculated from independent samples of the same matrix, analyzed on different days and was estimated as 7.5–16.7% RSD_{IR} (Table 3).

The instrumental LOD was estimated by serial dilution of the AFM₁ standard solution until a S/N of 3 was obtained, yielding a measured value of 0.01 µg/L. This value is equivalent to an LOD of 0.0025 µg/kg in SMP and WMP, 0.0072 µg/kg in ANP,

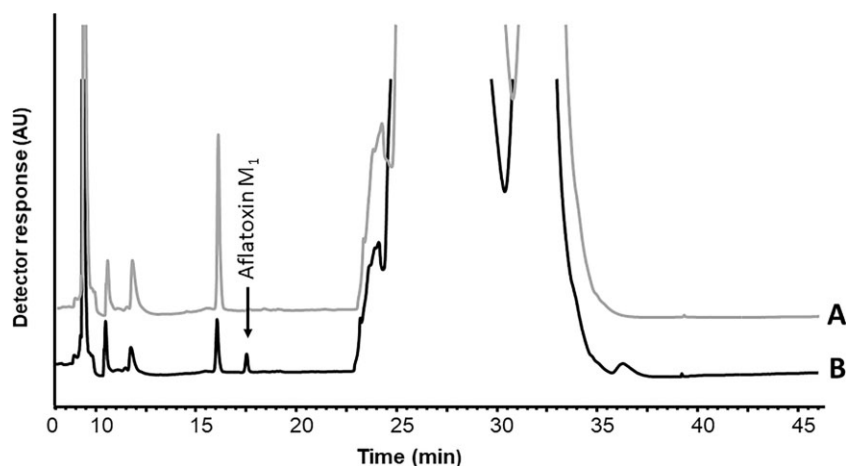


Figure 1. Overlaid chromatograms showing either unspiked skim milk powder (A) or spiked with 0.010 µg/kg of aflatoxin M₁ (B).

0.002 µg/kg in yogurt, 0.001 µg/kg in milk, and 0.0084 µg/kg in whey powders.

Method robustness was investigated by performing a Plackett–Burman trial as previously described (41). The seven factors assessed were: extract volume (9.5, 10.5 mL), centrifuge speed (2450, 2550 × g), shaker time (80, 100 min), Triton drops (2, 0.5), reconstitution volume (1.9, 2.1 mL), aflatoxin spike amount (0.045, 0.055 µg/L), vortex before shaking (yes, no), and a dummy factor. The method was found to be robust for the parameters evaluated, and the results obtained were normally distributed, with variances conforming to that expected by chance (Figure 3). As with all external standard-based methods, critical method parameters included accurate measurement of the sample weight, extract volume, and aliquot volume.

A WMP interlaboratory proficiency scheme sample was analyzed using this method and yielded an AFM₁ concentration of 0.021 µg/kg. This sample has previously been analyzed for AFM₁ content with a range of methods, including ELISA, LC–MS, HPLC–fluorescence, and immunoaffinity optical biosensor, which gave a range of AFM₁ concentrations (0.016–0.036 µg/kg), with an average of 0.026 µg/kg and a SD of 0.008 µg/kg, as shown in Table 4 (29). Single-factor analysis of variance suggested that there was no difference between methods ($P=0.58$). The

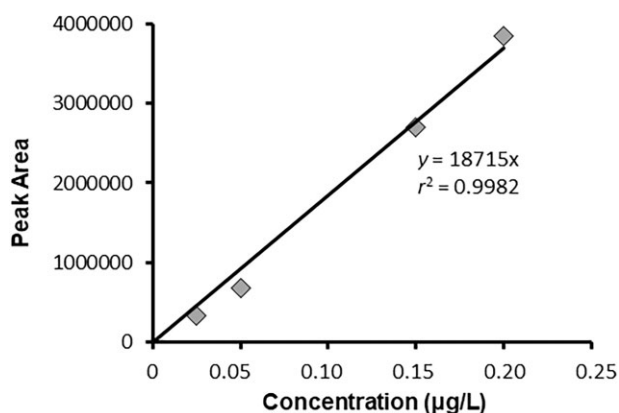


Figure 2. Calibration curve with linear detector dose response (forced through zero).

equivalence of quantitative data from independent analytical methods is generally considered to be indicative of an unbiased estimate of analyte content. Despite the fundamentally different analytical principles used in the method comparison, all techniques yielded a comparable estimate of AFM₁ content with no significant overall differences, confirming that each method provides a reliable estimate of the AFM₁ content in dairy products.

The present study describes an enhancement of the chromatographic separation protocol and extends the scope of the

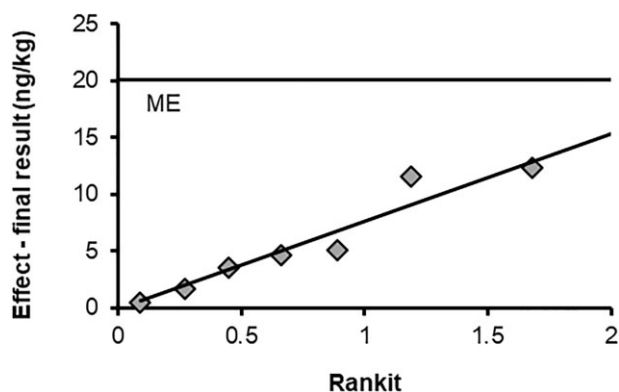


Figure 3. Half-normal plot demonstrating robustness of the aflatoxin M₁ method in liquid milk.

Table 4. Comparison of aflatoxin M₁ content in an interlaboratory proficiency trial sample (µg/kg)

| Method | Concentration mean (range) |
|---|----------------------------|
| Immunoaffinity HPLC–fluorescence ^a | 0.021 |
| Optical immunoassay ^b | 0.027 (0.019–0.036) |
| ELISA ^b | 0.026 (0.020–0.036) |
| LC–MS/MS ^b | 0.026 |
| HPLC–fluorescence ^b | 0.023 (0.016–0.030) |

^aCurrent method.

^bFrom reference 29 with permission from Springer Nature.

Table 3. Recovery and precision of aflatoxin M₁ from a range of matrixes spiked at 0.05 µg/L and 0.1 µg/L

| Sample | 0.05 µg/L average recovery, % | 0.1 µg/L average recovery % | Repeatability RSD, %, (HorRat) | Intermediate precision RSD, % |
|-------------------|-------------------------------|-----------------------------|--------------------------------|-------------------------------|
| WP ^a | 82.8 (n=6) ^b | 87.2 (n=3) | 8.7 (0.4) | 7.5 (d=3) ^c |
| WPI ^d | 93.0 (n=6) | 83.7 (n=3) | 8.9 (0.4) | 13.4 (d=3) |
| WPC ^e | 95.4 (n=8) | 90.1 (n=3) | 10.6 (0.5) | 9.8 (d=3) |
| Adult nutritional | 97.1 (n=3) | ND ^f | 7.1 (0.4) | 16.7 (d=2) |
| Liquid milk | 83.9 (n=6) | 83.6 (n=3) | 6.6 (0.3) | 7.9 (d=3) |
| Yogurt | 87.1 (n=6) | 84.8 (n=3) | 6.7 (0.3) | 9.4 (d=3) |
| WMP ^g | 82.6 (n=6) | 84.4 (n=3) | 9.8 (0.5) | 9.4 (d=3) |
| SMP ^h | 88.0 (n=6) | 87.3 (n=3) | 11.2 (0.5) | 9.1 (d=3) |

^aWP = Whey protein.

^bn = Number of replicates.

^cd = Number of days.

^dWPI = Whey protein isolate.

^eWPC = Whey protein concentrate.

^fND = Not determined.

^gWMP = Whole milk powder.

^hSMP = Skim milk powder.

previously published method (33). This method has several advantages as it (1) complies with the recommended regulatory procedures for the quantitative analysis of AFM₁ (34, 35), (2) incorporates online, selective immunoaffinity purification facilitating a high throughput of samples, (3) utilizes highly sensitive fluorescence detection, and (4) uses a modified HPLC system that is significantly less costly and simpler in operation compared with alternative LC-MS systems.

The automated RIDACREST ICE-HPLC-fluorescence coupled platform allows for high-throughput analysis of the AFM₁ content in manufactured dairy products in the analytical range below and above regulatory limits (0.5 µg/kg for Codex and 0.05 µg/kg for European regulations) for milk and milk-based products (12, 13).

Conclusions

An improved chromatographic method, intended for use in high-throughput laboratories as part of routine product compliance release testing to demonstrate that dairy products contain less than the maximum regulatory levels of AFM₁, is described. The method was subjected to single-laboratory validation and was determined to be accurate, precise, and fit for purpose.

CRedit Author Statement

Jackie Wood: Conceptualization, Formal analysis, Methodology, Validation, Writing—original draft. **Brendon Gill:** Conceptualization, Formal analysis, Visualization, Writing—review & editing. **Iain McGrail:** Methodology, Resources. **Harvey Indyk:** Conceptualization, Writing—review & editing.

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Conflict of Interest

None of the authors have any conflict of interest.

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