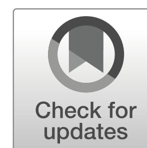
Food Analytical Methods
https://doi.org/10.1007/s12161-019-01621-5



Development and Application of an Optical Biosensor Immunoassay for Aflatoxin M_1 in Bovine Milk

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Received: 15 April 2019 / Accepted: 31 July 2019
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Abstract

An automated optical biosensor-based immunoassay exploiting surface plasmon resonance detection for the quantitation of aflatoxin M_1 (AFM_1) in milk and milk powders is described. A monoclonal antibody and an immobilized protein–AFM_1 conjugate are utilized in a simple inhibition format following aqueous extraction and immunoaffinity clean-up of the sample, thereby avoiding the need for signal amplification techniques. The sensor surface is stable over multiple regeneration cycles, and the technique yields a method detection limit of 0.1 ng g^{-1}, which is five times lower than the European Commission maximum residue limit. The described antibody-based biosensor technique provides the advantages of quantitative data, automation, and real-time and non-labeled detection of AFM_1. The method therefore facilitates routine quantitative threshold-level screening for the identification of potential non-compliance of AFM_1 content prior to confirmatory analysis by reference chromatographic methods and may be considered to complement the enzyme-linked immunosorbent assay technique.

Keywords Aflatoxin M_1 · Milk · Biosensor · Surface plasmon resonance · Immunoassay

Introduction

The aflatoxins B_1, B_2, G_1, and G_2 are mycotoxins produced as secondary metabolites of *Aspergillus* molds, which can contaminate feed and food with potentially severe health consequences. Aflatoxin M_1 (AFM_1) is produced in ruminants through hepatic hydroxylation of AFB_1 and is expressed in the milk of dairy cows that have ingested contaminated feed. AFM_1, if present in raw milk, is persistent in manufactured dairy products due to its thermal stability. Among the mycotoxins, the presence of aflatoxins in dairy products is regarded with the greatest concern, as has been reviewed (Benkerroum 2016; Campagnollo et al. 2016; Iqbal et al. 2015; Ketney et al. 2017; Mohammadi 2011).

As significant components of the human diet, milk and milk products are regulated internationally with respect to AFM_1 content in view of its profound toxicity, with the European Commission establishing a maximum limit for liquid milk of 50 ng L^{-1} (0.5 μg kg^{-1} milk powder), whereas the USA and China have adopted a maximum limit for liquid milk of 500 ng L^{-1} (5 μg kg^{-1} milk powder), with more restrictive limits set for infant foods (Campagnollo et al. 2016; Ketney et al. 2017). Recognition of the risks associated with aflatoxin consumption via milk and milk products has resulted in a rapidly expanding published global literature (Klingelhöfer et al. 2018), and the many studies of the occurrence and worldwide distribution of AFM_1 in dairy products have been comprehensively reviewed (Campagnollo et al. 2016; Iqbal et al. 2015; Ketney et al. 2017; Mohammadi 2011).

Currently, the overwhelmingly predominant methods deployed for the analysis of AFM_1 in dairy products are based either on (1) solid-phase extraction (SPE) or, more commonly, immunoaffinity column purification followed by high-performance liquid chromatography (HPLC) coupled with fluorescence or mass spectrometric detection, or on (2) immunochemical techniques exploiting primarily the biospecificity of antibodies (with recent developments in molecular-imprinted-polymer and aptamer biorecognition), of which the enzyme-linked immunosorbent assay (ELISA) is by far the most commonly implemented. The latter methods are generally deployed as qualitative or semi-quantitative routine screening methods, in view of their operational simplicity compared with the HPLC methods that are internationally mandated for confirmatory analysis. Recent reviews report that the many commercially available ELISA kits generally exploit a competitive assay format, in view of the low

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Published online: 10 August 2019

molecular mass of AFM_1, and are acknowledged to be vulnerable to analytical variance and false-positive results, attributed to cross-reactivity and sample matrix non-specific binding interferences (Campagnollo et al. 2016; Iqbal et al. 2015; Ketney et al. 2017; Li et al. 2014; Maragos 2004; Shephard 2016). Nonetheless, ELISAs typically exhibit high sensitivity due to inherent enzyme signal amplification, whereas alternative label-free biosensor platforms have advantages of speed, real-time and direct detection, automation, reusability, and avoidance of potential label influences (Maragos 2016).

The continuing evolution of antibody-based affinity biosensors represents a complementary analytical strategy for the screening of low-molecular-mass food contaminants for possible regulatory non-compliance. Biosensors combine a biological recognition entity interfaced with a physiochemical transducer exploiting spectrophotometric, optical, piezoelectrical or electrochemical principles, and optical evanescent wave techniques, including the fiber optic and surface plasmon resonance (SPR) devices which have been reviewed (Maragos 2004). Although biosensors exploiting electrochemical transduction have been the most commonly described platform, real-time and label-free optical SPR transduction techniques that detect changes in refractive index, associated with biomolecular binding events at a regenerable sensor surface, have proliferated for food-contaminant screening (McGrath et al. 2012; Thompson et al. 2017). The magnitude of refractive index change due to interaction between analyte in solution and immobilized ligand at the sensor surface can be related to concentration under controlled conditions. These techniques have predominantly been applied to the analysis of AFB_1 in food and feed, with fewer applications described for AFM_1 in milk and dairy products (Vidal et al. 2013). An emerging technology exploiting an aptamer-based biosensor incorporating a silicon-based photonic ring resonator has also demonstrated potential for the detection of AFM_1 (Guider et al. 2015). SPR-based biosensor methods configured in an inhibition format have been reported for the determination of AFB_1, although no data for samples were presented and surface regeneration difficulties were described (Daly et al. 2000; van der Gaag et al. 2003). Two SPR-based biosensor methods for the quantitation of AFM_1 in milk exploiting different novel amplification strategies to enhance the limited analytical sensitivity of conventional SPR platforms have been reported (Karczmarczyk et al. 2016; Wang et al. 2009).

The present study describes the development of an SPR-based quantitative screening immunoassay for the determination of AFM_1 in milk that avoids the requirement for amplification techniques and may therefore be applied in a routine compliance testing environment.

Materials and Methods

Instrumentation

The Biacore® Q SPR biosensor instrument and data processing software version 3.0.5 and CM5 sensor chips were from GE Healthcare (Uppsala, Sweden). The multi-flow cell addressable Biacore® T200 biosensor and Series S CM5 chips were also used during method development.

Reagents

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–HCl (EDC, 0.4 mol L^{-1}), *N*-hydroxysuccinimide (NHS, 0.1 mol L^{-1}), ethanolamine–HCl (1 mol L^{-1}, pH = 8.5), sodium acetate buffer (10 mmol L^{-1}, pH = 4.5 and 5.0), and HBS-EP buffer (10 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl, 3.4 mmol L^{-1} EDTA, 0.005% surfactant P20, pH = 7.4) were obtained from GE Healthcare (Uppsala, Sweden). All water used was of > 18 MΩ resistivity.

AFM_1 (CRM46319, 10 μg mL^{-1} in acetonitrile), BSA–AFM_1 conjugate (A6412, 1 mg), bovine serum albumin (BSA; A7030), and phosphate-buffered saline (PBS; pH = 7.4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Four commercially available affinity-purified antibodies to AFM_1 were sourced: (1) rat monoclonal (AB 685, 1 mg mL^{-1}, clone 1C6, Abcam, Cambridge, UK), (2) lyophilized rabbit polyclonal (ABIN 343758, Antibodies-online, Aachen, Germany), (3) lyophilized goat polyclonal (ASI5 2827, Agrisera, Vannas, Sweden), and (4) lyophilized rabbit polyclonal (ASI5 2828, Agrisera, Vannas, Sweden). Stock antibody solutions (1 mg mL^{-1}) were prepared in water and were stored for up to 6 months at − 18 °C. Intermediate antibody solutions (10 μg mL^{-1}) were prepared in HBS-EP and were stable for at least 4 months at 4 °C.

Aflaprep M Wide immunoaffinity columns (P124) with an AFM_1 capacity of at least 100 ng were obtained from R-Biopharm Rhone (Glasgow, Scotland) and were stored at 4 °C.

Biosensor Surface Preparation

Immobilization of BSA and BSA–AFM_1 conjugate to separately assigned flow cells of a CM5 sensor chip was achieved by conventional amine-coupling at 25 °C. Each surface was activated with a mixture of EDC and NHS (1:1 *v*/*v*) at 10 μL min^{-1} for 7 min, followed by ligand (40 μg mL^{-1} in 10 mmol L^{-1} sodium acetate, pH = 4.5) at 10 μL min^{-1} for 7 min. A reference flow cell was prepared by omitting the ligand immobilization step. Following immobilization, unreacted ester functionalities were deactivated with ethanolamine (1 mol L^{-1}, pH = 8.5) at 10 μL min^{-1} for 7 min.

Between analyses, functionalized chips were stored in a sealed container over silica gel desiccant at 4 °C.

Standards

An AFM_1 stock standard (10 μg mL^{-1}) was prepared by evaporating to dryness 100 μL of AFM_1 stock under nitrogen and re-dissolving in 100 μL of HBS-EP. An intermediate AFM_1 standard (1000 ng mL^{-1}) was prepared by dilution in HBS-EP and was stored at 4 °C for up to 4 months. Daily calibration standards (10, 1, 0.75, 0.5, 0.25, 0.1, and 0.01 ng mL^{-1}) were prepared by serial dilution of the intermediate standard in HBS-EP. These procedures were performed under low-level incandescent light.

Samples

Whole milk powders and liquid milks were obtained locally in New Zealand. In addition, milk powder samples were sourced from two rounds of the Fapas proficiency program (York, UK), with assigned AFM_1 content estimated from diverse sample extraction and end-point quantitation methods used by multiple participants.

Sample Preparation

Sample preparation was performed under conditions of low level incandescent light. Milk powder (1–2 g) or liquid milk (10–20 mL) samples were weighed accurately into 50 mL disposable polycarbonate centrifuge tubes, and powders were dissolved in approximately 20 mL of water at 37 °C with vortex mixing, followed by sonication for 15 min. Samples were then made to 50 mL with water, cooled for 10 min at 10 °C, centrifuged at 3000×*g* for 15 min, and the upper fat layer removed.

Immunoaffinity columns, previously stored at 4 °C, were allowed to equilibrate to room temperature, mounted on an SPE manifold, and the liquid drained under gravity to the upper column bed level. The sample extract (50 mL) was allowed to pass through the column under gravity, supplemented with manual syringe pressure, at an approximate rate of 1–2 mL min^{-1}. The column was sequentially washed to waste with 4 mL of PBS buffer (0.01 M) and 20 mL of water and was dried with forced syringe air and application of slight vacuum. AFM_1 was then eluted with 3× 1.25 mL aliquots of methanol/acetonitrile (40:60 v/v) with back-flushing, collected in a vial, and clarified through a 0.45 μm PTFE membrane filter (at this stage, the sample may be stored at 4 °C for up to 3 days if required). The extract was evaporated to dryness under nitrogen at ≤ 40 °C, immediately re-dissolved in 1.0 mL of HBS-EP buffer, and passed through a low volume 0.45 μm PTFE membrane filter prior to analysis.

Biosensor Assay

Reagents and functionalized sensor chips were equilibrated to ambient temperature. Calibration standards and sample extracts (120 μL) were dispensed in a 96-well microtiter plate and sealed with an adhesive foil. Automated analysis was routinely accomplished with the single-flow cell addressable Q instrument under optimized assay conditions (running buffer: HBS-EP; injection time: 600 s; flow rate: 10 μL min^{-1}; temperature: 25 °C). Monoclonal anti-AFM_1 (0.5 μg mL^{-1}) antibody was mixed (1:1 v/v) with calibrant or sample extract and 100 μL was injected over the active sensor chip surface. At the end of each cycle, bound antibody was removed with sequential regeneration of 12 μL of 100 mM HCl and 7 μL of 25 mM NaOH at 50 μL min^{-1}.

Relative binding responses were acquired 30 s after the commencement of the dissociation phase and were used for both AFM_1 calibration and quantitation of AFM_1 content. A four-parameter regression [$y = R_{hi} - ((R_{hi} - R_{lo})/1 + (Conc/A_1)^{A2})$], where R_{hi} = response at infinite concentration, R_{lo} = response at zero concentration, $A_1 = IC_{50}$, A_2 = slope factor, and *Conc* = concentration of AFM_1 (ng mL^{-1}), was software generated and used to construct the dose–response calibration.

Following analysis, the remaining AFM_1 solutions were deactivated with sodium hypochlorite solution (10% v/v adjusted to pH = 7) prior to disposal.

Results and Discussion

Method Development

AFM_1 predominantly occurs unbound in fluid milk but is not homogeneously distributed, with evidence that association with the casein fraction elevates its concentration in cheese (Benkerroum 2016; Campagnollo et al. 2016; Mohammadi 2011). Despite its relatively low solubility in water (10–30 μg mL^{-1}), the analysis of AFM_1 in milk powder can be expediently accomplished after aqueous dissolution and removal of fat, consistent with the protocols commonly recommended for liquid milk analysis by LC, ELISA, and related immunoassay techniques (Iqbal et al. 2015; Ketney et al. 2017). Such an approach is in contrast to the more exhaustive organic solvent strategies required for cheese. Following extraction, immunoaffinity column clean-up is commonly used in many LC-based methods in order to both concentrate and fractionate the crude extract. This strategy was adopted presently to provide an extract consistent with the lower inherent sensitivity of SPR relative to ELISA, and thereby avoid the requirement for signal amplification techniques that would otherwise be needed (Karczmarczyk et al. 2016; Wang et al. 2009).

Several significant factors that influence biosensor immunoassay performance were evaluated, including antibody selection, assay format, ligand affinity, coupling chemistry, buffer conditions, contact time, and regeneration. The low molecular mass of AFM_1 necessitates an indirect solution competition (inhibition) assay format, whereby solution AFM_1 competes with immobilized AFM_1 for antibody binding. Unless structurally modified, AFM_1 is expediently immobilized to the carboxymethyldextran chip surface by standard covalent amine-coupling of its BSA conjugate, as has been described previously for both AFM_1 and AFB_1 (Daly et al. 2000; Karczmarczyk et al. 2016; Wang et al. 2009). A surface ligand density of approximately 12,000 response units (RU, where 1 RU is a change in refractive index of 10^{-6}, equivalent to 1 pg mm^{-2} protein) was typically achieved for BSA–AFM_1, representing approximately 175 fmol of immobilized BSA–AFM_1 conjugate.

Four commercially available high-affinity antibodies with claimed specificity to AFM_1 were evaluated by determining their relative binding characteristics to three independently functionalized high-density BSA–AFM_1, BSA, and reference surfaces. All four antibodies bound to BSA–AFM_1 with no non-specific binding to the reference surface. However, although the three polyclonal antibodies also bound significantly to the BSA surface, suggesting cross-reactivity to the BSA hapten protein of the immunogen, the rat monoclonal antibody (mAb) did not cross-react to BSA and was therefore selected for further method development. The reported properties of the selected mAb cite limited cross-reactivity to AFB_1, but not to BSA and other irrelevant antigens, by ELISA, and this mAb has also been applied in a previous SPR-immunoassay study (Wang et al. 2009). A mAb-binding inhibition of > 99% over the active surface was achieved when equilibrated with AFM_1 (100 ng mL^{-1}) in solution, representing a further demonstration of both specificity of the selected mAb and absence of its non-specific binding. The BSA–AFM_1 functionalized surface yielded a maximum uninhibited mAb-binding capacity (R_{max}) of approximately 4000 RU and facilitated concentration analysis under mass transport-limiting conditions, whereby antibody binding progresses at a constant rate. Under routine uninhibited assay conditions, less than 5% of the active surface is bound by antibody. As a further confirmation of the suitability of the selected mAb, it was immobilized as ligand, and binding of AFM_1 in competition with BSA–AFM_1 conjugate was demonstrated in the alternative indirect surface competition format.

Bulk refractive index shifts upon injection were minimized under optimized assay conditions whereby calibrants, sample extracts, and mAb were prepared in HBS-EP running buffer. Despite their bivalency, antibodies are generally exploited as the detecting species in competitive immunoassays, whereby both paratopes are required to be occupied by antigen for effective inhibition. Both antibody affinity and concentration determine the assay working range, as illustrated for AFM_1 in Fig. 1; an mAb concentration of 0.5–1.0 μg mL^{-1} was found to provide optimal response while conserving antibody, with the specific concentration dependent on the individual batch. A 10-min injection time was also implemented to optimize assay sensitivity.

Although previous studies have reported difficulty in regenerating the immobilized ligand surface subsequent to antibody binding (Daly et al. 2000), this was achieved effectively with sequential low pH and high pH reagents, consistent with (1) common strategies reported to overcome the attractive enthalpic and entropic forces between immobilized ligand and analyte (Goode et al. 2015) and (2) studies specific to AFM_1 determination (Karczmarczyk et al. 2016; Wang et al. 2009). The ligand regeneration protocol yielded a stable within-assay absolute baseline response after 40 injection cycles (relative standard deviation, $RSD_r = 0.15\%$) and a drift of approximately 2 RU $cycle^{-1}$. Each singly addressable immobilized surface was stable over > 250 injection cycles, as indicated by a negligible loss of mAb-binding capacity across multiple runs.

A sensorgram schematic, typical dose–response curve, and associated sensorgrams derived under optimum assay conditions are presented in Fig. 2 for a set of AFM_1 calibration standards (0.01–10 ng mL^{-1}), with calibration curves effectively described by a four-parameter regression over this concentration interval, consistent with general immunoassay protocols. Although accurate quantitation is restricted to approximately one order of magnitude in AFM_1 concentration, the relatively narrow linear dynamic range is comparable to

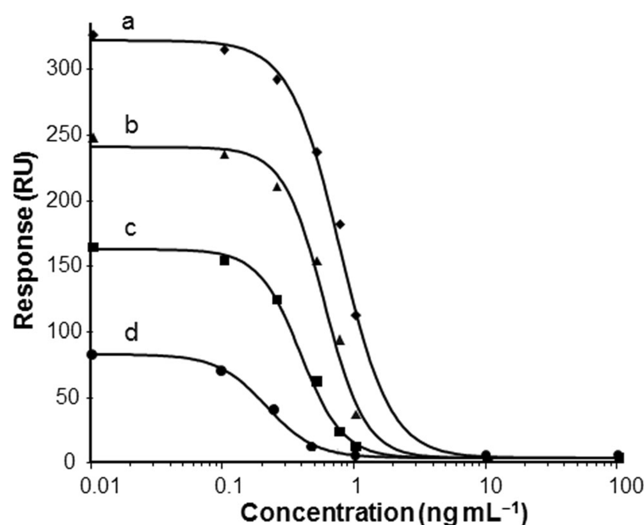

Fig. 1 Influence of selected monoclonal antibody concentrations on sensitivity and working range. Dose–response of aflatoxin M_1 with antibody concentrations (IC_{50} ng mL^{-1}) of **a** 2.0 μg mL^{-1} (0.80), **b** 1.5 μg mL^{-1} (0.60), **c** 1.0 μg mL^{-1} (0.40), and **d** 0.5 μg mL^{-1} (0.22) over immobilized BSA–AFM_1 under the described inhibition assay conditions

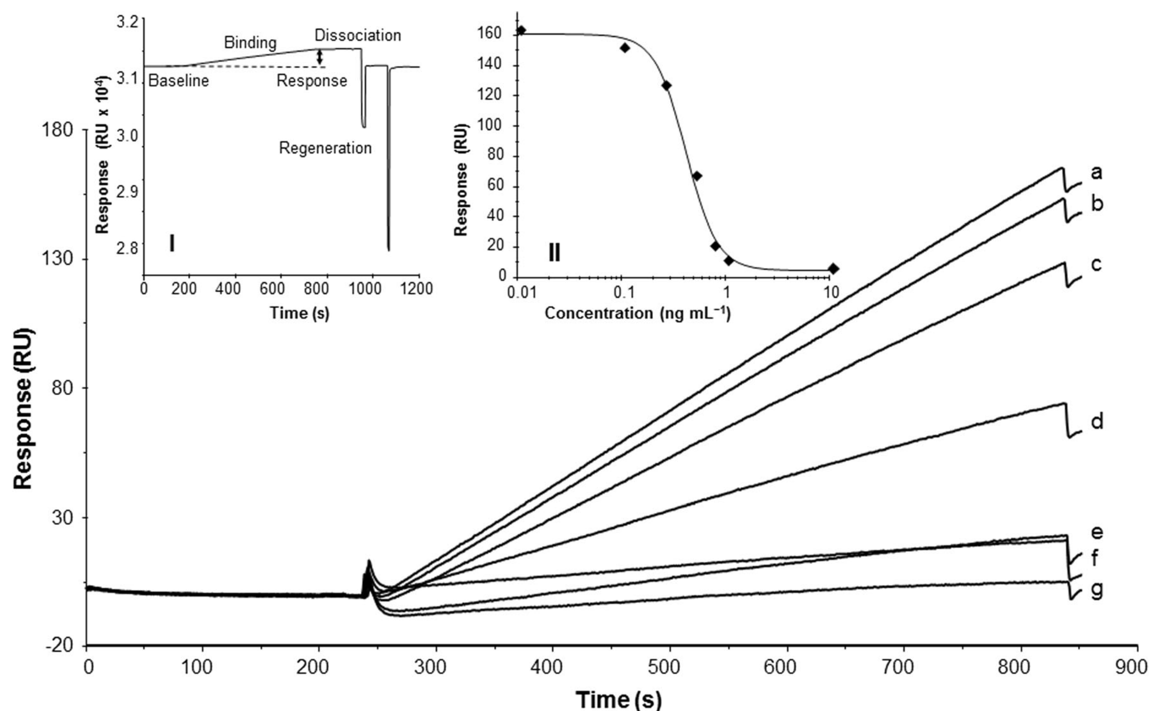

Fig. 2 Overlaid sensorgrams obtained for the concentration series of aflatoxin M_1 **a** 0.01 ng mL^{-1}, **b** 0.10 ng mL^{-1}, **c** 0.25 ng mL^{-1}, **d** 0.50 ng mL^{-1}, **e** 0.75 ng mL^{-1}, **f** 1.0 ng mL^{-1}, and **g** 10.0 ng mL^{-1} under optimized inhibition assay conditions (regeneration phase of each cycle removed for clarity). Inset I: schematic of a typical uninhibited complete sensorgram cycle defining its regions; II: dose−response calibration derived from relative binding response (RU)

previously reported SPR-based immunoassays for aflatoxins (Daly et al. 2000; Karczmarczyk et al. 2016; van der Gaag et al. 2003; Wang et al. 2009).

Instrument intermediate precision (RSD_{iR}) of the mean IC_{50} value (0.43 ng mL^{-1}) from replicate calibration curves ($n = 34$) obtained from multiple active surfaces was 9.8%, and the regression model yielded acceptable bias and recovery of < 9% and 95–109% ($n = 21$), respectively, for calculated versus actual calibration concentration values over the effective quantitation range (0.1–1.0 ng mL^{-1}). Instrument stability was further monitored by the performance of a check standard (0.25 ng mL^{-1}) across multiple independent sequences, yielding a mean of 99.1% of the actual value and an RSD_{iR} of 6.6% ($n = 20$). The instrumental limit of detection from replicate ($n = 6$) analysis of uninhibited mAb was estimated to be 0.1 ng mL^{-1} (3 × SD) and the limit of blank (LoB) was estimated to be 0.05 ng g^{-1} from replicates ($n = 12$) of a blank milk powder sample (Armbruster and Pry 2008). The method detection limit (MDL) was estimated to be 0.1 ng g^{-1} from replicate analysis ($n = 8$) of a quality control whole milk powder containing a low level of incurred AFM_1 (Su 1998). Complementary to the MDL, the cut-off value for a contaminant screening test method is the assigned concentration threshold above which the sample (1) is considered to be suspect positive, (2) is considered to be potentially non-compliant, and (3) requires confirmatory analysis (EU Commission 2014). Based on the intermediate precision performance of the described method, the cut-off value at a screening target concentration of 0.5 × EU regulatory limit (equivalent to the lower EU limit for infant formula) is estimated to be 0.18 ng g^{-1} and facilitates a reliable quantitative screening for potential non-compliance at the EU regulatory limit for AFM_1 of 0.5 ng g^{-1} (type II error < 5%).

The mean recovery, as estimated by dosing an uncontaminated whole milk powder with AFM_1 at 0.5×, 1.0×, and 2× the EU maximum regulatory limit (0.5 ng g^{-1}) was 96.7% (89.6–101.0%), supporting the suitability of the proposed biosensor method for the determination of the AFM_1 content of milk products. The within-run repeatability RSD_r for a whole milk powder containing incurred AFM_1 at approximately 0.5× the EU limit was determined to be 4.92% ($n = 8$), and the between-run intermediate precision RSD_{iR} was 18.7% ($n = 12$), which yields a Horwitz value (observed RSD_{iR}/predicted RSD_{iR}, where predicted $RSD_{iR} = 2^{(1-0.5\log C)}$) of 0.34, which complies with the accepted guidance limits of 0.3–1.3 at these low concentrations (Horwitz and Albert 2006).

Despite the acknowledged specificity of antibody–antigen interactions, all ligand-binding platforms, including SPR-based immunoassays, are vulnerable to potential non-specific binding influences that are predominantly associated with highly complex and variable foods or biological matrices (Situ et al. 2008; Vaisocherová et al. 2015; Visentin et al. 2018). The magnitude of such non-specific interactions from milk in the presently described SPR biosensor immunoassay

of AFM_1 was, in general, analytically negligible relative to the analytical signal (< 5.0%). However, minor and artefactual negative detector responses were observed with some milk powder samples that were, in the absence of mAb, of equivalent magnitude over both active and reference surfaces, suggesting a physical distension of the hydrophilic carboxymethyl dextran support, rather than a non-specific binding interaction with immobilized ligand (Daly et al. 2000). If required, this effect may be accounted for by the reference flow cell subtraction technique, expediently accomplished when the described assay is implemented on the multi-flow cell addressable Biacore T200 instrument.

Application to Milk Powders

Comparison against independent reference analytical methods provides additional confidence in the reliability of a candidate method. Non-perishable milk powder samples were acquired (1) from an international proficiency scheme (Fapas) with assigned consensus values, and (2) in-house, with comparison analysis by alternative techniques. The AFM_1 content of these samples were determined by the described biosensor method, with the data summarized in Table 1.

With the unavailability of an appropriate certified reference material, Fapas proficiency scheme milk powders with assigned mean and range values (consensus mean of participants' data with a z-score of $< \pm 2$ (z-scores of $> \pm 2$ were outliers) were analyzed. Although not certified reference values, proficiency scheme-assigned values are acknowledged to be reliable indicators of content, despite the relatively high variance around assigned values due to both low AFM_1 concentration and the wide range of disparate sample preparation, extraction, and quantitation techniques applied by multiple participants.

Apparent uncontaminated samples can yield trace levels by any analytical technique (type I error), as was occasionally observed with milk powder blank samples by the described biosensor immunoassay. These trace positives were nonetheless below the LoB and MDL, where the LoB is the highest apparent concentration statistically expected from replicate analysis of a nominal blank sample (Armbruster and Pry 2008). Despite the profoundly different analytical principles, the comparative analytical data support the suitability of the described biosensor immunoassay for the quantitative screening determination of the AFM_1 content of milk and milk powders. Given the scarcity of AFM_1-contaminated milk products in New Zealand, method accuracy is further supported by a mean spike recovery of 96.7%, as reported above.

The described SPR-based biosensor immunoassay complements ELISA as a threshold-level screening technique for the identification of potential non-compliance of AFM_1 content, prior to confirmatory analysis by reference chromatographic techniques. However, the inherently label-free and direct detection of AFM_1–antibody binding, the generation of quantitative data, and ease of automation provide certain advantages compared with conventional ELISA.

Conclusions

Although many biosensor studies have been restricted to proof-of-concept only, an optical biosensor antibody-based competitive inhibition immunoassay for the quantitation of AFM_1 in milk and milk powder was developed and comprehensively validated, supporting its application to regulatory compliance. The technique relies on a prior immunoaffinity sample clean-up step that obviates the requirement for complex biosensor amplification strategies. Sample extraction and clean-up require approximately 30 min and instrumental

Table 1 Comparison of AFM_1 content of milk powders by biosensor and alternative methods (ng g^{-1})

Sample[a]	Biosensor[b]	ELISA[c]	LC–MS/MS[d]	HPLC–FI[e]	Fapas[f]
A	0.03 (0.01–0.11)	na	na	0.02 (0.01–0.03)	na
B	0.27 (0.19–0.36)	0.26 (0.20–0.36)	0.26	0.23 (0.16–0.30)	na
C	0.10 (0.09–0.12)	0.11	na	na	0.10 (0.06–0.15)
D	0.10 (0.09–0.12)	0.11	na	na	0.07 (0.04–0.09)

na not applicable

[a] A: milk powder; B: in-house quality control milk powder; C: Fapas sample 1; D: Fapas sample 2

[b] SPR biosensor (mean, range in parentheses)

[c] R-Biopharm R1121 (mean, range in parentheses)

[d] Analytica Laboratories, Hamilton, New Zealand (acetonitrile extraction, LC–tandem mass spectrometry, three multiple reaction monitoring transitions, $^{13}C_{17}$-AFM_1 internal standard)

[e] AOAC 2000.08, modified; HPLC using fluorimetric detection

[f] Value assigned by Fapas based on consensus of participant data from multiple analytical methods (extraction, clean-up, ELISA, HPLC, LC–tandem mass spectrometry) (mean, range in parentheses with z-score < 2)

analysis requires 20 min per sample cycle, including surface regeneration.

Although relatively limited in concentration range, the estimated MDL of the biosensor method is five times lower than the EU maximum residue limit, providing assurance of reliability as a quantitative routine screening of milk and milk powders for AFM_1 non-compliance prior to confirmatory analysis. The described antibody-based biosensor method therefore complements ELISA, and alternative biosensor platforms, while providing the advantages of quantitative data, automation, and real-time and non-labeled detection of AFM_1 in milk.

Acknowledgments The authors acknowledge Dr. Terry Cooney (Analytica Laboratories Ltd., Hamilton, New Zealand) for LC–tandem mass spectrometry analyses and the support and encouragement of Dr. Robert Crawford and Fonterra Co-operative Group Ltd. throughout this study.

Compliance with Ethical Standards

Conflict of Interest Harvey Indyk declares that he has no conflict of interest. Sowmya Chetikam declares that she has no conflict of interest. Brendon Gill declares that he has no conflict of interest. Jackie Wood declares that she has no conflict of interest. David Woollard declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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