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Dual-function natural biopolymers for aflatoxin M_1: adsorption and diagnostic applications in milk

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Research Article

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Abstract

This study investigated the hypothesis that a natural biopolymer system could simultaneously mitigate and detect aflatoxin M_1 (AFM_1) contamination in milk. AFM_1, the hydroxylated metabolite of aflatoxin B_1, is a potent carcinogen that remains stable during milk processing and continues to pose significant food safety concerns. The research therefore aimed to determine the prevalence of moulds and AFM_1 in raw buffalo and cow milk from Giza Governorate, Egypt, and to evaluate the performance of a novel natural biopolymer complex composed of chitosan, β-cyclodextrin, AFM_1-specific antibodies and anthocyanins, designed for dual functionality in AFM_1 adsorption and diagnostic signal enhancement. Total mould counts were assessed following the ISO protocol, while milk composition and somatic cell count (SCC) were determined by Bentley 150 scan. AFM_1 detection was initially performed by thin-layer chromatography (TLC) and subsequently quantified by a validated reversed-phase high-performance liquid chromatography (HPLC) method. The HPLC method exhibited excellent performance with linearity ($R^2 = 0.99999$), sensitivity (LOD 0.002 µg/L, LOQ 0.007 µg/L) and precision (RSD ≤ 1.5%). The lowering in milk compositional quality was statistically significant ($P < 0.05$) with elevated SCC in AFM_1-positive samples. Screening revealed AFM_1 contamination in 28% of buffalo and 56% of cow milk samples, with mean concentrations of 0.084 µg/L and 0.188 µg/L, respectively. Application of the natural biopolymer achieved AFM_1 reductions of 65–73% in buffalo milk and 83–90% in cow milk, while anthocyanins within the polymer provided concentration-dependent diagnostic signals. Sensory evaluation indicated that treated milk maintained acceptable organoleptic properties without adverse effects on compositional quality. These findings emphasise the prospective use of natural biopolymers as dual-function analytical and mitigation tools for AFM_1 in milk, offering a reliable approach for routine milk safety monitoring.

Milk is a fundamental dietary component across all age groups, particularly for infants and children, due to its high nutritional value. However, its safety is increasingly compromised by the presence of mycotoxins such as aflatoxin M_1 (AFM_1), a hydroxylated metabolite of aflatoxin B_1 (AFB_1), which is excreted in the milk of lactating animals consuming contaminated feed. AFM_1 is classified as a Group 2B carcinogen by the International Agency for Research on Cancer (IARC) and has been linked to hepatotoxic, mutagenic and immunosuppressive effects (Abdallah *et al.*, 2024; Jahromi *et al.*, 2025).

Aspergillus flavus and *Aspergillus parasiticus* are the most common aflatoxin producers worldwide. These two species are consistently identified as the dominant sources of AFM_1 in milk. Recent reviews report high prevalence of AFM_1 in milk samples globally. Surveys show prevalence ranges from low percentages up to over 80%, depending on season, feed contamination and detection method (Shabeer *et al.*, 2022; Jahromi *et al.*, 2025). In regions like South Asia and the Middle East, cow and buffalo milk are widely consumed and are a substantial part of the human diet. Comparing both helps assess public health exposure risk across common dairy sources rather than just one species (Zheng *et al.*, 2022).

Mould count in cow and buffalo milk is an important indicator of milk quality and safety, as mould can cause spoilage and pose health risks through mycotoxins. Recent studies have reported mould contamination in raw milk from both cows and buffaloes, with variations in counts and species (Fusco *et al.*, 2020). Mould counts are routinely monitored to evaluate contamination levels, which can vary depending on geographical location, handling, storage and

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processing conditions. Counts above 3 log CFU/mL are considered indicative of significant fungal contamination that requires intervention (Garnier *et al.*, 2017).

The global prevalence of AFM_1 contamination in milk varies widely, with higher incidences reported in developing countries due to inadequate feed storage practices and limited regulatory enforcement (Kortei *et al.*, 2022). Studies have shown that between 0.3% and 6.2% of ingested AFB_1 is converted to AFM_1 in the liver and secreted into milk (Abdallah *et al.*, 2024; Adam *et al.*, 2025). Regulatory agencies have established maximum residue limits (MRLs) to mitigate exposure, with the European Union setting a stringent limit of 0.05 µg/L and the U.S. FDA allowing up to 0.5 µg/L (Jahromi *et al.*, 2025).

Detection of AFM_1 in milk has traditionally relied on chromatographic techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS/MS), which offer high sensitivity and specificity (Wood *et al.*, 2021). However, due to the thermal stability of AFM_1, conventional milk processing methods such as pasteurisation and sterilisation are ineffective in eliminating the toxin (Moussa *et al.*, 2020). If pre-pasteurisation milk contains AFM_1 above regulatory limits, pasteurisation or UHT will not guarantee compliance. Pasteurisation may slightly lower measured AFM_1 ($< 20\%$), but not consistently enough to be a control measure (Hassanpour *et al.*, 2019). This has prompted the exploration of alternative mitigation strategies, including the use of natural adsorbents.

Chitosan, a cationic polysaccharide, has been extensively studied for its ability to adsorb mycotoxins such as AFM_1 through electrostatic interactions and hydrogen bonding, thereby reducing toxin levels without impairing milk quality (Juarez-Morales *et al.*, 2017). Fungal chitosan often has a relatively low surface area and larger particle size, which means fewer available binding sites for aflatoxin molecules compared with nano- or composite adsorbents (Salama *et al.*, 2025). β-Cyclodextrin (β-CD), a cyclic oligosaccharide with a hydrophobic inner cavity, can encapsulate small toxic molecules such as AFM_1, enhancing stability and facilitating their removal from aqueous matrices (Gonzalez Pereira *et al.*, 2021; Musuc, 2024). Anthocyanins, natural flavonoid pigments of plant origin, provide diagnostic potential owing to their pH-sensitive colorimetric and fluorescence properties, enabling real-time, concentration-dependent detection of AFM_1 in milk (Khoo *et al.*, 2017). Such AFM_1-specific antibody selectivity is particularly relevant for dairy applications, where preservation of milk composition and complying with the European Food Safety Authority (EFSA) maximum limit of 0.05 µg/L in milk are required (EFSA, 2013). Highlighting these biomolecules individually emphasises their unique contributions and establishes the rationale for integrating them into a multifunctional biopolymer system.

Given the public health and regulatory significance of AFM_1 contamination, this study aims to (i) detect and quantify mould and AFM_1 levels in raw buffalo and cow milk using a validated HPLC method, (ii) assess the effect of AFM_1 on the chemical composition of the examined milk samples, (iii) evaluate the efficacy of selected natural biopolymers in reducing AFM_1 concentrations under controlled experimental conditions and (iv) examine the impact of these biopolymers on the sensory and compositional properties of both milk types. The findings are expected to contribute to safer dairy production practices and support future strategies for mycotoxin mitigation in milk.

Materials and methods

Sample collection

The milk samples were collected between 2024 and 2025 from various markets in the Giza governorate of Egypt. The samples included 100 L of each of buffalo's and cow's raw milk, with each sample volume being 1 L. Each sample was stored in clean, dry and sterile sampling bottles and kept in an icebox at 4°C within 2 h before being transported to the Animal Health Research Institute in Dokki, Giza, for analysis. The samples were frozen after mould counting, and then thawed at room temperature (23°C) for 30 min before preparation for chromatographic analysis.

Sample preparation and mould content examination

Each sample was aseptically transferred into a sterile test tube at a volume of one millilitre. After adding 9 mL of 1% peptone water (Himedia, RM001), the mixture was homogenised for two minutes using a sterile blending machine (Lab-Blender/400-Seward, Worthing, UK). Following that, the homogenised mixture was serially diluted ten times (ISO, 2020). The total mould count (CFU/ml) was calculated by inoculating different dilutions using the pouring technique on duplicate plates of sterile Sabouraud dextrose agar (Himedia, MH063) supplemented with 0.05 mg/ml chloramphenicol (Himedia, CMS218). Thereafter, the inoculated plates were put in an incubator set at 25°C and left there for five days. Samples that recovered one or more viable mould colonies after incubation were considered analytically positive (ISO, 2008).

Determination of milk chemical components

To evaluate the compositional components of milk and the somatic cell count (SCC), a milk scanner (Bentley soma 150) was utilised, as explained by Saad and Attia (2021).

Standard preparation

An AFM_1 certified reference solution (50 mg/mL) was prepared in a methanol/chloroform mixture Sigma-Aldrich Co (81:19, v/v) and stored at −20 °C. Before use, the stock solution was diluted with methanol/chloroform (1:1, v/v). Intermediate solutions are crucial for the preparation of matrix-matched calibration standards and quality control (QC) samples. Serial dilution of a stock solution with an appropriate solvent mixture is employed to generate working standards, which are then used to fortify blank matrix material. This process establishes calibration points spanning a relevant analytical range. Consistent application of the extraction protocol to all fortified, QC and experimental samples ensure analytical comparability. This facilitates accurate quantification and reliable evaluation against the matrix-matched calibration curve outlined by Fallah (2010).

Biopolymer preparation

A multifunctional biopolymer was designed for the diagnosis and adsorption reduction of AFM_1 from milk. The biopolymer consisted of chitosan (medium molecular weight, Sigma-Aldrich, USA) as a matrix; β-CD (Merck, Germany) as inclusion sites; AFM_1 monoclonal antibodies (for specific binding) obtained from Abcam, UK, anthocyanin (purified extract from *Vitis vinifera*, Sigma-Aldrich, USA) as a colorimetric indicator; and genipin as a natural crosslinker (Fisher Scientific/Acros Organics, USA).

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Four formulations were prepared based on different component ratios to assess adsorption versus diagnostic performance (Table 1). Components were mixed on a dry weight basis to yield 1.0 g of each composite. Chitosan was dissolved in 1% acetic acid to form a cationic polymer solution, while β-CD was dispersed in deionised water with constant stirring to allow host-guest complexation with the hydrophobic moieties of AFM_1, as previously described (Barbosa *et al.*, 2019; Zhang *et al.*, 2019).

Anthocyanins (0.1% solution) were incorporated into the polymeric matrix to provide pH-dependent optical and fluorescence responses for diagnostic functionality (Khoo *et al.*, 2017). Antibodies were immobilised within the cross-linked polymeric network using genipin at pH 6.5, following protocols adapted from biosensor immobilisation studies (Vidal *et al.*, 2013). The resulting formulations were homogenised and lyophilised to obtain stable biopolymer powders stored at 4°C until use. This preparation approach ensured structural stability, high adsorption capacity and diagnostic responsiveness, aligning with previously reported chitosan-based mycotoxin mitigation systems (Juarez-Morales *et al.*, 2017; Hua *et al.*, 2023).

Adsorption and diagnostic evaluation

Three milk samples were collected from a supplier quality assurance farm and verified by HPLC to contain AFM_1 levels below the quantification limit of 0.007 µg/L, in compliance with EU standards. Each composite was incubated with three raw milk samples for each (cow and buffalo) that were collected fresh, defatted by centrifugation at 2,500 RPM for 10 min and artificially spiked with AFM_1 at 0.05 µg/L (close to the EU MRL). After 1 h contact at 25°C with gentle agitation, supernatants were analysed by the validated HPLC-FLD (fluorescence detection) to quantify residual AFM_1. The best formulation was assigned by the adsorption capacity (µg AFM_1/g composite), which was calculated from the reduction in AFM_1 concentration. Moreover, the diagnostic sensitivity was evaluated by visual colorimetric intensity (anthocyanin response, semi-quantitative) and by HPLC on captured toxin. Each test was repeated in triplicate (n = 3).

Detection of AFM_1 in milk samples by thin-layer chromatography (TLC)

The presence of AFM_1 in milk samples was detected using a modified TLC technique (Scussel, 2003). The skimmed milk samples were warmed to 37 °C and then filtered through Whatman No. 4 paper (International Ltd., UK). AFM_1 was extracted by adding an equal volume of chloroform (Sigma-Aldrich, USA) with vigorous shaking for 15 min. The organic layer was evaporated under nitrogen or using a rotary evaporator (Rotavapor, Büchi, Switzerland) and reconstituted in chloroform: methanol (9:1, v/v). Aliquots (10–20 µL) of sample and standard were applied to pre-activated silica gel 60 F254 TLC plates (Sigma-Aldrich, USA; 110 °C, 30 min). Plates were developed in toluene: acetone: chloroform (60:30:10, v/v/v), air-dried and examined under UV light at 365 nm. AFM_1 was identified by blue fluorescence with an RF of 0.5–0.6. Semi-quantitative analysis was performed through visual comparison with known standards, and as required, densitometric measurements were utilised. The method demonstrated satisfactory recovery rates (94–97%) and relative standard deviations (7–16%), consistent with previously validated protocols.

Quantification of AFM_1 in milk samples by HPLC

Quantification of AFM_1 was performed by HPLC following Kolarič and Šimko (2023). Ten millilitres of raw milk were diluted, centrifuged to remove fat and the supernatant applied to an AFM_1-specific immunoaffinity column. After washing with water, AFM_1 was eluted with methanol, evaporated under nitrogen and the residue reconstituted in water: acetonitrile (80:20, v/v). A 20 µL aliquot was injected onto a C18 column (250 × 4.6 mm, 5 µm; Sigma-Aldrich, USA) maintained at 25 °C for isocratic separation. The ratio (80:20) of water to acetonitrile mobile phase was utilised at a flow rate of 1 mL/min. Fluorescence detection occurred at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. This method has been verified following the guidelines set by the International Council for Harmonisation (ICH, 2005) and the United States Pharmacopeia (USP, 2024).

Sensory and compositional quality parameters evaluation of biopolymer-supplemented milk samples

Three employees from Cairo University's Food Hygiene Department were given 50 mL plastic containers containing milk samples that had been pasteurised in a lab at 90°C for 10 minutes and then abruptly cooled. The milk samples had previously been analysed and AFM_1 was within permissible limit (0.05 µg/L). Ten points were awarded for flavour, five for colour and five for appearance in a 20-point scoring system. Before examination, all assessors attested to having completely washed their mouths with distilled water. A specific scorecard was used to document the outcomes (Alvarez, 2023). The research committee authorised all methods, including sensory evaluation (no. 55,429). All milk samples of both animal species and control samples (three samples for each species and each control) were examined. A milk scanner (Bentley soma: 150) was utilised to measure the compositional components of milk and SCC.

Determination of residual chitosan in milk

Residual chitosan in milk following treatment with the chitosan-based composite was assessed using acid hydrolysis followed by colorimetric quantification of glucosamine, as a marker of chitosan presence. Milk samples (10 mL) were collected immediately after removing the composite sheets and clarified through centrifugation (4,000 × *g*, 10 min, 4 °C) to remove fat and particulate matter. The skimmed fraction was hydrolysed with 6 M hydrochloric acid at 105 °C for 6 h to depolymerise any residual chitosan into free glucosamine. After cooling, the hydrolysates were neutralised with sodium hydroxide and filtered prior to analysis. Glucosamine concentration was determined using a colorimetric assay based on the Elson–Morgan reaction with absorbance measured at 530 nm using a UV-visible spectrophotometer (Peak Instruments, Houston, TX, USA). Quantification was performed against external glucosamine standards prepared in a treated milk matrix and subjected to the same hydrolysis procedure. No glucosamine attributable to chitosan was detected in the treated milk samples above the detection limit (Smith and Gilkerson, 1979).

Statistical analysis

Utilising SPSS v.25, the *t*-test and one-way ANOVA were used to determine the *p*-value for a significant difference between the items

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Table 1. Composition ratios of chitosan-based biopolymer for AFM_1 adsorption/diagnosis (parts by weight)

Formulation	Chitosan	β-CD	Antibodies	Anthocyanin	Purpose
F1 (adsorption-focused)	90	6	1	3	Maximise adsorption
F2 (diagnostic focused)	82	8	2	8	Maximise detection
F3 (balanced)	85	8	1	6	Adsorption + detection
F4 (minimal antibodies)	92	6	0.5	1.5	Cost effective adsorption

under investigation at a 95.0% confidence interval. Less than 0.05 was the statistical significance *P-value*.

Results

Prevalence of moulds and results of AFM_1 by thin-layer chromatography (TLC) in the examined samples

The number of positive samples was 35 (35.00%) for buffalo milk and 66 (66.00%) for cow's milk. A total of 100 raw milk samples from both buffalo and cow sources were analysed for AFM_1 detection using TLC (Table 2). AFM_1 was found in 28 of 100 buffalo milk samples, indicating a contamination rate of 28%. It was also detected in 56 of 100 cow milk samples, corresponding to a contamination rate of 56%.

Influence of AFM_1 on chemical milk characteristics

The mean value of fat, lactose, proteins, salt percentages and SCC (cells/ml) in the examined AFM_1-positive buffalo milk samples was calculated as 5.3 ± 0.04, 3.4 ± 0.04, 2.6 ± 0.03, 0.8 ± 0.005 and $8.6 \times 10^5 \pm 1.4 \times 10^4$, respectively, while in AFM_1-positive cow's milk samples, it was 2.7 ± 0.04, 3.2 ± 0.03, 2.5 ± 0.03, 0.8 ± 0.005 and $8.9 \times 10^5 \pm 1.3 \times 10^4$, respectively. The lowering in fat, protein and lactose percentages was a statistically significant ($p < 0.05$) difference with high SCC and salt percentages in AFM_1-positive samples (Table 3).

HPLC method verification for AFM_1 quantification

The HPLC method for quantifying AFM_1 in milk was validated following ICH (2005) and USP (2024) guidelines. Linearity was established over a concentration range of 0.015–1.000 ug/L, yielding a correlation coefficient (R) of 0.99999 and a regression equation of $y = 5.5975x + 3.994$ (Fig. 1). Specificity was confirmed by the absence of matrix interference in chromatograms of both standard and spiked samples at 0.05 ug/L with AFM1 consistently eluting at a retention time of 1.071 minutes (Fig. 2).

Recovery rates at three fortification levels (½ MRL, MRL and 2 × MRL) ranged from 97.8% to 102.4%, meeting acceptable criteria. Precision analysis showed intraday and intermediate RSDs (relative standard deviation) between 0.5–0.9% and 0.8–1.5%, respectively. The method demonstrated high sensitivity, with a limit of detection (LOD) of 0.002ug/L and a limit of quantification (LOQ) of 0.007ug/L. Robustness was verified under slight variations in chromatographic conditions, yielding individual RSDs ≤ 2% and a pooled RSD of 0.58%. System suitability testing confirmed method reliability, with retention time RSD at 0.3%, a tailing factor of 1.10 ± 0.04 and a theoretical plate count averaging 10,018 ± 101.

Concentrations of AFM_1 by HPLC

The contaminated raw milk samples were analysed for AFM_1 concentrations using HPLC. The mean AFM_1 concentration in buffalo milk was 0.08 µg/L, while cow's milk exhibited a significantly higher mean level of 0.19 µg/L. The standard errors were 0.018 and 0.023 for buffalo and cow milk, respectively. The concentrations ranged from 0.04 to 0.79 µg/L in buffalo milk and from 0.03 to 0.82 µg/L in cow milk.

Regarding regulatory thresholds, 6 buffalo milk samples and 14 cow milk samples exceeded the US permissible limit of 0.5 µg/L, whereas 27 buffalo and 54 cow milk samples surpassed the European Union maximum residue limit of 0.05 µg/L. These findings highlight a higher prevalence and intensity of AFM_1 contamination in cow milk compared to buffalo milk, emphasising the need for targeted mitigation strategies.

Application of the biopolymer

Four formulations (Table 1) were successfully obtained with uniform appearance and stability. The spiked blank three milk samples (buffalo and cow) for each with the permitted level (PL) of AFM_1 set by EU regulation (0.05 µg/L) were treated with all four formulations to decide the best formulation. This was detected through their reduction affinity and adsorption, and diagnostic sensitivity provided a perspective on their action. F1 (90:6:1:3) achieved the highest adsorption (>90% reduction), suggesting suitability for milk detoxification. F2 (82:8:2:8) yielded the strongest diagnostic signal, with an HPLC LOQ of 0.007 µg/L, indicating high sensitivity for AFM_1 detection. F3 (85:8:1:6) offered a balance of adsorption and detection, suitable for integrated sensor–detoxification systems. F4 (92:6:0.5:1.5) reduced AFM_1 but with a weaker diagnostic response, though it was cost-effective due to reduced antibody usage.

Reduction affinity

Formulation F1 showed the greatest adsorption efficiency in both buffalo and cow milk, achieving a 65–73% reduction in AFM_1 for buffalo milk and an 83–90% reduction for cow milk. Other formulations demonstrated lower but consistent adsorption (Table 4).

Adsorption capacity and diagnostic sensitivity

Formulations were further assessed for adsorption capacity and detection sensitivity. F1 again outperformed others, showing the highest adsorption (78.4 µg/L) while maintaining excellent diagnostic sensitivity (LOD = 0.002 µg/L). The other 3 formulations (F2, F3 and F4) attained less adsorption (65.1, 70.5 and 60.2 µg/L), respectively, on the same diagnostic sensitivity (LOD = 0.002 µg/L, Fig. 3).

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Table 2. Total mould count (CFU/mL) in the examined milk samples and prevalence of AFM_1 residues examined in raw milk samples by TLC

Item	Milk species			
	Buffalo (n = 100)		Cow (n = 100)	
Positive samples for mould, No (%)	35	(35.00)	66	(66.00)
Mean of mould count ±	3.5×10^{5a}	$\pm 4.4 \times 10^{4}$	7.5×10^{5b}	$\pm 9.0 \times 10^{4}$
AFM_1 residues, No (%)	28	(28.00)	56	(56.00)

n = number of examined samples. No. = number of positive samples. ± = standard error.
For each milk species, mean values with different superscripts (a, b) in the same row differ significantly ($P < 0.05$).

Table 3. Chemical parameters analysis results of the examined buffaloes' and cows' milk samples (n = 200)

Item Samples	Fat	Lactose	Proteins	Salts	SCC (cells/ml)
Buffalo's milk					
P = 28	$5.3^{a} \pm 0.04$	$3.4^{a} \pm 0.04$	$2.6^{a} \pm 0.03$	$0.8^{a} \pm 0.005$	$8.6 \times 10^{5a} \pm 1.4 \times 10^{4}$
N = 72	$7.2^{b} \pm 0.06$	$4.5^{b} \pm 0.02$	$3.4^{b} \pm 0.01$	$0.6^{b} \pm 0.006$	$3.6 \times 10^{5b} \pm 2.0 \times 10^{4}$
Cow's milk					
P = 56	$2.7^{a} \pm 0.04$	$3.2^{a} \pm 0.03$	$2.5^{a} \pm 0.03$	$0.8^{a} \pm 0.005$	$8.9 \times 10^{5a} \pm 1.3 \times 10^{4}$
N = 44	$4.3^{b} \pm 0.02$	$4.5^{b} \pm 0.02$	$3.4^{b} \pm 0.01$	$0.5^{b} \pm 0.007$	$2.9 \times 10^{5b} \pm 5.0 \times 10^{3}$

Values equal percentage, ± = standard error, P: Positive for AFM_1, N: Negative for AFM_1.
SCC: Somatic Cell Count.
For each milk species, mean values with different superscripts (a, b) in the same row differ significantly ($P < 0.05$).

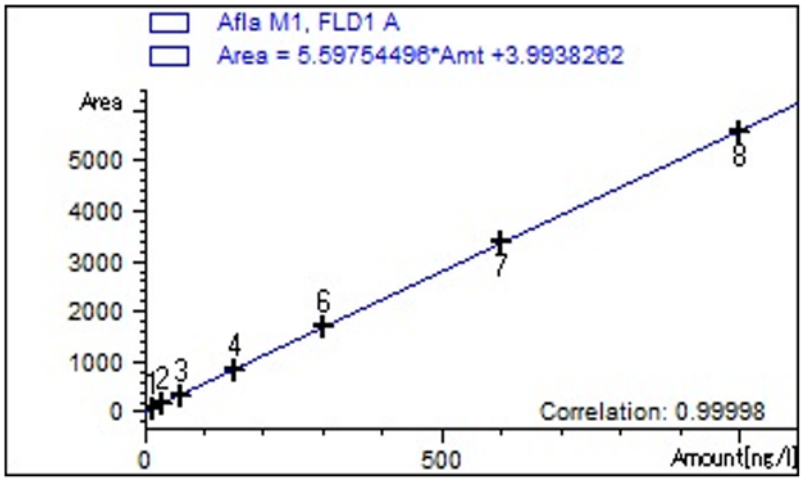


Figure 1. Calibration plot for AFM_1 standard in milk demonstrating method linearity for quantitative determination by HPLC ($R^2 = 0.99998$).

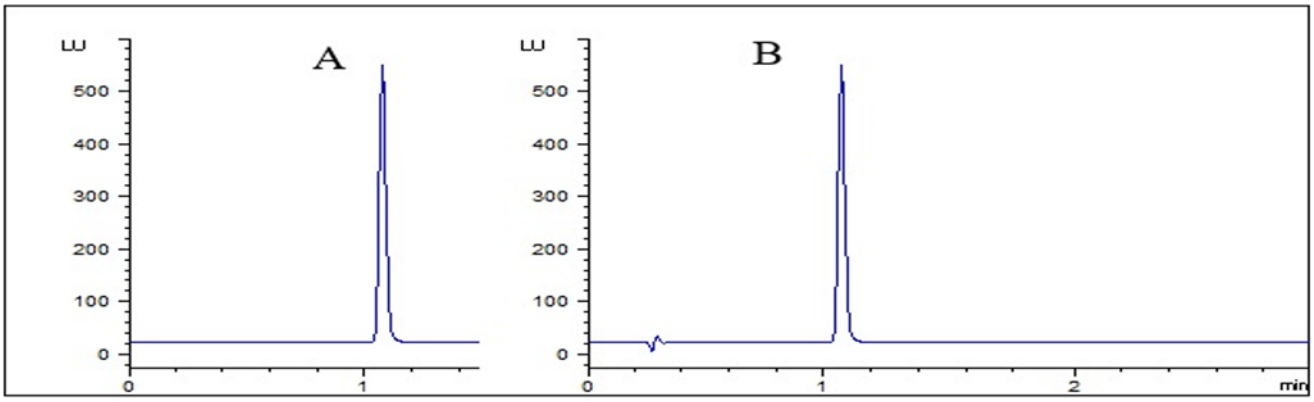


Figure 2. HPLC chromatograms for AFM_1 at 0.05 μg/L in methanol/chloroform (1/1) [A] and in milk [B]. The identical retention times and sharp peaks confirm high specificity and absence of matrix interference.

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Table 4. AFM_1 reduction by biopolymer formulations in spiked buffalo and cow blank milk, showing residual toxin concentrations and corresponding percentage reduction (mean ± SD, n = 3)

Formulation	Buffalo milk (µg/L, mean ± SD) n = 3	Reduction %	Cow milk (µg/L, mean ± SD) n = 3	Reduction %
F1	0.017–0.020 ± 0.002	65–73%	0.005–0.008 ± 0.001	83–90%
F2	0.025–0.028 ± 0.003	44–50%	0.018–0.021 ± 0.002	58–64%
F3	0.023–0.026 ± 0.002	48–54%	0.015–0.018 ± 0.001	64–70%
F4	0.030–0.033 ± 0.004	34–40%	0.022–0.025 ± 0.002	50–56%

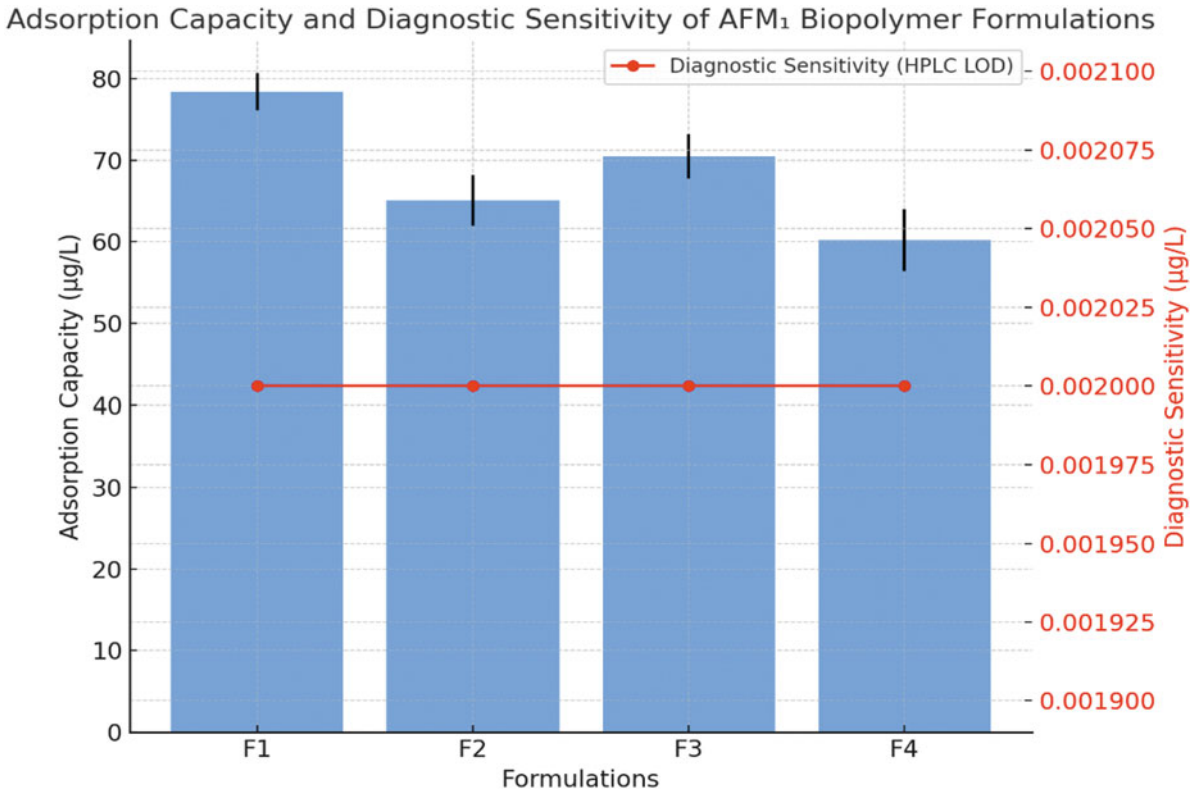


Figure 3. Comparative adsorption efficiency and diagnostic sensitivity of natural biopolymer formulations towards AFM_1, demonstrating their dual capability for toxin mitigation and analytical detection.

Effect of biopolymer treatment on AFM_1 in AFM_1-positive raw milk samples

Following treatment with F1, AFM_1 levels significantly decreased in both buffalo and cow milk samples. Post-treatment concentrations averaged 0.0455 µg/L in buffalo's milk and 0.0454 µg/L in cow's milk, with minimum detected levels at 0.014 µg/L and 0.010 µg/L, respectively. Maximum concentrations were 0.219 µg/L for buffalo and 0.1241 µg/L for cow. The estimated reduction was 65–73% in buffalo milk and 83–90% in cow milk, indicating greater efficacy in cow milk (Table 5).

Milk samples sensory quality parameters evaluation

As presented in Table 6, regarding the treated buffalo milk sensory parameters grading, the mean values of flavour, colour and appearance were 9.5, 4.6 and 4.6, respectively, while in cow's milk samples were 9.8, 5 and 5, respectively. There was a significant improvement in flavour, colour and appearance parameters for buffalo and cow milk samples before and after adsorbent supplementation. There was a non-significant ($P > 0.05$) effect on compositional parameters for buffalo and cow milk samples before and after adsorbent supplementation.

Residual chitosan in milk

The method showed a limit of detection of ≤2µg/L, mean recoveries of 95–102% for glucosamine spiked into milk and satisfactory repeatability with intra-assay coefficients of variation below 5.0%.

Discussion

The mean value of total mould count (TMC) in the examined buffalo and cow milk samples was calculated as $3.5 \times 10^5 \pm 4.4 \times 10^4$ and $7.5 \times 10^5 \pm 9.0 \times 10^4$ CFU/ml, respectively. According to a study conducted by Mohamed *et al.* (2024), cow milk showed a significantly higher TMC, averaging around 3.08 log CFU/mL, compared to buffalo milk, which had about 2.44 log CFU/mL. Mould contamination percentages were reported as 60% in cow milk and 40% in buffalo milk samples analysed. Both milk species support mould growth; buffalo milk seems to have comparatively lower

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Table 5. AFM_1 reduction % in positive surveyed milk after treatment with F1 biopolymer

Item milk species	Buffalo's milk	Cow's milk
Mean	0.045485	0.045366
Standard error (SE)	0.002436	0.002587
Minimum	0.014	0.010
Maximum	0.219	0.1241
Reduction %	65- 73	83- 90

contamination levels, potentially linked to differences in milk composition and antimicrobial factors (Habiba *et al.*, 2025).

The detection of AFM_1 in raw milk samples using TLC revealed a contamination rate of 28% in buffalo milk and 56% in cow milk, indicating a markedly higher prevalence in cow-derived samples. These findings align with global trends reported in recent surveillance studies. For instance, Zheng *et al.* (2022) reported AFM_1 positivity in 48.0% of buffalo milk and 65.7% of cow milk samples collected across China, with cow milk consistently exhibiting higher contamination levels. Similarly, De Roma *et al.* (2017) observed AFM_1 in 12.3% of cow milk and 7.2% of buffalo milk samples in Southern Italy.

The variation in AFM_1 contamination between buffalo and cow milk can be attributed to several interrelated factors. Cows demonstrate a higher metabolic conversion rate of AFB_1 to its hydroxylated form, AFM_1, with reported transfer efficiencies ranging from 0.3% to 6.2%, whereas buffaloes exhibit lower biotransformation capacity (Kortei *et al.*, 2022). Additionally, differences in feeding regimes contribute to contamination risk; cow farms more frequently utilise stored concentrates and silage, which are susceptible to fungal growth under suboptimal storage conditions (Abdallah *et al.*, 2024). Furthermore, inherent genetic and physiological disparities, particularly in hepatic enzymatic activity and milk production rates, may also influence the extent of AFM_1 excretion across species (Seid and Mama, 2019).

The mean fat percentage in the examined AFM_1-positive buffalo and cow milk samples was 5.3% ± 0.04 and 2.7% ± 0.04, respectively. Research on Holstein Friesian cows in Pakistan has shown that high levels of AFM_1 are directly related to decreased milk fat (3.82%) and compromised immune responses in cows, which further connects to fat reduction (Ullah *et al.*, 2023). The prevalence of AFM_1 in UHT milk showed higher mean levels (82.57 ng/kg) than raw milk, regardless of specific fat percentage reporting. Pasteurised milk samples had a median AFM_1 level of 24.89 ng/L compared to 13.54 ng/L in raw milk, indicating that processing may influence toxin stability in different fat compositions (Ghaffarian-Bahraman *et al.*, 2023).

Studies indicated that dietary aflatoxin contamination can lead to a reduction in milk fat %. Feeding dairy cows diets contaminated with aflatoxins was associated with lower milk fat% in some studies, although results can vary depending on the aflatoxin amount and sequestering agents used. Some research shows no significant impact on milk fat% when cows consume aflatoxin-contaminated feed. AFM_1, a metabolite of AFB_1 found in milk, has a strong affinity for milk proteins, particularly caseins. This interaction has been widely studied, suggesting that AFM_1 binds mainly through hydrophobic interactions, affecting the structure of milk proteins and potentially altering their properties (Rodrigues *et al.*, 2011; Jiang *et al.*, 2021; Harshitha *et al.*, 2023).

There is some indication that aflatoxin exposure via contaminated feed can alter mineral metabolism and concentration in milk. However, consistent data on changes in milk mineral content due to aflatoxins is sparse (Messina *et al.*, 2024). The result of this study about the effect of AFM_1 on milk sugars was considered preliminary information. The impact of aflatoxins on lactose content in milk is less well-documented. There is limited direct evidence indicating significant alterations in lactose levels due to aflatoxins contamination, implying lactose concentration might be relatively stable despite contamination. The literature shows no consistent correlation between SCC and AFM_1 level. SCC may only influence transfer transiently or in combination with very high milk yield (Zentai *et al.*, 2023).

The use of TLC, while less sensitive than HPLC or LC-MS/MS, remains a viable screening tool in resource-limited settings (Scussel, 2003). Given that 56% of cow milk samples exceeded the European Union's MRL of 0.05 µg/L, these results underscore a significant public health concern. The high prevalence in cow milk necessitates targeted interventions, including improved feed management, routine monitoring and the potential application of natural adsorbents to mitigate AFM_1 levels. The validated HPLC method demonstrated excellent analytical performance (Maggira *et al.*, 2021; Šimko and Kolarič, 2022). These studies confirm that HPLC-FLD, especially when paired with immunoaffinity cleanup, offers high sensitivity, precision and reproducibility for AFM_1 quantification in milk and dairy matrices.

The mean AFM_1 concentrations in our study, 0.0841 µg/L in buffalo's milk and 0.18753 µg/L in cow's milk, are consistent with global surveillance data. For example, Wood *et al.* (2021) reported similar contamination levels in European milk samples, with cow milk more frequently exceeding regulatory thresholds.

Our findings revealed that 27 buffalo and 54 cow milk samples exceeded the EU MRL of 0.05 µg/L, while 6 buffalo and 14 cow samples surpassed the US FDA limit of 0.5 µg/L. These exceedance rates underscore the urgent need for targeted mitigation strategies, particularly in cow milk production systems. Abdallah *et al.* (2024) and Jahromi *et al.* (2025) emphasise that AFM_1 contamination remains a persistent global challenge, especially in regions with inadequate feed storage and monitoring infrastructure.

Post-treatment analysis showed significant reductions in AFM_1 concentrations in both buffalo and cow milk. In buffalo's milk, AFM_1 levels were decreased by 65–73%, resulting in a post-treatment average of 0.0455 µg/L. In contrast, cow milk showed a greater reduction of 83–90%, with a comparable average of 0.0454 µg/L. These results suggest that natural adsorbents could effectively reduce AFM_1 contamination in milk.

Abdelnaby *et al.* (2022) reported a 68% reduction in AFM_1 using bentonite and date pit materials, with minimal changes in the milk's composition. Similarly, Womack ED (2015) found 98.4% removal efficiency with granular activated carbon while maintaining the nutritional integrity of fat, protein and lactose. Additionally, Allen *et al.* (2019) observed a 60% reduction in AFM_1 carry-over to milk when using bentonite clay in animal diets. The improved adsorption efficiency in cow milk may be due to compositional factors such as lower fat content and altered protein structures, which enhance interactions with adsorbents (Carraro *et al.*, 2013). The most advantageous one of these biopolymer preparations was F1 (Table 1). It demonstrated superior multifunctionality, combining efficient AFM_1 adsorption with simultaneous diagnostic capability. Unlike traditional mineral adsorbents such as bentonite and kaolin, which achieve detoxification primarily through non-specific binding and may impair the nutritional quality of

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Table 6. Sensory grading and compositional quality parameters of the examined milk before and after the treatment

Sample	Flavour (10)	Colour (5)	Appearance (5)	Fat%	Lactose%	Proteins%	Salts%	SCC (cells/ml)
Buffalo's milk (n = 3)	$9.5^{a} \pm 0.0$	$4.6^{a} \pm 0.1$	$4.6^{a} \pm 0.1$	$7.0^{a} \pm 0.06$	$4.7^{a} \pm 0.03$	$3.4^{a} \pm 0.03$	$0.6^{a} \pm 0.03$	$3.4 \times 10^{5a} \pm 8.8 \times 10^{3}$
Cow's milk (n = 3)	$9.8^{a} \pm 0.1$	$5.0^{a} \pm 0.0$	$5.0^{a} \pm 0.0$	$3.5^{b} \pm 0.03$	$4.3^{b} \pm 0.1$	$3.4^{ab} \pm 0.0$	$0.6^{a} \pm 0.0$	$2.4 \times 10^{5b} \pm 4.4 \times 10^{3}$
Control buffalo's milk (n = 3)	$7.1^{b} \pm 0.1$	$3.5^{b} \pm 0.2$	$3.3^{b} \pm 0.1$	$7.0^{a} \pm 0.03$	$4.6^{a} \pm 0.03$	$3.3^{ab} \pm 0.03$	$0.6^{a} \pm 0.03$	$3.5 \times 10^{5a} \pm 7.9 \times 10^{3}$
Control cow's milk (n = 3)	$7.5^{b} \pm 0.2$	$3.3^{b} \pm 0.1$	$3.1^{b} \pm 0.1$	$3.4^{b} \pm 0.03$	$4.3^{b} \pm 0.03$	$3.3^{b} \pm 0.03$	$0.6^{a} \pm 0.03$	$2.5 \times 10^{5b} \pm 6.0 \times 10^{3}$

n = number of the examined samples. ± = Standard error (SE).
Different superscript (a and b) letters in the same column indicate a significant difference ($P < 0.05$).
The same superscript (a and a) or (b and b) letters in the same column indicate a non-significant difference ($P > 0.05$).

milk proteins, fat and lactose (Damato *et al.*, 2022; Hamad *et al.*, 2023).

Formulation F1 enabled selective removal of aflatoxin M_1 without altering milk composition, owing to the complementary binding mechanisms of its constituents. Chitosan contributed electrostatic and hydrogen-bonding interactions, consistent with reported AFM_1 detoxification studies (Abdelnaby *et al.*, 2022), while β-CD enhanced sequestration through inclusion of hydrophobic aflatoxin molecules (Fliszár-Nyúl et al., 2019; Šimko and Kolarič, 2022). Immobilised monoclonal antibodies further improved selectivity through specific antigen-antibody recognition, limiting non-specific adsorption (Vidal *et al.*, 2013). These synergistic interactions resulted in the highest adsorption capacity observed (78.4 ± 2.3 μg/L). Anthocyanins additionally provided a plant-based visual signal for rapid indication of AFM_1 presence, while stabilising the chitosan matrix through polyphenolic interactions (Khoo *et al.*, 2017; Juarez-Morales *et al.*, 2017). Together, this dual adsorption-diagnostic functionality offers a practical advantage over single-component adsorbents for field-supported AFM_1 management in milk. In contrast, F2 and F3 showed moderate adsorption (65.1 ± 3.1 and 70.5 ± 2.7 μg/L), respectively, indicating partial but less synergistic interactions compared to F1.

The improved efficiency of cow milk adsorption, compared to buffalo milk, may be due to compositional factors such as lower fat content and altered protein structures. These factors enhance interactions with adsorbents (Harshitha *et al.*, 2023). Overall, these findings reinforce the viability of using natural biopolymers to reduce AFM_1 contamination in dairy products. Chitosan mainly improves sensory parameters by promoting beneficial microbial activity and metabolite production, enhancing flavour and texture (Ressutte *et al.*, 2022). β-CD is effective as a flavour masker and texture improver, reducing undesirable tastes and improving mouthfeel. Anthocyanins maintain and protect sensory quality via antioxidant activity that prevents lipid oxidation and flavour degradation (Enaru *et al.*, 2021).

The sensory evaluation showed significant improvements in attributes like reduced off-flavours and higher overall sensory scores when anthocyanin was used in milk (Tian *et al.*, 2022). These compounds (chitosan, β-CD and anthocyanin) can complement each other, showing promise for enhanced stability and multifunctional sensory improvements in milk. Chitosan and β-CD can interact physically and chemically to form functional complexes that improve the delivery, stability and sensory attributes when applied in milk (Ding *et al.*, 2019).

For field application alongside laboratory validation, the biopolymer was fabricated as a sheet that shows clear applicability at milk collection and intake points. This enables controlled, short-term contact with raw milk (1 g/L; 1 h at 25 ^{0}C with gentle agitation) followed by complete physical removal. Under these conditions, milk chemical composition and sensory attributes were preserved, and no chitosan residues were detected (LOD ≤ 2 μg/L). This supports its use as a preprocessing screening and adsorption aid to inform batch acceptance, diversion decisions or further testing prior to industrial processing. In parallel, the same material enhances laboratory workflows as a matrix clean-up and preconcentration tool before chromatographic confirmation, thereby strengthening analytical robustness. This approach is therefore positioned as a complementary intervention aligned with established regulatory testing frameworks rather than a substitute for gold-standard methods.

From an environmental perspective, the use of natural biopolymers and plant-derived anthocyanins provides a sustainable and biodegradable alternative to synthetic or mineral adsorbents, aligning with the growing demand for eco-friendly food safety interventions (Juarez-Morales *et al.*, 2017; Oladzadabbasabadi *et al.*, 2022). Minimising exposure to AFM_1 is crucial for human health, as Wang *et al.* (2024) reported strong evidence linking AFM_1 to hepatocarcinogenicity. Economically, the application of biopolymer-based technologies in dairy production can reduce losses associated with contamination-related recalls and enhance compliance with strict international food safety regulations, particularly in export markets where the European Union maximum residue limit (0.05 μg/L) is enforced (Van Egmond *et al.*, 2017; Hamad *et al.*, 2023). This triple advantage (environmental sustainability, public health protection and economic resilience) highlights the potential of multifunctional biopolymers as practical tools for integrated dairy safety management.

In conclusion, this study highlights the novelty of employing a natural biopolymer formulation, particularly F1, as a dual-function system for both adsorption and diagnostic detection of AFM_1 in milk. By integrating chitosan, β-CD, anthocyanins and monoclonal antibodies, the biopolymer achieved superior toxin removal while providing a plant-derived fluorescence signal for real-time monitoring. AFM_1 contamination was shown to adversely affect milk compositional parameters, whereas biopolymer treatment significantly improved sensory quality without compromising nutritional value. The validated HPLC method ensured regulatory compliance and analytical reliability, and when combined with TLC

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screening, provided a robust framework for AFM_1 detection and quantification. These findings support biopolymer technology as a sustainable, eco-friendly intervention to mitigate AFM_1, enhance dairy safety and strengthen alignment with evolving milk safety regulations.

Given the demonstrated absence of detectable residues and preservation of milk quality, future work should focus on validating scale-up in bulk systems (≥1,000–5,000 L). This includes optimisation of mixing dynamics, contact time and material handling. Additional studies should address repeat-use performance, disposal of AFM_1-loaded sheets and cost–benefit analyses to support industrial feasibility. From a regulatory standpoint, further engagement is needed to clarify classification as a processing aid or screening tool. This should be consistent with EFSA and FDA principles, which require removal and no technological effect in the final food. Expanded residue datasets and operational validation under commercial conditions will facilitate the integration of this technology into risk-based AFM_1 control strategies across the dairy supply chain.

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Data availability. The corresponding author can relay requests for data sharing upon reasonable requests.

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