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Aflatoxins M1 and M2 in Raw Milk and Milk Powder: Seasonal and Geographic Occurrence with Evaluation of β-Cyclodextrin Polymers for AFM1 Removal

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ABSTRACT

Aflatoxins M1 (AFM1) and M2 (AFM2) pose public health risks, necessitating monitoring and mitigation strategies. Climate change increases the risk of aflatoxin contamination in animal feed, particularly maize silage, which may lead to the carry-over of these toxins into milk. This study evaluated the occurrence of AFM1 and AFM2 in raw milk from Polish conventional dairy farms during different feeding seasons and milk powder samples (n = 70) purchased from retail markets in Poland (35), the Czech Republic (6), Slovakia (9), Hungary (10), and Iran (10). Additionally, the efficacy of β-cyclodextrin bead polymers (BCPs) was investigated. Results showed an excellent safety profile of milk powder in the V4 countries (Czech Republic, Slovakia, and Hungary), remaining below the LOQ. Of the 284 raw milk samples tested, AFM1 was detected above the LOQ in 4 samples, whereas AFM2 was detected above the LOQ in 3 samples. Polish raw milk was generally pure, although a few isolated positive findings were recorded, mainly in winter and spring. Among the Polish milk powder samples, AFM1 was detected in 2 samples at concentrations of 0.077 and 0.101 µg/kg, and AFM2 was detected in 1 sample at a level of 0.049 µg/kg. In the Iranian samples, AFM1 was detected in 2 cases at 0.042 and 0.049 µg/kg, whereas AFM2 was detected in 5 samples at concentrations ranging from 0.011 to 0.026 µg/kg. Decontamination assays demonstrated that BCPs can effectively remove AFM1 from dairy matrices. The physicochemical properties of milk significantly influenced binding capacity; superior removal efficiency was observed in raw and skim milk compared with homogenized milk. These results underscore the need for region-specific monitoring and suggest that BCP-based technological interventions may represent a complementary safety approach. Nevertheless, further studies are needed to evaluate its applicability in dairy processing.

Key words: aflatoxin M1 and M2, raw and powder milk, seasonal variation, geographical origin

INTRODUCTION

Milk and dairy products play an essential role in human nutrition, providing high-quality proteins, fats, vitamins, and minerals such as calcium, potassium, and vitamins A, D, and B-complex. They contribute to bone health, cardiovascular function, and gastrointestinal microbiota, and are particularly important for children during growth and development (De Lamas et al., 2019; Sharifan et al., 2025; Pereira, 2014; Rizzoli, 2022). Both organic and conventional milk contain comparable essential nutrients, making dairy products a cornerstone of balanced diets worldwide (Ryabova et al., 2023).

Due to its extended shelf life and global transportability, milk powder is an indispensable dairy commodity. Although evaporation and spray-drying improve microbiological safety, they can concentrate chemical contaminants present in raw milk. Therefore, milk powder safety depends on raw milk quality and processing conditions (Ryabova et al., 2023; Schuck, 2002).

Aflatoxins (AF) are among the most toxic and extensively studied mycotoxins. Under high temperature and humidity, several toxigenic species, notably *Aspergillus flavus* and *Aspergillus parasiticus*, can colonize agricultural commodities before harvest and during storage and

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-26. Nonstandard abbreviations are available in the Notes.

produce aflatoxins (Nazhand et al., 2020; Shekhar et al., 2025).

Dairy cows consuming aflatoxin B1 (AFB1)-contaminated feed rapidly metabolize it into aflatoxins M1 (AFM1) and M2 (AFM2), which are excreted into milk (Figure 1). With carry-over rates reaching up to 6%, milk contamination is directly driven by AFB1 concentrations in feed, animal health and climatic factors (Min et al., 2021; Zentai et al., 2023).

Aflatoxins pose a severe public health risk. The International Agency for Research on Cancer classifies AFM1 as a Group 1 human carcinogen, and the less-studied AFM2 exhibits similar toxicological properties (IARC, 2002). Infants and children are particularly vulnerable due to high dairy consumption. Their thermal stability is a critical challenge, as they resist degradation during pasteurization and spray-drying. Consequently, dehydration can concentrate aflatoxins in milk powder. Regulatory limits vary significantly, ranging from 0.05 µg/kg in the European Union to 0.5 µg/kg in the United States (FDA, 2007; Zheng et al., 2013; Nguyen et al., 2020; EU 2023/915).

Climate change is expanding the distribution of aflatoxin-producing fungi into previously low-risk temperate regions. Consequently, as dairy cattle consume locally sourced feed, mycotoxin carry-over into milk and milk powder varies significantly worldwide, depending directly on regional climate and agricultural practices (Battilani et al., 2016; Van der Fels-Klerx et al., 2019). Despite extensive research, data gaps remain regarding AFM2, as current literature focuses mainly on AFM1. Moreover, updated cross-regional studies evaluating mycotoxin profiles in raw milk and milk powder across contrasting climatic zones and different production systems remain limited, particularly because feed contamination patterns are expected to change (Nguyen et al., 2020; Kolarič et al., 2024; Malissiova et al., 2024).

Given the persistence of AFM1, recent research has increasingly focused on post-harvest mitigation strategies, as summarized by Kolarič et al. (2024). That review describes chemical, physical, biological, and physicochemical decontamination approaches, summarizes patents related to AFM1 elimination from milk and compares methods using the SCUFEERS criteria (Safety, Capacity, Universality, Finance, Ecology, Efficiency, Rapidity, and Selectivity). This framework provides a structured basis for assessing the practical applicability, although none of the currently available methods fulfills all of these criteria. In recent years, cyclodextrins (CDs) and their derivatives have emerged as environmentally friendly adsorbents capable of removing contaminants from various matrices. They are valuable in water purification, food applications, and packaging materials, enabling toxin elimination without negatively affecting

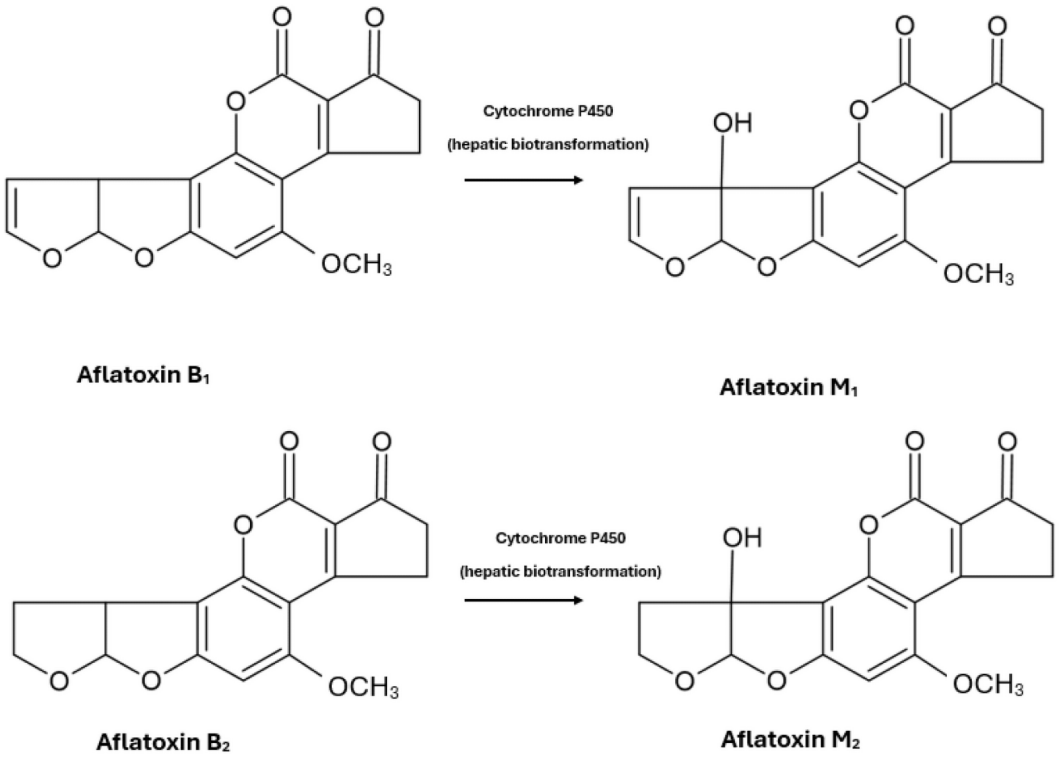

Figure 1. Hepatic biotransformation of aflatoxins B1 and B2 to their respective metabolites (M1 and M2) involving cytochrome P450.

product quality (Matencio et al., 2021). Cyclodextrins can form inclusion complexes with mycotoxins, including ochratoxin A, aflatoxins, patulin, zearalenone, zearalenol, and citrinin. One study reported that adding 1% β-CD during apple juice processing reduced patulin by 70% and enzymatic browning by 75% (Gonzalez Pereira et al., 2021). Insoluble β-CD polymers (BCP) have also reduced mycotoxins in aqueous solutions, including beer, and can be regenerated using a 50:50 ethanol–water mixture (Poór et al., 2018). The formation of an inclusion complex between BCP and AFM1 in a model solution was investigated by Kolarič et al. (2025).

The aim of this study was to: (1) assess seasonal and regional trends in the occurrence of aflatoxins M1 and M2 in Polish raw milk; (2) determine their presence in commercial milk powder from the V4 countries (Poland, the Czech Republic, Slovakia, and Hungary) and Iran; and (3) characterize AFM1 removal using BCP in model Ringer's solution and apply the optimized conditions to raw milk.

MATERIALS AND METHODS

Raw milk – 2 research models

Two sampling schemes were used in the study, representing contrasting dairy production systems. In both models, the unit of statistical analysis was a single raw bulk tank milk sample. The first model corresponded to an intensive production system characteristic of the western regions of Poland, where animals were kept indoors throughout the year. Based on their large-scale operations, 9 farms (herd sizes ranging from 420 to 1,050 cows) were purposely selected for this model. These same 9 farms were sampled repeatedly, resulting in a total of 228 raw bulk tank milk samples collected at a frequency of one to 2 samples per farm per month, from January to December 2025. The cows were fed a TMR based primarily on maize silage and grass or alfalfa silage/haylage, supplemented with concentrate feeds and mineral additives, with no major differences between summer and winter feeding. After collection, milk samples were cooled to 4°C and transported to the laboratory, where they were stored at −18°C until analysis. Sampling over 12 mo allowed assessment of the potential impact of seasonality on the occurrence of AFM1 and AFM2 in milk.

The second model represented a semi-intensive production system located in the eastern regions of Poland (Lubelskie, Mazowieckie, and Podlaskie provinces). This region is characterized by a highly fragmented farm structure scattered over a large area, with small- and medium-scale dairy farms predominating. This model provides a typical and representative picture of conventional dairy cattle farming in Poland. Due to the high fragmentation of this sector, a total of 28 family-owned farms were included in this part of the study, with herd sizes typically ranging from approximately 20 to 120 cows. A total of 56 milk samples were collected directly from these farms (winter: n = 29; summer: n = 27). These samples were also transported under refrigerated conditions and handled in the same manner as those in Model 1. The exact number of samples, as well as the location and date of collection, are detailed in Table 1.

Milk powder

The second material tested consisted of whole and skim milk powder samples (n = 35) obtained from Polish producers and purchased from retail chains throughout the country. For comparative purposes, milk powder samples produced in the Visegrad Group countries, namely Slovakia, the Czech Republic, and Hungary (n = 25), as well as in Iran (n = 10), were also analyzed. All milk powder samples were purchased in 2025.

Samples for AFM1 removal

To complement the monitoring study on the occurrence of aflatoxins in milk, an additional experiment was conducted to evaluate the efficiency of AFM1 removal from different types of milk using native β-cyclodextrin and its derivatives. The tested matrices included whole homogenized cow milk (3.5% fat; Pilos, Lidl, Bratislava, Slovakia), skim milk (0.5% fat; K-Classic, Kaufland, Bratislava, Slovakia), and raw cow milk (4.0% fat; ÚSVIT pri Dunaji Agricultural Cooperative, Dunajská Lužná, Slovakia).

Chemicals and reagents

The certified reference material of aflatoxin M1 (AFM1) was supplied as a 0.1 µg/mL solution in acetonitrile by Romer Labs, whereas aflatoxin M2 (AFM2) was supplied as a 0.1 µg/mL solution in acetonitrile by LGC Standards. The certified purity of all CRMs was > 99.0%. High-performance liquid chromatography (HPLC)-grade water, acetonitrile (ACN), and methanol (MeOH) were purchased from Witko. For the AFM1 removal experiment, the following chemicals were used: β-cyclodextrin bead polymer (Cyclolab R&D Laboratory Ltd.), native β-cyclodextrin (Cyclolab R&D Laboratory Ltd.), heptakis(2,3,6-tri-O-acetyl)-β-CD (Sigma-Aldrich), triacetyl-β-CD (Sigma-Aldrich), succinyl-β-CD (Sigma-Aldrich), methyl-β-CD (Sigma-Aldrich), sodium hydroxide, p.a. (Mikrochem), adipic acid, 99% (Sigma-Aldrich), acetic acid, p.a. (Mikrochem), tartaric acid, ≥ 99.5% (Sigma-Aldrich), and Ringer tablets (Merck KGaA).

Extraction of AFM1 and AFM2 from milk and milk powder

Preparation of raw milk samples. Milk samples were thoroughly mixed before analysis. For each sample, 2 aliquots of 50 g were transferred into separate 50-mL Falcon tubes. The samples were then centrifuged for 10 min at 10,000 rpm using an MPW-380R centrifuge (MPW Med. Instruments). After centrifugation, the upper fat layer was removed. For analysis, 50 g of defatted milk was applied to an Afla M1 LC immunoaffinity columns (VICAM) at a flow rate of approximately 1 drop/s.

Preparation of milk powder samples. Five grams of milk powder were weighed into a 50-mL Falcon tube, and 20 mL of ACN was added. The mixture was shaken for 1 min and then centrifuged for 10 min at 10,000 rpm using an MPW-380R centrifuge (MPW Med. Instruments). Fifteen milliliters of the clear supernatant were transferred to a vacuum evaporation flask and evaporated to dryness at 45°C. The residue was reconstituted in 5 mL of purified water, sonicated, and the resulting extract was applied to an Afla M1 LC immunoaffinity columns (VICAM) at a flow rate of approximately 1 drop/s.

Elution from the IAC column. The column bed was dried by passing air through the column. The column was then washed with 2 × 10 mL of purified water and dried again with air. The analytes were eluted with 4 mL of ACN. The eluate was evaporated to dryness under vacuum at 45°C. Immediately before HPLC analysis, the dry residue was dissolved in 1 mL of the mobile phase.

Preparation of crosslinked β-Cyclodextrin

Crosslinked β-CD was prepared according to the method described by Han et al. (2007). Briefly, 50 g of powdered native β-CD was weighed, and 40 mL of distilled water was added. The suspension was stirred for 2 h at room temperature on a magnetic stirrer at 250 rpm. After mixing, 2.5 g of adipic acid was added, and the pH was adjusted to 10 using aqueous NaOH solution (c = 1 M/mL). After pH adjustment, the mixture was stirred for a further 1.5 h on a magnetic stirrer. The pH was then adjusted to 5 using 5% acetic acid. The resulting mixture was filtered through filter paper and washed 3 times with approximately 80 mL of distilled water. After washing, the filter paper together with the crosslinked β-CD was placed in a climatic chamber and dried for 20 h at 60°C and 10% relative humidity. The dried crosslinked β-CD was then stored until further use. The same procedure was applied for crosslinking with tartaric acid.

Table 1. The origin and number of samples collected from various regions of Poland at different times of the year

Origin of samples - province	Sampling season	Number of samples	Research model
Mazowieckie	winter	6	1
Wielkpolskie		7	
Opolskie		6	
Świętokrzyskie		4	
Kujawsko-pomorskie		5	
Śląskie		4	
Mazowieckie	spring	11	
Wielkpolskie		18	
Opolskie		12	
Świętokrzyskie		6	
Kujawsko-pomorskie		6	
śląskie		4	
Lubelskie		21	
Mazowieckie	summer	10	
Wielkpolskie		13	
Opolskie		10	
Świętokrzyskie		6	
Kujawsko-pomorskie		5	
śląskie		4	
Mazowieckie	autumn	7	
Wielkpolskie		20	
Opolskie		15	
Świętokrzyskie		7	
Kujawsko-pomorskie		7	
śląskie		7	
Lubelskie		7	
Mazowieckie	winter	5	2
	summer	5	
Podlaskie	winter	5	
	summer	5	
Lubelskie	winter	19	
	summer	17	

Removal of AFM_1 from the model solution

For the removal of AFM1 from the model Ringer's solution, 7 types of β-CD were used: native β-CD, heptakis(2,3,6-tri-O-acetyl)-β-CD, triacetyl-β-CD, succinyl-β-CD, methyl-β-CD, and laboratory-prepared crosslinked forms of β-CD with tartaric acid (TA) and adipic acid (AA). Ringer's solution (pH 6.5) was prepared by dissolving 1 Ringer tablet (Merck KGaA, Germany) in deionized water. AFM1 was removed from the model solutions using a modified procedure based on that described by Mohos et al. (2022). Aliquots of 5 mL of the prepared solution containing AFM1 at a concentration of 0.5 µg/kg were transferred into test tubes containing the appropriate type and amount of β-CD. The samples were then mixed on a magnetic stirrer for 40 min at 1,000 rpm at ambient temperature. After mixing, the solutions were centrifuged at 1,020 × g for 15 min. The supernatant was carefully collected from each test tube using a micropipette, transferred into vials for HPLC analysis, and analyzed under the conditions described above.

Removal of AFM1 from milk

For the removal of AFM1 from milk, native β-CD, BCP, and β-CD cross-linked with adipic acid and tartaric acid were used. Three types of milk were tested in the AFM1 removal experiments: whole homogenized milk (3.5% fat), skim milk (0.5% fat), and raw milk (4.0% fat). First, milk spiked with AFM1 at a concentration of 0.5 µg/kg was prepared. Aliquots of 40 mL were transferred into tubes, and β-CD was added at a concentration of 2% (wt/vol). The samples were mixed on a magnetic stirrer for 40 min at 1,000 rpm at ambient temperature. Subsequently, the milk samples were transferred into centrifuge tubes and centrifuged at 1,020 × g for 15 min. After centrifugation, the insoluble β-CD–AFM1 complex was separated from the remaining liquid phase. The recovered milk phase was then centrifuged again at 2,000 × g for 20 min to remove the fat layer. To determine the final AFM1 concentration, extraction of AFM1 from the milk matrix was performed before HPLC analysis.

Determination of aflatoxins using HPLC-FLD

Identification and quantification of AFM1 and AFM2 were performed using high-performance liquid chromatography with fluorescence detection (HPLC-FLD; Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was carried out on a C18 analytical column (4.6 × 250 mm; Waters, Milford, MA, USA). The column was maintained at a constant temperature of 40°C. Separation was performed under isocratic conditions using a mobile phase consisting of water, ACN, and MeOH (68:24:8, vol/vol) at a flow rate of 1.0 mL/min. The total run time was 20 min. Detection was performed at an excitation wavelength of 360 nm and an emission wavelength of 440 nm.

Method validation

To verify the analytical method, the calibration curve linearity range, limit of detection (LOD), limit of quantification (LOQ), recovery (R), and precision (repeatability expressed as relative standard deviation, RSD) were determined for individual analytes and matrices (milk and milk powder). Standard solutions of AFM1 and AFM2 at a concentration of 0.05 µg/mL were prepared by dilution of the reference solutions (0.1 µg/mL). Six-point calibration curves were prepared over the range of 0.125 to 2.0 ng/mL using Chromeleon 7.3.2 (Thermo Fisher Scientific, Pleasanton, CA, USA). The coefficients of determination (R^2) for the obtained curves were 0.9992 for AFM1 and 0.9994 for AFM2. The LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration curve, according to the ICH Q2(R1) guidelines. The LOD and LOQ values for AFM1 and AFM2 in milk and milk powder are shown in Table S1 (Supplemental Material). Recovery and method precision were determined on the basis of analyses of 3 spiked samples for each matrix and concentration level. The fortification levels, recovery values, and RSD values for each matrix and analyte are presented in Table S2 (Supplemental Material).

The European Commission has established performance criteria for analytical methods for various mycotoxins, including AFM1, in Commission Implementing Regulation (EU) 2023/2782 (EU 2023/2782). No criteria have been established for AFM2. Therefore, the analytical parameters obtained for AFM2 in this study were compared with the criteria established for AFM1. For all analyzed aflatoxin concentrations, the regulation recommends recovery within the range of 70% to 120%, whereas method precision expressed as RSD should not exceed 20%. The method developed in this study met these criteria, with recovery values ranging from 71% to 97% for all tested concentrations and matrices and RSD values not exceeding 20%.

The developed method was also verified, at least with respect to AFM1, in an interlaboratory proficiency test. The proficiency test focused on AFM1 in milk powder and was conducted between February and March 2025 by FAPAS® (Sand Hutton, York, UK) and Fera Science Ltd. The result obtained was 0.185 µg/kg, compared with the assigned value of 0.241 µg/kg, corresponding to a z-score of −1.1.

Statistical analyses

Statistical analysis was performed using Statistica 13.1 software (StatSoft, Carlsbad, CA, USA). Inferential statistical analysis was applied exclusively to the decontamination experiments. In this part, data from 3 independent replications were subjected to one-way ANOVA (ANOVA) followed by Tukey's post hoc test, and differences were considered significant at $P < 0.05$. For the monitoring data, no inferential statistical analysis was performed because positive results above the LOQ were rare and limited to a few individual samples; therefore, these results were interpreted descriptively.

RESULTS AND DISCUSSION

Seasonal and regional dynamics of AFM1 and AFM2 occurrence in raw milk

The method developed for the determination of aflatoxins in both milk and milk powder enabled assessment of the safety of raw milk produced on Polish farms and of milk powder available in selected countries. To confirm the specificity and reliability of the IAC/HPLC-FLD methodology, particularly for the detection of AFM2 at trace levels, representative chromatograms are presented in Figure 2. The chromatograms demonstrate clear peak resolution and the absence of matrix interferences, comparing the analytical standards and a fortified sample with a naturally contaminated milk powder sample positive for both AFM1 and AFM2.

In the first study model, 228 raw milk samples were analyzed and divided into 4 seasons: winter (n = 32), spring (n = 78), summer (n = 48), and autumn (n = 70). Table 2 shows the type, origin, and sampling date of the samples in which the levels of aflatoxins M1 and M2 were above the LOQ.

A very low frequency of both aflatoxins was recorded in the analyzed samples, and none exceeded the European Union limit of 0.05 µg/kg for AFM1. Concentrations above the LOQ of 0.002 µg/kg for AFM1 were detected in only 4 samples throughout the year, representing approximately 1.7% of all raw milk samples tested. In winter, 1 positive sample was detected in the Wielkopolskie Province at a level of 0.004 µg/kg. In spring, AFM1 was detected in 3 samples from the Świętokrzyskie, Mazowieckie, and Kujawsko-Pomorskie provinces at levels of 0.010, 0.019, and 0.014 µg/kg, respectively. In nearly 99% of the samples, AFM2 concentrations did not reach the LOQ. During the spring and autumn seasons, none of the samples exceeded the LOQ, indicating high raw material purity during these periods. In winter, 1 positive sample was detected, whereas in summer 2 samples exceeded the LOQ, highlighting the sporadic nature of AFM2 contamination in Polish milk on an annual basis. In Model 1, despite the use of a Total Mixed Ration (TMR) with consistent ingredients throughout the year, positive AFM1/AFM2 findings were sporadic, with several observations occurring in the winter–spring period. However, given the very small number of positive samples, no robust inference on seasonality can be drawn. It is important to note that while the dietary composition remains stable, the microbiological quality of the silage used in the TMR may fluctuate. Prolonged storage of silage, potential aerobic instability due to damaged silage piles, or improper management of the face of the silo can lead to localized mold growth. These factors, often exacerbated by the high humidity characteristic of the transition from winter to spring, may plausibly contribute to sporadic aflatoxin occurrence even in highly controlled intensive systems; however, this explanation should be regarded as exploratory. In a study of the dairy market in Bosnia and Herzegovina, Kesić et al. (2021) conducted a comparative analysis to evaluate the impact of seasonality on AFM1 contamination in raw milk. The authors indicated that the highest prevalence of this mycotoxin was observed during the winter months, which was directly linked to the use of feed stored for long periods and therefore more susceptible to mold growth. This is broadly consistent with the limited observations from Model 1; however, due to the very low number of positive samples, these findings should be treated as preliminary and descriptive. They may indicate that the winter–spring feeding period warrants continued attention within Good Agricultural Practices (GAP), rather than demonstrating a definitive seasonal effect.

In the second study model, a total of 56 raw milk samples were analyzed, collected directly from Polish farms engaged in conventional dairy farming. The study considered key environmental variables, including the winter and summer seasons and 3 geographically and agriculturally diverse provinces: Lubelskie, Mazowieckie, and Podlaskie. The inclusion of samples from the Mazowieckie and Podlaskie provinces is particularly relevant because these regions are among the leading milk-producing areas in Poland. In contrast, Model 2 reflected a more diverse feeding regimen where silage-based winter diets were supplemented or replaced by fresh pasture grass during the summer months. In all analyzed samples, the concentrations of both AFM1 and AFM2 were below the LOQ. The absence of detectable aflatoxin levels, particularly during the winter season, confirms the safety of the analyzed samples. According to Tomašević et al. (2015), the autumn–winter period is widely recognized as a time of increased risk of AFM1 occurrence in milk. During this period, cows are mainly fed preserved and stored feed, such as silage (e.g., maize silage) and compound feed. Improper storage conditions,

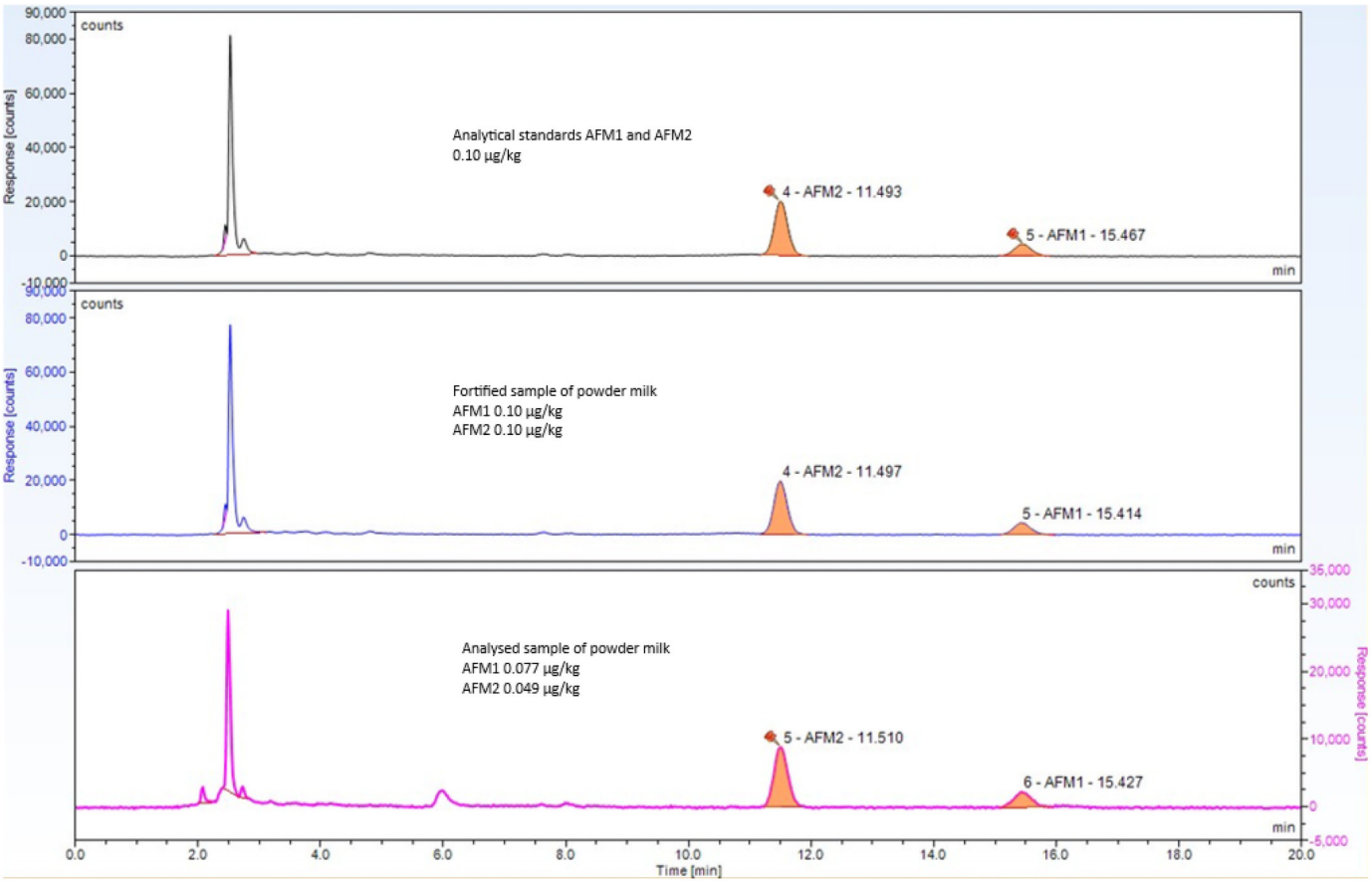

Figure 2. A chromatogram of analytical standards of AFM1 and AFM2 at a concentration of 0.10 µg/kg and a selected sample of powder milk in which the AFM1 (0.077 µg/kg) and AFM2 (0.049 µg/kg) content was determined.

including high humidity and limited air access in silos, favor the growth of Aspergillus molds and the synthesis of AFB1, which is subsequently metabolized to AFM1 in the cow's body. In a comprehensive 2-year study conducted between 2013 and 2014, Tomašević et al. (2015) analyzed 678 raw milk samples and 438 heat-treated milk samples from Serbia. The authors reported substantial contamination levels, with 56.2% of raw milk samples and 32.6% of heat-treated milk samples exceeding the maximum residue limit established by the European Union. The mean AFM1 concentration in winter milk samples were 0.358 µg/kg, which did not differ significantly from the spring mean of 0.375 µg/kg. Conversely, AFM1 levels in raw milk were significantly lower during the summer (0.039 µg/kg) and autumn (0.103 µg/kg) seasons. Notably, seasonal fluctuations in AFM1 concentrations in heat-treated milk closely mirrored the trends observed in the raw material. In a comprehensive report by Womack et al. (2016), the global occurrence of AFM1 in milk between 2010 and 2015 was evaluated.

Table 2. Raw milk samples in which AFM1 and/or AFM2 levels exceeded the limit of quantification (LOQ)

Origin	No. of samples / positive samples / percentage of total	Season of sampling	Sample	AFM1 (µg/kg) ± SD	AFM2 (µg/kg) ± SD
Kujawsko-pomorskie	6 / 1 / 17%	Spring	1	0.014 ± 0.003	<LOQ
Mazowieckie	11 / 1 / 9%	Spring	1	0.019 ± 0.004	<LOQ
Świetokrzyskie	6 / 1 / 17%	Spring	1	0.010 ± 0.002	<LOQ
Wielkopolskie	7 / 1 / 15%	Winter	1	0.004 ± 0.001	0.002 ± 0.001
	13 / 2 / 16%	Summer	1	<LOQ	0.078 ± 0.016
			2	<LOQ	0.012 ± 0.003

Abbreviation: LOQ - limit of quantification. SD - standard deviation.

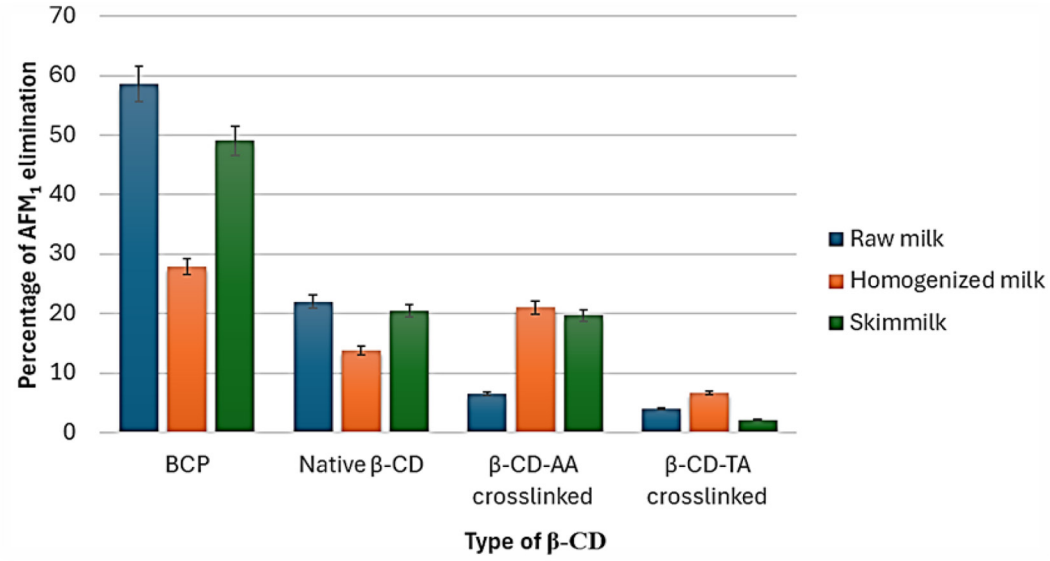

Figure 3. Measure of aflatoxin M_1 (AFM1) removal from different processed milk using different type of β-cyclodextrin (β-CD). BCP – β-cyclodextrin bead polymer, AA – adipic acid, TA – tartaric acid

Among the 7,841 samples analyzed, 75% tested positive for AFM1. Furthermore, 26% of these samples exceeded the strict European Union maximum residue limit of 0.05 µg/kg, whereas 1.53% exceeded the less stringent limit of 0.5 µg/kg established by the US Food and Drug Administration (FDA). A substantial body of literature now confirms the widespread and persistent presence of AFM1 in dairy products worldwide, emphasizing the need for continuous monitoring.

Research by Nile et al. (2016) on 600 milk samples from India revealed AFM1 contamination in 33.2% of goat milk samples and 45.3% of cow milk samples, with 44% of the latter exceeding the European Union limit of 0.05 µg/kg. Significantly higher concentrations in urban and peri-urban farms indicate that regional climate and feed management strongly influence mycotoxin risk. Similarly, Goncalves et al. (2017) demonstrated a markedly different safety profile in Brazilian smallholder farms, detecting AFM1 in 100% of raw milk samples, with concentrations reaching up to 3.385 µg/kg. Because 60% of the samples exceeded the European Union limit, the authors attributed this severe contamination primarily to the lack of Good Agricultural Practices during feed storage. Lee and Lee (2015) were among the first to comprehensively analyze both liquid and powdered milk for the concurrent presence of AFM1 and AFM2 in Seoul, South Korea. In the analyzed liquid milk samples, 8 cases of aflatoxin contamination were recorded (6 positive for AFM1 and 2 for AFM2), with a mean concentration of 0.021 µg/kg. However, the overall detection frequency of the less frequently investigated AFM2 remained relatively low, ranging from 2% to 10% across all analyzed dairy products.

To better illustrate the scale of mycotoxicological safety in the Polish dairy sector, the obtained results were compared with literature data from other regions of the world (Table 3). A marked disparity in the incidence and concentrations of AFM1 across geographical zones is evident. While raw materials originating from Central Europe, including the V4 countries, are characterized by marginal contamination levels (<2% for raw milk) and an almost negligible risk of exceeding the strict European Union limit, dairy chains in warm and subtropical climate zones are exposed to chronic mycotoxicological pressure. As shown in Table 3, in countries such as Iran, Pakistan, China, and Brazil, the percentage of positive samples often reaches 100%, and the recorded concentrations may exceed the European Union limit of 0.05 µg/kg many times over. This highlights the important role of integrated Good Agricultural Practice systems in EU member states, which, together with a cooler climate, effectively limit the development of *Aspergillus* fungi during feed storage.

Occurrence of AFM1 and AFM2 in commercial milk powder from different geographical regions

Table 4 presents the results obtained for positive samples from Poland, other Visegrad Group countries, and Iran, in which the levels of AFM1 and AFM2 exceeded the LOQ. The limits of quantification (LOQ) for the applied analytical method were 0.020 µg/kg for AFM1 and 0.010 µg/kg for AFM2.

Table 3. Global variation in the occurrence of AFM1 in raw/pasteurized milk

Country (Region)	Number of samples (n)	Incidence (>LOQ)	Range [µg/kg]	Exceeding EU limit (0.05 µg/kg)	Reference
Poland	228	1.7%	<LOQ-0.019	0%	Present study (model 1)
Poland	56	0%	<LOQ	0%	Present study (model 2)
Germany	20	0%	>LOD- < LOQ	0%	El-Khatib et al., 2025
Iran (Northwest)	60	100%	0.057–0.271	100%	Mokhtari et al., 2022
Iran	111	77%	<LOQ-0.28	40%	Kamkar 2005
Turkey	176	30%	<LOQ-1.01	17%	Golge, 2014
Brazil	52	100%	<LOQ-3.385	40%	Goncalves et al., 2017
Pakistan (Sindh)	315	100%	0.49–0.72	100%	Bhuptani et al., 2022
China	189	44%	<LOQ-0.017	0%	Xiong et al., 2022
Lebanon	712	59%	<LOQ-0.219	28%	Daou et al., 2020
Turkey	120	89%	<LOQ-0.079	3.3%	Eker at al. 2019
Serbia	1793	87%	<LOQ-0.05	0%	Udovicki et al., 2019
Greece	285	56%		0%	
Egypt	10	60%	<LOQ-0.125	66%	Younis et al., 2016

Based on the obtained data, significant variation in the contamination profile was observed depending on the product's region of origin. In samples representing the Visegrad Group countries (the Czech Republic, Slovakia, and Hungary; n = 25), none of the investigated mycotoxins were detected at concentrations exceeding the method LOQ, meaning that 100% of the results were < LOQ. For samples originating from Poland (n = 35), a predominant proportion of negative results (approximately 91.4%) was recorded; nevertheless, the presence of outliers was identified. Quantifiable AFM1 concentrations were detected in 2 samples, reaching 0.077 µg/kg and the highest value recorded in the study, 0.101 µg/kg. Additionally, 1 Polish sample exhibited the presence of AFM2 at a level of 0.049 µg/kg.

The significantly higher AFM1 concentrations recorded in a subset of the milk powder samples can be explained from a technological perspective, as reported by Iqbal et al. (2015). As the authors indicated, AFM1 is a highly thermostable compound that resists degradation during standard heat treatments such as pasteurization, UHT sterilization, and spray drying. Consequently, intensive evaporation of water leads to a pronounced concentration effect of the toxin in the dry matter. This phenomenon clearly explains why raw milk that complies with stringent safety regulations in its liquid form may exhibit elevated contamination levels after processing. Conversely, there are also literature reports suggesting that thermal treatments can induce a partial reduction in AFM1 levels, ranging from 12% to 35% (Prandini et al., 2009; Kabak, 2012).

A significantly different distribution profile was observed in the group of samples imported from Iran (n = 10). The presence of AFM1 was identified in 20% of the samples, with values of 0.042 and 0.049 µg/kg, whereas AFM2 was quantified in as many as 50% of the tested samples, with concentrations ranging from 0.011 to 0.026 µg/kg. The occurrence of AFM2 in dairy products from the Middle Eastern region appears to be recurrent. As confirmed by Iqbal et al. (2015), the Middle East and other warm climate zones are areas with a substantially elevated risk of aflatoxin contamination compared with the European Union. This results from both temperature and humidity conditions that favor toxin biosynthesis by *Aspergillus* spp. in the field and challenges in maintaining rigorous cold chains and feed control in these regions. This geographical vulnerability is well documented in the literature. Fallah et al. (2011) conducted a study to

Table 4. Milk powder samples in which AFM1 and/or AFM2 levels exceeded the limit of quantification (LOQ)

Origin	No. of samples / positive samples / percentage of total	Sample	AFM1 [µg/kg] ± SD	AFM2 [µg/kg] ± SD
Poland	35 / 2 / 6%	1	0.077 ± 0.016	0.049 ± 0.010
		2	0.101 ± 0.021	<LOQ
Czech Republic, Slovakia, Hungary	25 / 0 / 0%	—	<LOQ	<LOQ
Iran	10 / 5 / 50%	1	0.049 ± 0.010	0.026 ± 0.006
		2	<LOQ	0.019 ± 0.004
		3	0.042 ± 0.009	0.023 ± 0.005
		4	<LOQ	0.014 ± 0.003
		5	<LOQ	0.011 ± 0.003

Abbreviation: LOQ - limit of quantification. SD - standard deviation.

determine AFM1 contamination in milk in Iran and reported a mean aflatoxin level of 0.323 μg/L. Similarly, a high incidence of AFM1 in milk, with a mean concentration of 0.252 μg/L, was reported by Sadia et al. (2012) in Pakistan. Comparable mean levels (0.212 μg/L) in Pakistani milk were also documented by Iqbal and Asi (2013). Furthermore, in a study conducted by Tomašević et al. (2015), the mean concentration of AFM1 in milk powder samples purchased in Serbia reached 0.847 μg/kg. High contamination rates are particularly evident in processed products. Milk powder analyzed by Lee and Lee (2015) emerged as the most contaminated product in their comprehensive assessment, with AFM1 confirmed in as many as 74% of the analyzed samples. The mean AFM1 concentration in this matrix reached 0.271 μg/kg, providing strong evidence of the mycotoxin concentration effect during water evaporation from the raw material. Additionally, the powdered product exhibited the presence of AFM2 at a mean level of 0.030 μg/kg. In the same context, other analyses of milk powder from Egypt (n = 10) demonstrated that 80% of the samples contained AFM1, with concentrations exceeding the permissible EU limits in all positive samples.

The comprehensive analysis of milk and milk powder samples from the V4 countries and Iran indicates that aflatoxins M1 and M2 remain detectable contaminants in the global dairy supply chain despite long-standing regulatory limits and established monitoring programs aimed at minimizing exposure. Although most European samples typically comply with current legislation, the presence of measurable aflatoxin concentrations confirms a persistent exposure risk and highlights significant variability across countries, production batches, and product types. These findings underscore the critical need not only for continued surveillance but also for the evaluation of processing-level interventions that could further reduce aflatoxin levels in milk. In this context, assessing the technological feasibility of potential decontamination strategies becomes highly relevant as a complementary approach to the primary preventive measures implemented at the feed level.

The development of a comprehensive strategy for eliminating aflatoxins from the dairy food chain

Although the monitoring results indicated a very low occurrence of aflatoxins in Polish milk, numerous reports from regions with warmer climates indicate that aflatoxin contamination remains a persistent issue in the global dairy supply chain. Therefore, the development and evaluation of technological strategies capable of reducing AFM1 levels in milk represent an important complementary approach to primary preventive measures applied at the feed level.

The previous published work (Kolarič et al., 2025) showed that AFM1 can effectively form inclusion complexes with BCP. In this study, several other types of β-CD (native β-CD, chemical derivatives – heptakis(2,3,6-tri-O-acetyl)-β-CD, triacetyl-β-CD, succinyl-β-CD, methyl-β-CD, and laboratory-prepared crosslinked forms of β-CD with tartaric (TA) and adipic acid (AA)) were evaluated for AFM1 removal from a model Ringer's solution. The aim was to determine how the structure and solubility of individual CD forms affect the formation of the inclusion complex and the efficiency of AFM1 removal from the aqueous environment. The results are shown in Table 5. Under the selected mixing conditions, native β-CD dissolved in Ringer's solution, and therefore the formed inclusion complex between AFM1 and β-CD could not be effectively separated, making it impossible to evaluate adsorption efficiency. Unsatisfactory results were also obtained using the chemical derivatives of β-CD. In this system, the derivatives did not form, or formed only a very unstable complex with AFM1, and thus had no effect on AFM1 removal from the model system. The crosslinked forms performed somewhat better. However, the binding efficiency of AFM1 to β-CD crosslinked with AA (14.2%) or TA (12.7%) was still lower compared with BCP (63.1%) as shown in our previous study (Kolarič et al., 2025). The higher efficiency of AFM1 removal achieved with BCP, compared with crosslinked forms of β-CD, can be attributed to several factors. BCP possesses a substantially larger specific surface area and a more porous structure, which enhances the accessibility of cyclodextrin cavities for mycotoxin adsorption (Crini, 2014). In contrast, crosslinking with AA or TA produces ester or amide bonds that generate denser polymer networks, potentially leading to partial blockage of cyclodextrin cavities (i.e., steric hindrance) or alterations in their spatial arrangement. Moreover, BCP maintains a more favorable balance between hydrophobicity and hydrophilicity, which increases its affinity for the moderately hydrophobic AFM1 molecule (Crini et al., 2018).

Next, the 4 types of β-CD (native β-CD, BCP, β-CD-AA crosslinked and β-CD-TA crosslinked) were applied in the experiments of AFM1 removal from real cow milk samples. The complexity of the milk matrix greatly limits the solubility of native β-CD, what was described also in the studies related to cholesterol elimination from milk (Kolarič and Šimko, 2022). Therefore, the application of native β-CD for AFM1 removal from milk was limited. The efficiency of AFM1 removal using 4 types of β-CD was also assessed in different types of processed milk, including raw, homogenized, and skim milk. In non-homogenized milk, fat globules naturally merge and rise to the surface as cream, whereas homogenization reduces their size and ensures uniform distribution throughout

the volume (Shetty and Sharma, 2022). By comparing raw (non-homogenized), whole (homogenized), and skim milk, the study explored how differences in milk processing and matrix related physical properties are associated with variations in AFM1 elimination efficiency. The results indicated differences in AFM1 removal efficiency among the tested milk matrices, suggesting that milk composition and processing-related factors, including fat content and fat distribution, may influence the adsorption process. The highest AFM1 elimination rates using native β-CD (22.0%) and BCP (58.6%) were observed in raw, non-homogenized milk. Comparable removal efficiencies were obtained in skim milk (20.5% for native β CD and 49.1% for BCP), whereas lower efficiencies were recorded in whole homogenized milk (13.8% for native β CD and 27.9% for BCP). These results indicate that differences in milk matrix characteristics, associated with milk processing and fat content, are accompanied by measurable variations in AFM1 removal efficiency. In particular, matrices with either non homogenized fat (raw milk) or very low fat content (skim milk) showed higher adsorption efficiencies compared with whole homogenized milk. A possible explanation for this observation is that, in whole homogenized milk, the fine dispersion of fat globules may increase the availability of lipophilic components, such as cholesterol, which can compete with AFM1 for binding to β cyclodextrin cavities. This competitive interaction may reduce the number of adsorption sites accessible for AFM1. A similar competitive effect may also occur for native β CD, which is known to exhibit affinity toward various hydrophobic milk constituents released or redistributed during homogenization. However, given that the tested milk types originated from different sources, these interpretations should be regarded as indicative rather than definitive, and additional controlled studies using standardized milk matrices would be required to isolate the individual contributions of fat content, homogenization, and other compositional factors.

During the evaluation of the crosslinked β-CD derivatives, both polymers demonstrated only limited AFM1 removal in raw milk. In contrast, in whole homogenized milk, the β-CD crosslinked with adipic acid exhibited substantially higher removal efficiency, reaching up to 21%. This difference is likely attributable to the distinct structural properties of the crosslinking agents. The flexible, linear architecture of adipic acid may facilitate more effective inclusion of AFM1 into the cyclodextrin cavity. However, in raw milk, the presence of large fat globules may form a physical barrier that restricts the interaction between AFM1 and the polymer, thereby reducing binding efficiency even when the polymer's structural characteristics are favorable (Kwak et al., 2011). This suggests that matrix-related physical barriers can override the intrinsic affinity of the polymer toward AFM1.

From a practical perspective, the effectiveness of AFM1 removal must be interpreted in relation to initial contamination levels and regulatory thresholds. In cases where the initial AFM1 concentration is close to the European Union maximum limit of 0.05 µg/kg, the observed removal efficiency of BCP (up to 58.6% in raw milk) could theoretically reduce AFM1 to concentrations well below the regulatory limit. This suggests that, under specific contamination scenarios, β-cyclodextrin-based sorbents may contribute to achieving compliance with food safety regulations. From a regulatory and technological standpoint, particular attention must be paid to the type of sorbent used. While β-cyclodextrin bead polymers exhibited the highest AFM1 removal efficiency, their application in dairy processing would require validation regarding complete sorbent removal, absence of residues, and potential effects on milk composition and sensory quality. In contrast, native β-cyclodextrin is already approved for food applications and has GRAS status, which makes it more attractive from a regulatory perspective.

Table 5. The effect of the type of β-cyclodextrin (β-CD) on the elimination of aflatoxin M1 (AFM1) from a model Ringer's solution (c_{AFM1} = 0.5 µg/kg)

Type of β-CD	Amount of β-CD [%, wt/vol]	Final concentration of AFM1 [µg/kg][a]	Percentage of removal of AFM1 [%]
Native	4	0.47 ± 0.01	6.2
BCP[b]	4	0.19 ± 0.01*	63.1
heptakis(2,3,6-tri-O-acetyl)-β-CD	4	0.52 ± 0.00	—
triacetyl-β-CD	4	0.51 ± 0.00	—
succinyl-β-CD	4	0.50 ± 0.00	—
metyl-β-CD	4	0.51 ± 0.00	—
β-CD-AA crosslinked	4	0.43 ± 0.01*	14.2
β-CD-TA crosslinked	4	0.44 ± 0.01*	12.7

[a] – the results are expressed as mean ± standard deviation, n = 3 (independent replications);.

[b] – the results according to previous study (Kolarič et al., 2025);.

* - the results are statistically different at $P < 0.05$ from initial concentration of AFM1; BCP - β-CD polymer; AA – adipic acid; TA – tartaric acid.

Therefore, future research should focus on improving the AFM1 binding efficiency of native β-cyclodextrin under milk processing conditions, for example by optimizing dosage, contact time, or process integration, to combine regulatory acceptability with sufficient detoxification performance.

CONCLUSION

The comprehensive evaluation of raw milk and commercial milk powder demonstrated that the occurrence of aflatoxins M1 and M2 was generally low, although clear differences were observed depending on geographical origin, product type, and milk matrix. In raw milk from Poland, positive findings were sporadic and limited to a few individual samples, confirming a high overall safety level; however, these observations were too infrequent to support robust conclusions regarding seasonality. In milk powder, samples from the Visegrad Group countries showed a very favorable safety profile, whereas products from Iran exhibited a higher frequency of aflatoxin occurrence, indicating regional differences in contamination patterns. These findings suggest that climatic conditions, feed quality, and production practices may influence the occurrence of aflatoxins in the dairy chain. Among the tested decontamination strategies, β-cyclodextrin bead polymers (BCPs) showed the highest AFM1 removal efficiency, while adsorption performance varied according to the type of milk tested. Overall, the results confirm the importance of continued region-specific monitoring and indicate that processing-level decontamination strategies may serve as a complementary measure to primary prevention at the feed level in efforts to reduce aflatoxin exposure in dairy products.

NOTES

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Conflict of interest The authors declare that they have no conflict of interest.

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