Country report on the presence of aflatoxin M1 in milk

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1. National legislation in Slovakia

In Slovakia, the regulation of aflatoxin M_1 (AFM_1) in milk is primarily based on European Union legislation, which is directly applicable, and complemented by national laws governing food safety, official controls, and public health protection.

1.1. EU legislative framework

The regulation of AFM_1 in milk within Slovakia is based on a harmonised European Union (EU) legislative framework, which establishes common rules on food safety and contaminant control across all Member States. These regulations are directly applicable and ensure a consistent level of consumer protection throughout the EU.

Previously, the maximum levels for AFM_1 in milk were established by Commission Regulation (EC) No 1881/2006, which defined strict limits for contaminants in foodstuffs. However, this regulation was repealed and replaced by Regulation (EU) 2023/915, which currently represents the main legal act governing contaminants in food, including mycotoxins (Commission Regulation (EU) 2023/915).

Regulation (EU) 2023/915 consolidates and updates previous legislation while maintaining a high level of public health protection. It sets maximum permissible levels for AFM_1 in milk at 0.05 µg/kg for raw milk, heat-treated milk and milk for the manufacture of milk-based products, and 0.025 µg/kg for infant formulae, follow-on formulae and young-child formulae. These limits reflect a precautionary approach, particularly for vulnerable population groups such as infants, and are based on the principle that contaminant levels should be kept as low as reasonably achievable (ALARA). Although Regulation (EU) 2023/915 simplified and restructured the legislative framework, it did not introduce major changes in the maximum levels for AFM_1 compared to the earlier regulation but rather consolidated them into a clearer and more coherent legal instrument.

The EU framework is further complemented by subsequent amendments and implementing measures. In particular, Regulations (EU) 2024/1756 introduced modifications related mainly to other mycotoxins, sampling procedures, and clarification of food categories, while the limits for AFM_1 in milk remained unchanged. The amendment primarily introduces changes to Annex I, where it more precisely defines to which food categories and at which stages of production or distribution the individual limits apply. At the same time, it supplements and modifies maximum levels for several contaminants, including ergot sclerotia, T-2 and HT-2 toxins, tropane alkaloids, and hydrocyanic acid, with the changes mainly affecting cereals, oats, rice, and foods intended for young children. The amendment also includes an update of sampling procedures and analytical methods in order to ensure uniform and comparable controls across EU Member States (Commission Regulation (EU) 2024/1756). This demonstrates the dynamic nature of the EU regulatory system, which is continuously updated based on new scientific evidence and risk assessments.

In addition to maximum limits, the EU legislative framework includes controls and enforcement mechanisms. Regulation (EU) 2017/625 on official controls establishes harmonised rules for monitoring and verification of compliance with food and feed law across the entire agri-food chain. It introduces risk-based controls, defines the responsibilities of competent authorities, and ensures that contaminants such as AFM_1 are systematically monitored in food products, including milk and dairy products.

Analytical requirements for the determination of AFM_1 were previously defined in Commission Regulation (EC) No 401/2006, which laid down specific methods of sampling and analytical performance criteria for the official control of mycotoxins in foodstuffs. This regulation included detailed provisions for sample collection, preparation, and validation of analytical methods, ensuring reliability and comparability of results across laboratories. It explicitly addressed mycotoxins, including aflatoxins, and established criteria such as limits of detection, recovery rates, and precision requirements for their determination. This regulation was repealed and replaced by Commission Implementing Regulation (EU) 2023/2782, which introduced a revised and more harmonised framework for sampling and analysis. Unlike the earlier regulation, the new act adopts a horizontal approach and does not explicitly list individual mycotoxins such as AFM_1. Instead, it defines generalised sampling procedures and analytical performance criteria applicable to all mycotoxins across defined food categories, including milk and dairy products.

1.2. Slovak national legislation

In Slovakia, the control of AFM_1 in milk is governed by a combination of directly applicable European Union legislation and national legal acts that define responsibilities for food safety, veterinary supervision, and official controls. The basic legal framework is established by **Act No. 152/1995 Coll.** on foodstuffs, which lays down general requirements for food safety, responsibilities of food business operators, and procedures for official controls. This act ensures that food placed on the market must comply with EU requirements, including maximum levels for contaminants such as AFM_1.

In addition, **Act No. 39/2007 Coll.** on veterinary care regulates the production of raw milk and animal health, including the control of feed contamination, which is a critical factor in the occurrence of AFM_1 in milk. The protection of public health is further supported by Act No. 355/2007 Coll. on the protection, support and development of public health, which covers monitoring of chemical contaminants in food and assessment of their health risks.

The system of official control is implemented by several competent authorities. The main authority responsible for food safety and control of food of animal origin, including milk and dairy products, is the **State Veterinary and Food Administration of the Slovak Republic** (SVPS SR). This authority carries out official inspections, sampling, and laboratory analysis of food products, and supervises compliance with both EU and national legislation.

Another key institution is the **Public Health Authority of the Slovak Republic** (UVZ SR), which focuses on the protection of consumer health and assesses risks related to contaminants in food, including mycotoxins. It also conducts monitoring programmes and provides expert opinions in the field of food safety.

At the regional level, these activities are supported by **Regional Veterinary and Food Administrations** and **Regional Public Health Authorities**, which perform local inspections, sampling, and enforcement measures.

In practice, the control of AFM_1 in milk is based on a coordinated system combining prevention and monitoring. Food business operators are required to implement HACCP-based systems and ensure the safety of feed and raw materials, while competent authorities carry out regular official controls, including sampling of milk and dairy products, to verify compliance with EU maximum limits.

Overall, the Slovak system reflects the harmonised EU approach, with clearly defined responsibilities and a structured control framework ensuring effective monitoring and management of AFM_1 in the dairy sector.

1.2.1. Aflatoxin M_1 monitoring in Slovakia

Monitoring of AFM_1 in Slovakia is carried out based on an annual plan, within which the SVPS SR allocates financial resources at the beginning of each year to cover analyses throughout the entire year.

The role of Veterinary and Food Institute in Bratislava, Košice and State Veterinary and Food Institute in Dolný Kubín is based on a mandate from the **Ministry of Agriculture and Rural Development of the Slovak Republic** (MPRV SR), and as the national reference laboratories, they are responsible for the analysis of AFM_1 samples from across the whole country.

Sampling is performed at several levels of the food chain - most commonly at farms (primary production), dairy processing plants, and within the retail network. This multi-level approach allows monitoring of AFM_1 contamination throughout the entire process, from production to final sale. AFM_1 analyses were performed at the Veterinary and Food Institute in Bratislava (VPÚ BA) until the end of 2024. Since 2025, following the specialisation of the State Veterinary and Food Institutes, this analysis has been carried out by the Veterinary and Food Institute in Košice.

The main objective of the monitoring system is to ensure the protection of public health in the Slovak Republic by controlling the safety of milk and dairy products.

Data on analysed milk samples for the period 2015–2025, provided by the State Veterinary and Food Institute in Dolný Kubín, are shown in Table 1.

Tab. 1 Summary of analysed milk samples for aflatoxin M_1 in Slovakia during the period 2015–2025.

Year	No. of samples	No. of positive samples	No. of negative samples
2015	13	0	13
2016	12	0	12
2017	15	2	13
2018	13	0	13
2019	10	1	9
2020	10	0	10
2021	10	1	9
2022	12	1	11
2023	5	1	4
2024	6	0	6
2025	49	0	49

Source: Data provided by the State Veterinary and Food Institute in Dolný Kubín

1.2.2. Key institutions and their roles in aflatoxin M_1 monitoring and research in Slovakia

The State Veterinary and Food Administration of the Slovak Republic (SVPS SR) is the central authority responsible for food safety and official control of food and feed in Slovakia. It operates under the Ministry of Agriculture and Rural Development and coordinates national monitoring programmes, including those focused on contaminants such as aflatoxin M_1. SVPS SR supervises compliance with both EU and national legislation, organises official inspections, and ensures that food of animal origin, including milk and dairy products, meets safety requirements.

At the regional level, the official control system is implemented by the **Regional Veterinary and Food Administrations** (RVPS). These authorities are responsible for carrying out inspections directly in the field, including farms, dairy processing plants, and retail establishments. They organise and perform sampling of milk and dairy products, verify compliance with hygiene and safety standards, and enforce corrective measures in cases of non-compliance.

The laboratory analysis of aflatoxin M_1 is performed by specialised institutions within the network of the **State Veterinary and Food Institutes** (SVPI). These laboratories play a crucial

role in the official control system by providing reliable analytical results for contaminants in food. As a national reference laboratory, selected institutes are responsible for the analysis of AFM_1 in milk samples collected across Slovakia. In recent years, analytical activities have been reorganised and partially centralised to designated laboratories in order to enhance efficiency and ensure consistent analytical performance.

The **Public Health Authority of the Slovak Republic** (UVZ SR) is responsible for the assessment of risks related to food contaminants from a public health perspective. It evaluates the impact of mycotoxins such as aflatoxin M_1 on human health, participates in national monitoring programmes, and provides expert opinions and recommendations to support food safety policies.

Dairy research institute (Výskumný ústav mliekarenský) focuses on applied research in the field of milk production, processing, and dairy product quality. Its activities include the development of technological processes, quality control methods, and evaluation of contaminants in milk and dairy products. Within the context of food safety, the institute contributes to research on the occurrence and prevention of contaminants such as aflatoxin M_1, particularly in relation to dairy processing and product stability.

The Faculty of Chemical and Food Technology of the Slovak University of Technology in Bratislava (FCHPT STU) is one of the leading academic institutions in Slovakia in the field of food chemistry, food safety, and analytical methods. Research carried out at the faculty includes the study of mycotoxins, development and validation of analytical methods, and assessment of chemical contaminants in food. The faculty plays an important role in both research and education, contributing to scientific knowledge and training specialists in food safety and analysis.

The National Agricultural and Food Centre (Národné poľnohospodárske a potravinárske centrum, NPPC) is a key state research institution focusing on agriculture, food production, and food safety. Its research activities cover crop production, feed safety, and risk factors influencing the occurrence of mycotoxins in the food chain. Since aflatoxin M_1 in milk originates from contaminated feed, NPPC plays an important role in studying primary sources of contamination and preventive measures at the agricultural level.

The Department of Hygiene, Technology and Food Safety at the University of Veterinary Medicine and Pharmacy in Košice (UVLF Košice) is involved in research and teaching related to food hygiene, veterinary public health, and food safety control. The department

conducts studies on microbiological and chemical hazards in food, including mycotoxins, and focuses on risk assessment, monitoring, and prevention strategies. It also contributes to the education of veterinarians and food safety professionals who later participate in official control systems.

2. Former research and research results

The presence of AFM_1 in the dairy supply chain represents a persistent challenge for public health and food safety in Slovakia. In recent years, research in this field has gained increased importance due to shifting climatic patterns in Central Europe, which favour the growth of aflatoxigenic fungi in feed crops.

Given the strict European regulatory limits, Slovak research has strategically pivoted towards two critical areas:

1. **Development of advanced analytical methods**: Enhancing the sensitivity, speed, and cost-effectiveness of AFM_1 detection to ensure robust monitoring of raw milk and dairy products.
2. **Mitigation and reduction strategies**: Investigating innovative processing and decontamination techniques aimed at reducing the AFM_1 concentrations without compromising the nutritional and sensory quality of the milk.

The following sections summarize the key research activities and publications conducted in these areas, highlighting the main findings and their practical implications for the Slovak dairy industry. Furthermore, the research conducted in Slovakia is not only aimed at ensuring compliance with national safety standards but also at contributing to the collective food security of the Visegrad Four (V4) region. By focusing on both diagnostic precision and practical mitigation, this work provides a scientific foundation for harmonizing control mechanisms across borders. The integration of these research findings is crucial for developing proactive recommendations that can protect the dairy value chain against the increasing risks of mycotoxin contamination in Central Europe.

2.1. Development of analytical methods for aflatoxin M_1 determination

2.1.1. Introduction

Several analytical approaches are used for the determination of AFM_1 concentrations in milk and dairy products, including thin-layer chromatography (TLC), high-performance liquid

chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). The first method officially recognized by the International Association of Official Analytical Chemists (AOAC) for AFM_1 determination was TLC. However, it is no longer used today because its detection limit is relatively high (more than 50 ng/kg) and its accuracy low. ELISA represents a simple and user-friendly technique, although its results sometimes require confirmation by additional analytical methods. Currently, the official AOAC method for determining AFM_1 in dairy products is HPLC with fluorescence detection (**HPLC-FLD**) (Sarmast et al., 2021).

Before AFM_1 detection itself, samples must be properly prepared. This process typically includes the removal of fat and other undesirable components, followed by centrifugation and filtration, as well as extraction or sample clean-up. In cheese analysis, AFM_1 extraction may also involve preparing a suspension. Rapid and simple sample pretreatment can be achieved using immunoaffinity columns (**IAC**), which combine the extraction and clean-up steps into a single procedure. Alternatively, traditional techniques such as solid-phase extraction (SPE), liquid–liquid extraction, or single-step multifunctional clean-up columns such as MycoSep may also be used (Iqbal et al., 2015). General steps of sample preparation prior to the determination of AFM_1 concentration can be summarized according to the **Fig. 1**.

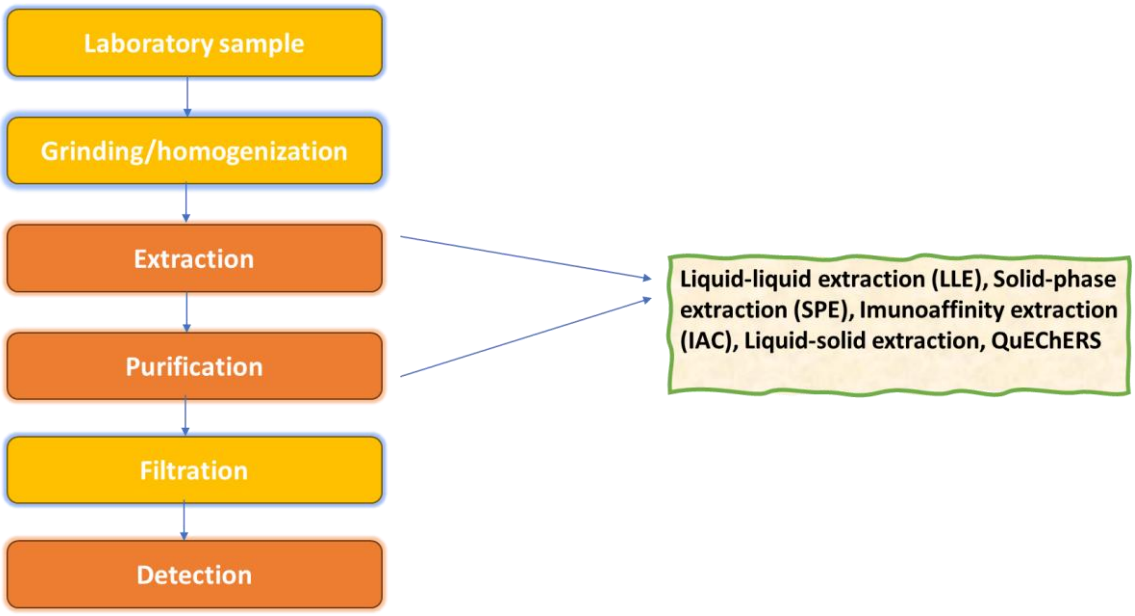

Fig. 1. Summary of the basic steps of sample preparation prior to aflatoxin M_1 determination.

IAC occupy a special position among immuno-based analytical approaches, as they have been used for many years as a method for sample clean-up and concentration in aflatoxin analysis.

The principle of IAC lies in the immobilization of an antibody (polyclonal or monoclonal) that recognizes the analyte onto a solid support, such as agarose or silica, in a phosphate buffer within a small column. Aflatoxins are low-molecular-weight compounds and are immunogenic only when bound to a protein carrier. Antibodies specific to aflatoxins are produced, bound to agarose, sepharose, or dextran, and immobilized in the column. Matrix components do not interact with the antibodies, and the washing step removes most potential interferences. The toxin can be eluted using a solvent that denatures the antibodies. Both single-analyte and multifunctional columns for simultaneous determination of multiple mycotoxins are available. The main disadvantages include high cost and the fact that the column can be used only once due to antibody denaturation during elution (Hussain, 2011).

IAC columns are preconditioned with a buffer solution, usually phosphate-buffered saline. The sample is slowly applied to the column, after which the gel is washed, and finally the analyte is eluted from the IAC by disrupting the antibody–antigen interaction. Liquid matrices are often defatted and/or filtered before direct passage through the IAC, since extraction and clean-up occur simultaneously within the column. For solid matrices (e.g., cheese), solvent extraction is required, typically performed using a blender or shaking. Elution solvents must be aqueous to ensure compatibility with IAC (Li et al., 2011).

Three main methods for AFM_1 detection include **biological**, **analytical**, and **immunological** procedures. Biological tests are non-specific, time-consuming, and qualitative. The main immunochemical assay is the widely used ELISA enzyme test. Other methodologies base their effectiveness on electrochemical and optical principles, such as chromatography, UV absorption, spectrometry, fluorescence, and immunochemical assays. Among new approaches are biosensors, electrokinetics, electrochemical transduction, amperometric detection, and adsorptive stripping voltammetry. Each methodology has its own advantages and limitations depending on sensitivity, ease of use, and cost-effectiveness (Espinosa-Calderón et al., 2011). Rapid methods for monitoring AFM_1 concentrations in milk or dairy products may be based on the use of special **test strips**. These can provide qualitative results (confirmation of the analyte's presence in the sample) or semi-quantitative results (when combined with a specific detector). The strip-test format is based on an indirect competitive immunoassay. The intensities of the lines displayed on the strip membrane (test line and control line) are measured using a photometric sensor. The test response is defined as the ratio between the signal intensity of the test line and that of the control line, and it is converted into toxin concentration using a batch-specific calibration curve (Pecorelli et al., 2020).

2.1.2. Advances in aflatoxin M_1 detection methods: Research by Slovak institutions

This section reviews key studies focused on the development, optimization, and validation of analytical techniques for AFM_1 monitoring, conducted by Slovak researchers and academic bodies.

1. *Kolarič, L., & Šimko, P. Development and validation of HPLC-FLD method for aflatoxin M1 determination in milk and dairy products. Acta Chimica Slovaca, 2023, 16(1), 99 - 108.*

The study aimed to develop and validate a reliable analytical method for determining AFM_1 in milk and dairy products using HPLC-FLD combined with IAC clean-up. To optimise the chromatographic conditions, the authors tested several mobile phase compositions, column temperatures, and fluorescence wavelengths. The best separation was achieved with a mobile phase consisting of water and acetonitrile (80/20, v/v), a C_{18} stationary phase maintained at 25 °C, and fluorescence detection at 360 nm excitation and 440 nm emission. These settings produced sharp peaks and appropriate retention times, confirming their suitability for AFM_1 analysis. The extraction and purification step was optimised by evaluating different elution solvents for the IAC columns. The study found that the composition of the elution agent had a significant impact on AFM_1 recovery. The most effective elution was obtained using 1.25 mL of acetonitrile/methanol (3/2, v/v) followed by 1.25 mL of water, yielding a recovery of 90.5%.

The method was validated according to European legislation and Eurachem guidelines using certified reference material. The calibration curve showed excellent linearity ($R^2 = 0.99$), and the limits of detection and quantification were 0.002 and 0.007 µg/kg, respectively - well below the EU maximum limit for AFM_1 in milk. Recovery tests demonstrated good accuracy, and both repeatability and intermediate precision met the required criteria, with low relative standard deviations and acceptable HorRat values.

In addition to the chromatographic validation, the authors compared the HPLC-FLD method with a rapid strip-based screening test (MilkSafe™ AflaM1), which is commonly used in Slovak dairy industry settings. The results showed good agreement: the HPLC-FLD method achieved recoveries between 82.9% and 103.6%, while the rapid test produced recoveries between 92.3% and 103.6%. The main findings suggest that while HPLC-FLD remains the essential reference method for legislative confirmation and detailed laboratory analysis due to its high accuracy and recovery rates, the lateral flow test strips offer a time-saving and cost-effective solution for "first-line" monitoring. This rapid approach is particularly suitable for

dairy processing plants during raw milk intake, enabling immediate decision-making and enhancing the overall safety of the Slovak dairy supply chain.

Finally, the validated HPLC-FLD method was applied to 25 milk and dairy product samples collected in Slovakia. In all cases, the AFM_1 concentration was below the limit of quantification.

2. *Kolarič, L., Vinohradská, E., & Šimko, P. Aflatoxin M1 in cheese: situation on the Slovak market. Acta Chimica Slovaca, 2025, 18(1), 32 - 40.*

This study focuses on the development, validation, and application of a rapid and reliable analytical method for determining AFM_1 in cheese products available on the Slovak market. The method developed in this study consists of three main steps: extraction of AFM_1 from cheese using an acetonitrile–water mixture, purification of the extract with IAC columns, and quantification by HPLC-FLD. The method was validated using both laboratory-prepared cheese made from milk spiked with AFM_1 and commercial cheese samples fortified with known AFM_1 concentrations.

The research included an analysis of 36 cheese samples (including ripened, unripened, and pasta filata types). The findings confirmed that all analysed samples from the Slovak market were compliant with the strict European legislative requirements, as none of the samples exceeded the maximum limit. However, the study highlighted a critical "enrichment factor" during the production process. It was observed that AFM_1 has a high affinity for casein, leading to a concentration of the toxin in the cheese curd compared to the initial whey.

The main findings of this research emphasize that while the Slovak cheese market remains safe for consumers, the concentration of AFM_1 can increase by 3 to 5 times during the cheese-making process, depending on the cheese type and moisture content. This study provides essential data for "national controlling bodies" regarding the necessity of monitoring not only raw milk but also processed dairy products, where the toxin levels may be higher due to the physical-chemical changes during processing.

3. *Hriciková, S., Kožárová, I., McGoldrick, D., & McCaul, O. Detection of antibiotic residues and Mycotoxins in milk using competitive immunochromatographic tests. Folia Veterinaria, 2023, 67(1), 35 - 44.*

This study investigated the presence of AFM_1 in raw cow's milk collected from dairy farms in Slovakia and Ireland using a rapid competitive immunochromatographic assay (AflaM1 Scan),

which quantifies AFM_1 based on the inverse intensity of a test line read by an automated IRIS device.

A total of 69 milk samples were analysed - 40 from Slovakia and 29 from Ireland. The results revealed an exceptionally high prevalence of AFM_1 contamination. Overall, 59 samples (85.5%) were non-compliant, meaning their AFM_1 concentration exceeded the regulatory limit of 50 ng/kg. Only 10 samples (14.5%) met the compliance criteria. Country-specific results showed that 32 of 40 Slovak samples (80%) and 27 of 29 Irish samples (93.1%) were non-compliant. AFM_1 concentrations in positive samples ranged from 69 ng/kg to > 200 ng/kg, with most samples exceeding the upper detection limit of the test (> 200 ng/kg).

These findings indicate a widespread contamination problem at the farm level in both countries. When compared with international studies, the prevalence observed here is unusually high. The authors emphasize that AFM_1 contamination poses serious risks to public health due to its carcinogenicity and stability during processing. They stress the need for improved feed management, stricter monitoring, and better farmer education to prevent contaminated milk from entering the food chain.

4. *Izzo, L., Mikušová, P., Lombardi, S., Sulyok, M., & Ritieni, A. Analysis of mycotoxin and secondary metabolites in commercial and traditional Slovak cheese samples. Toxins, 2022, 14(2), 134.*

This study represents the first comprehensive investigation of mycotoxins and fungal secondary metabolites in Slovak cheeses. A total of 68 commercial and traditional cheese samples made from cow's, sheep's, and goat's milk were analysed using a highly sensitive LC-ESI-MS/MS multi-mycotoxin method. The aim was to evaluate the presence of regulated and emerging fungal metabolites, given that cheese is a matrix highly susceptible to fungal growth and toxin production.

A key finding of the study is that AFM_1 was not detected in any of the analysed samples. This suggests that the raw milk used in Slovak cheese production was free of aflatoxin contamination at the time of sampling. Despite the absence of AFM_1, the study revealed a diverse spectrum of 13 fungal metabolites across the cheese samples. The most prevalent compound was enniatin B (ENN B), detected in 100% of samples (n = 68), though at low concentrations (0.02–0.71 µg/kg). ENN B is considered an "emerging mycotoxin" produced mainly by *Fusarium* species and is not currently regulated in food. Its universal presence indicates widespread exposure of dairy animals to ENN B through contaminated feed. The second most frequently detected

metabolite was tryptophol, found in approximately one-third of both commercial and traditional cheeses. Concentrations varied widely, reaching up to 7930 µg/kg in some traditional cheeses. Tryptophol is a fungal and yeast metabolite not previously reported in cheese, making its detection a novel finding. Other metabolites identified included andrastins (A–D), roquefortine C and D, mycophenolic acid, 3-nitropropionic acid, and several clavine alkaloids (isofumigaclavine, festuclavine, chanoclavine). Many of these compounds are associated with *Penicillium roqueforti* and related species used in the ripening of blue cheeses. Their presence, especially in Niva-type cheeses, reflects natural fungal activity during maturation.

Overall, the study demonstrates that while AFM_1 contamination is not a concern in Slovak cheeses, a variety of non-regulated fungal metabolites are present, some at relatively high levels. The findings highlight the need for further toxicological evaluation of emerging mycotoxins and secondary metabolites in cheese, particularly those produced by ripening fungi.

5. *Karapetis, S., Nikolelis, D., & Hianik, T. Label-free and redox markers-based electrochemical aptasensors for aflatoxin M1 detection. Sensors, 2018, 18(12), 4218.*

The article presents a comparative study of two electrochemical aptasensor platforms designed for the sensitive detection of AFM_1 in milk. Traditional methods such as HPLC and ELISA are accurate but "expensive, time-consuming and require qualified staff," motivating the development of faster biosensor-based alternatives.

Two aptamer immobilization strategies were evaluated:

1) Label-free aptasensor: amino-modified aptamers immobilized on PAMAM dendrimers, detected by electrochemical impedance spectroscopy (EIS).
2) Redox-based aptasensor: biotinylated aptamers immobilized on a neutravidin–ferrocene layer, detected by differential pulse voltammetry (DPV).

Both platforms showed high sensitivity, with limits of detection around 8–9 ng/L, well below EU limits for AFM_1 in milk. The sensors performed reliably in spiked milk samples, achieving recoveries above 78%. Their sensitivity was comparable to that of immunosensors, while offering advantages in stability, cost, and ease of fabrication. Overall, the study demonstrates that electrochemical aptasensors are promising tools for rapid, sensitive AFM_1 monitoring in dairy products.

6. *Kulikova, T. N., Porfireva, A. V., Evtugyn, G. A., & Hianik, T. Electrochemical Aptasensor with Layer-by-layer Deposited Polyaniline for Aflatoxin M1 Voltammetric Determination. Electroanalysis, 2019, 31(10), 1913-1924.*

This study introduces a novel electrochemical aptasensor for the rapid and reagent-free detection of AFM_1 in water and milk. The sensor is based on a layer-by-layer polyaniline (PANI) structure, where a DNA aptamer selective for AFM_1 is entrapped between two thin PANI films electrodeposited on a glassy carbon electrode. This configuration stabilizes the aptamer, protects its conformation, and enhances signal reproducibility.

AFM_1 binding alters the redox activity of PANI, specifically its emeraldine form, leading to a measurable decrease in voltammetric or impedance signals. Calibration curves were linear between 3–90 ng/L, with limits of detection between 1–5 ng/L, depending on the measurement mode. These values are well below the EU regulatory limit of 50 ng/L for milk.

The aptasensor demonstrated good selectivity, showing minimal response to aflatoxin B_1 or ochratoxin A, and its performance was validated using a simple milk pretreatment protocol. AFM_1 could be reliably detected at concentrations close to regulatory thresholds (≈20 ng/L), making the sensor suitable for practical food-safety monitoring. Overall, the work presents a sensitive, fast, and low-cost alternative to chromatographic and immunochemical methods for AFM_1 detection.

7. *Smolko, V., Shurpik, D., Porfireva, A., Evtugyn, G., Stoikov, I., & Hianik, T. Electrochemical aptasensor based on poly (neutral red) and carboxylated pillar [5] arene for sensitive determination of aflatoxin M1. Electroanalysis, 2018, 30(3), 486-496.*

This article presents a highly sensitive electrochemical aptasensor for detecting AFM_1 in milk and dairy products. The sensor is built on a glassy carbon electrode coated with electropolymerized Neutral Red (NR) in the presence of a polycarboxylated pillar[5]arene (P[5]A-COOH) macrocycle. The macrocycle provides multiple carboxyl groups that enable covalent attachment of both the AFM1-specific DNA aptamer and additional NR labels via EDC/NHS chemistry. Upon binding AFM_1, the aptamer undergoes structural changes that suppress the redox activity of the NR units and increase the charge-transfer resistance of the sensing layer. These changes are monitored using cyclic voltammetry and electrochemical impedance spectroscopy (EIS).

Under optimized conditions, the aptasensor achieves a limit of detection of 0.5 ng/L, with a linear response between 5–120 ng/L. This sensitivity is significantly below the EU regulatory limit for AFM_1 in milk (50 ng/kg). The sensor was successfully validated in spiked cow milk, sheep milk, and kefir, following simple methanol-based sample preparation, enabling reliable detection of AFM_1 at concentrations between 40–160 ng/kg.

Overall, the study demonstrates that combining poly(Neutral Red) with carboxylated pillar[5]arene provides a stable, reproducible, and highly sensitive platform for AFM_1 detection, offering a practical alternative to more complex chromatographic or immunochemical methods.

2.2. Aflatoxin M_1 mitigation and reduction strategies

2.2.1. Introduction

The continuous presence of AFM_1 in milk and dairy products worldwide is causing a strong effort to develop an effective method for its removal, as to date no method exists that could be efficiently applied at the industrial level while simultaneously achieving a significant reduction in AFM_1 concentration without compromising the nutritional composition of milk.

One way to reduce the concentration of AFM_1 in milk and dairy products is through **pre-harvest** and **post-harvest** interventions, which prevent the accumulation of aflatoxin B_1 (AFB_1) in crops and raw materials intended for the production of feed for dairy animals. Such methods may focus on preventing toxin formation where possible by inhibiting mold growth, on the application of good agricultural practices, including pest control and the proper use of fungicides. The most common approach to preventing AFB_1 contamination of feed before harvest is the implementation of biological control (Marshall et al., 2020).

With regard to the direct removal of AFM_1 from milk and dairy products, the detoxification methods identified so far can be divided into four groups:

1. **Physical methods** for AFM_1 removal: thermal processing, irradiation (pulsed light, ultraviolet radiation, gamma radiation, electron beams), cold plasma, electrolyzed water, etc.
2. Removal of AFM_1 using **chemical additives**: ammonia and hydrogen peroxide, ozonization, addition of acids/bases, plant extracts.
3. **Biological methods** for AFM_1 removal: enzymatic degradation, biotransformation, removal of AFM1 using microbial cells.

4. **Physicochemical removal** of AFM_1: adsorption onto organic and inorganic sorbents (bentonite, activated carbon, cyclodextrins, etc.).

Chemical processes can be used to degrade or inactivate AFM_1 in milk through chemical reactions such as ammoniation, ozonization, peroxidation, hydrolysis, or oxidation. In recent years, chemical degradation of AFM_1 has largely not been used, mainly due to concerns regarding the nutritional quality of milk, and it is also inconsistent with European food safety legislation. In addition, residues of chemical agents may directly harm animal and human health, promote reactions leading to the formation of hazardous contaminants, or require technologically complex and financially demanding equipment (Muaz et al., 2021).

Physical approaches may be based on thermal or non-thermal processes. However, studies have shown that AFM_1 exhibits high thermal stability, and its degradation occurs only at temperatures of approximately 237–306 °C, which are values that cannot be practically achieved in dairy processing (Campagnollo et al., 2016; Muaz et al., 2021). Among the available non-thermal methods, ultrasound appears to contribute to reducing aflatoxin levels by altering their molecular structure. Such effects may lead to hydrolysis of the lactone ring or disruption of the difuran core of the toxin. A decrease in AFM_1 concentration has also been observed following irradiation; however, the safety of this approach has not yet been unequivocally confirmed (Hernández-Falcón et al., 2018). The combined effect of ultraviolet, visible, and infrared spectra leads to damage of microbial cell walls and nucleic acids and simultaneously promotes the degradation of aflatoxins in foods. Current results suggest that pulsed light can effectively degrade aflatoxins without the formation of toxic by-products (Mostashari et al., 2022). Another promising physical method is plasma application. Reported results indicate that high-voltage atmospheric cold plasma (HVACP) can reduce AFM_1 content in skim milk to a safe level (below 0.5 $\mu g\ kg^{-1}$). The mechanism of AFM_1 degradation in this process is likely associated with the disruption of double bonds in the furan or lactone ring, which subsequently leads to reduced toxicity of the toxin after treatment (Nguyen et al., 2022).

Biological principles are based on the ability of native microorganisms to reduce aflatoxin concentrations through biodegradation or bioadsorption, with most microbial approaches relying primarily on sorption mechanisms that result in the formation of stable complexes with the toxin. Compared to bioadsorption, biodegradation has certain disadvantages—it occurs more slowly and may alter the chemical structure of aflatoxins, which can lead to the formation of undesirable and potentially toxic metabolites. Biodegradation methods employ microorganisms or enzymes derived from them to break down aflatoxins. These biological

systems are capable of catabolizing aflatoxins or transforming them into less toxic or completely non-toxic products (Giovati et al., 2015).

Physicochemical approaches represent a group of techniques that use physicochemical processes to remove contaminants from food and water. Among the most commonly used are adsorption-based methods, which rank among the most widespread approaches to contaminant elimination and are applied across many industrial sectors (Tapia-Orozco et al., 2016). Interactions between adsorbents and AFM_1 molecules are currently being intensively studied. Compared with other available methods, AFM_1 decontamination through adsorption is considered economically advantageous, highly effective, and at the same time minimizes the risk of forming secondary toxic substances. *In vitro* studies have identified several types of adsorbents capable of binding aflatoxins, particularly clay-based materials such as activated carbon, bentonite, zeolite, or hydrated calcium sodium aluminosilicate. The adsorption of AFM_1 onto a given sorbent primarily depends on its surface properties, especially surface morphology (surface area and porous structure) and the nature of surface chemical functional groups (Giovati et al., 2015).

2.2.2. Advances in aflatoxin M_1 mitigation methods: Research by Slovak institutions

This section reviews key studies focused on the mitigation strategies for AFM_1 in milk and dairy products, conducted by Slovak researchers and academic bodies.

1. *Kolarič, L., Minarovičová, L., Laukova, M., Kohajdova, Z., & Šimko, P. (2024). Elimination of aflatoxin M1 from milk: Current status, and potential outline of applicable mitigation procedures. Trends in Food Science & Technology, 150, 104603.*

This reviewed article critically evaluates chemical, physical, biological, and physicochemical mitigation strategies and assesses their applicability using the **SCUFEERS** concept, which encompasses the criteria of Safety, Capacity, Universality, Finance, Ecology, Efficiency, Rapidity, and Selectivity.

The main results can be summarized in these points:

- Chemical procedures have demonstrated the ability to degrade or inactivate AFM_1 under laboratory conditions. However, these approaches fundamentally conflict with food safety legislation, particularly within the European Union, due to the prohibition of adding reactive chemical agents to milk. In addition, chemical treatments may compromise the nutritional and sensory quality of milk, leave harmful residues, or

generate secondary toxic compounds. Consequently, chemical methods fail primarily in terms of the Safety, Selectivity, and Ecology criteria of the SCUFEERS framework and are therefore unsuitable for practical dairy application.

- Non-thermal technologies such as ultraviolet irradiation, pulsed light, ultrasound, gamma irradiation, and high-voltage atmospheric cold plasma have shown promising reduction efficiencies. Nevertheless, these methods raise concerns regarding the formation of degradation products with unknown toxicological properties, limited penetration in the milk matrix, and the lack of available industrial-scale equipment. As a result, physical methods face significant limitations under the Safety, Capacity, Finance, and Selectivity criteria, and their application is constrained by the precautionary principle.
- Biological approaches, based on enzymatic degradation or microbial activity, represent an environmentally attractive alternative and can transform AFM_1 into less toxic compounds. However, their practical use is hindered by several factors, including long processing times, strict requirements for pH and temperature, incomplete understanding of degradation pathways, and, most importantly, the absence of officially approved enzymes for AFM1 detoxification in foods. Consequently, biological procedures currently do not comply with the Safety criterion of the SCUFEERS concept and remain inaccessible for industrial implementation.
- In contrast, physicochemical procedures—primarily adsorption-based methods—emerge as the most promising strategy for AFM_1 mitigation. Adsorbents such as bentonite, activated carbon, chitin, chitosan, cyclodextrins, molecularly imprinted polymers, and magnetic nanoparticles have demonstrated high AFM_1 removal efficiencies while largely preserving milk quality. Importantly, many of these materials are of natural origin or are already approved as food or feed additives, ensuring compliance with food safety regulations. Physicochemical methods generally exhibit favourable performance across multiple SCUFEERS criteria, including Safety, Finance, Rapidity, Universality, and Selectivity, while minimizing the risk of secondary toxic compound formation. Although some adsorbents may induce minor changes in milk composition, these effects are considered manageable through process optimization.

Overall, the SCUFEERS evaluation clearly indicates that physicochemical adsorption-based approaches possess the highest potential for future industrial application. While all mitigation strategies remain at the laboratory stage, adsorption technologies align most closely with

legislative requirements, economic feasibility, and technological practicality in the dairy sector. Future research should therefore focus on optimizing adsorbent selectivity, minimizing impacts on milk composition, and developing innovative solutions such as active packaging systems capable of continuously reducing AFM_1 levels during storage. Such advances will be increasingly important as climate change is expected to exacerbate aflatoxin contamination risks in the coming decades.

2. *Šimko, P., & Kolarič, L. (2022). Decrease in aflatoxin M1 concentration in milk during cholesterol removal by application of β-cyclodextrin. Toxins, 14(6), 379.*

This article focuses on reducing contamination of milk by AFM_1, while the authors explore a novel mitigation approach based on the use of β-cyclodextrin (β-CD), a compound already widely applied in the dairy industry to remove cholesterol from milk.

To test this hypothesis, pasteurized cow's milk samples were artificially spiked with AFM_1 at concentrations ranging from 0.20 to 2.00 µg/kg. Cholesterol removal was then carried out using 2.0% (w/w) β-cyclodextrin under processing conditions already optimized for cholesterol reduction. After treatment, cholesterol and AFM_1 concentrations were measured by validated HPLC methods.

The results showed that cholesterol removal was highly effective, with an average reduction of 92.3%, confirming the efficiency and reliability of the β-cyclodextrin process. Importantly, AFM_1 concentrations also decreased in all treated samples. On average, the AFM_1 content was reduced by 39.1%, despite the fact that the process was not specifically optimized for AFM_1 removal. This reduction is attributed to the formation of an AFM_1–β-cyclodextrin inclusion complex, which can be separated from milk by centrifugation, similarly to the cholesterol complex.

The authors note that cholesterol was removed much more efficiently than AFM_1, mainly because AFM_1 is present at much lower concentrations (µg/kg versus mg/kg levels) and because the processing conditions were tailored for cholesterol, not for mycotoxins. Statistical analyses confirmed that the reductions in both cholesterol and AFM_1 were significant. The findings suggest that further optimization of processing parameters (such as β-CD dosage, mixing conditions, temperature, and separation steps) could substantially improve AFM_1 removal efficiency.

In conclusion, the study demonstrates for the first time that AFM_1 can be partially removed from milk as a beneficial side effect of cholesterol removal using β-cyclodextrin. Although the current efficiency does not allow complete detoxification, the method shows strong potential as a practical and "gentle" decontamination step in milk processing. With further optimization, this approach could significantly improve milk safety, reduce economic losses caused by regulatory non-compliance, and contribute to lowering chronic consumer exposure to aflatoxin M_1 worldwide.

3. *Kolarič, L., Rusinková, K., & Šimko, P. (2025). Characterization of the physicochemical procedure of aflatoxin M1 elimination from Ringer's solution as a model system and raw milk using beta-cyclodextrin polymer. Applied Food Research, 101395.*

This article investigates a physicochemical method for eliminating AFM_1 from milk using an insoluble β-cyclodextrin polymer (BCP), with the aim of developing a practical, food-safe decontamination technique suitable for dairy processing.

Firstly, the study characterizes and optimizes the AFM_1 elimination process in Ringer's solution as a simplified model system, and then applies the optimized conditions to raw cow's milk. The core of the method is the use of insoluble β-cyclodextrin polymer, which can bind AFM_1 through a combination of host–guest inclusion complex formation inside the cyclodextrin cavities and surface adsorption on the polymer matrix. After binding, the BCP–AFM_1 complex is removed from the liquid by centrifugation.

In the model system, the authors systematically examined the influence of key processing parameters, including mixing speed and time, settling time, centrifugation conditions, BCP dosage, and initial AFM_1 concentration. They identified mixing time and centrifugation speed as the most critical factors. Under optimized conditions—mixing at 1000 rpm for 40 minutes, centrifugation at 1020 g for 15 minutes, using 6% (w/v) BCP and an initial AFM_1 concentration of 0.5 ng/mL - the maximum AFM_1 elimination reached 71.9%.

To better understand the mechanism of interaction between AFM_1 and BCP, adsorption data were fitted to Langmuir and Freundlich isotherm models. Although both models described the experimental data reasonably well, the Freundlich isotherm provided a better fit, indicating non-ideal sorption on heterogeneous binding sites. This supports the conclusion that AFM_1 removal occurs through a combined mechanism, involving both selective inclusion complexation and non-specific multilayer adsorption on the polymer surface.

The optimized parameters from the model system were then applied to raw milk spiked with AFM_1 at two concentration levels. In milk, AFM_1 elimination was lower than in Ringer's solution, reaching up to 58%, depending on the initial toxin concentration and the amount of added BCP. The reduced efficiency is attributed to the complex composition of milk, where AFM_1 competes with other components, particularly cholesterol and proteins such as casein micelles, for binding sites on the polymer. At higher BCP dosages, polymer aggregation and steric effects may also limit the accessibility of cyclodextrin cavities.

Importantly, the study shows that the decontamination procedure does not significantly alter the nutritional composition or key physicochemical properties of milk, including protein, lactose, total solids, density, and freezing point. Only a minor reduction in fat content was observed, which is consistent with the known affinity of cyclodextrins for cholesterol. This confirms that the method meets essential requirements for food processing, preserving milk quality while improving safety.

In conclusion, the article demonstrates that β-cyclodextrin polymer is a promising, gentle, and food-compatible adsorbent for AFM_1 removal from milk. While complete decontamination is not yet achieved, the results represent a substantial improvement compared to many existing methods and outperform previous approaches based on native β-CD. The process is safe, environmentally friendly, and technologically feasible, with strong potential for further optimization.

3. Recommendations at country (and V4) level regarding legislation and/or control, and research areas

Based on the current legislative framework, monitoring data, and research activities, several recommendations can be proposed for Slovakia and the wider Visegrad Four (V4) region regarding the control of AFM_1 in milk.

The existing EU legislative framework provides a well-established and harmonised system for the control of AFM_1, and no major changes to maximum limits are currently required. However, at the national level, it is recommended to further strengthen the integration between feed safety and milk monitoring systems. Since AFM_1 originates from aflatoxin B_1 contamination in animal feed, greater emphasis should be placed on preventive measures at the primary production level, particularly through enhanced control of feed quality and storage conditions.

From a control perspective, the Slovak monitoring system is well structured and based on a multi-level sampling approach covering farms, dairy processing plants, and retail. Nevertheless, it is recommended to increase the number of samples and improve the consistency of sampling across years, as variability in sample numbers may affect the representativeness of monitoring results. In addition, the wider implementation of rapid screening methods directly at the dairy processing stage could enable faster decision-making and reduce the risk of contaminated milk entering the production chain.

At the V4 regional level, closer cooperation between countries is recommended, particularly in the areas of data sharing, harmonisation of monitoring strategies, and joint risk assessment. Given the similar climatic and agricultural conditions in Central Europe, establishing a coordinated regional monitoring framework could improve early detection of contamination trends associated with climate change and seasonal variability.

In terms of research, further efforts should focus on the development of sensitive, rapid, and cost-effective analytical methods suitable for routine monitoring, including on-site testing technologies. At the same time, greater attention should be paid to mitigation strategies, especially adsorption-based approaches, which currently show the highest potential for practical application in dairy processing. Future research should also address the impact of climate change on mycotoxin occurrence in feed crops and its subsequent transfer into milk.

Overall, the effective management of AFM_1 in milk requires a comprehensive approach combining preventive measures, reliable monitoring, and continuous research. Strengthening cooperation between regulatory authorities, laboratories, and research institutions at both national and regional levels will be essential to ensure long-term food safety and consumer protection in Slovakia and the V4 region.

Key future challenges in AFM_1 control (Slovakia/V4 region) should be focused on:

1. <u>Climate change and increasing mycotoxin risk</u>

Changing climatic conditions in Central Europe (higher temperatures, drought periods followed by humidity) create more favourable conditions for the growth of aflatoxin-producing fungi in feed crops, increasing the risk of AFM_1 occurrence in milk.

2. <u>Feed contamination as a primary source</u>

The main challenge remains effective prevention of aflatoxin B_1 contamination in feed, particularly during storage and transport, which directly determines AFM_1 levels in milk.

3. Variability and representativeness of monitoring data

Differences in the number of analysed samples between years may limit the ability to identify long-term trends and emerging risks.

4. Need for faster and more flexible monitoring

Traditional laboratory analyses are reliable but time-consuming; there is increasing demand for rapid, on-site screening methods (e.g., lateral flow tests, biosensors) for real-time decision-making in dairy production.

5. Limited availability of effective mitigation methods

Despite extensive research, there is still no widely accepted industrial method for AFM_1 removal from milk that fully meets safety, regulatory, and technological requirements.

6. Gap between research and practical application

Many promising methods (adsorption, biosensors, plasma treatment) remain at laboratory level and are not yet implemented in real dairy processing conditions.

7. Need for stronger integration between control systems

Better linkage between feed control, veterinary supervision, and milk monitoring systems is necessary for more effective prevention of AFM_1 contamination.

8. Harmonisation within the V4 region

Although EU legislation is harmonised, differences in monitoring strategies and data reporting still exist between V4 countries, limiting comparability and regional risk assessment.

9. Emerging mycotoxins and combined exposure

Increasing attention is needed not only for AFM_1 but also for other (non-regulated) mycotoxins and their combined effects in milk and dairy products.

10. Consumer protection and risk communication

Maintaining public confidence requires transparent communication of monitoring results and risks associated with mycotoxins in food.

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