Journal of Food Composition and Analysis 149 (2026) 108794

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

A simple and sensitive methodology for quantification of aflatoxins in milk and butter by HPLC: Method development, validation and application to real samples

Busra Aslan Akyol [a], Cengiz Gokbulut [b,*]

[a] Balikesir University, Institute of Health Sciences, Department of Veterinary Pharmacology and Toxicology, Balikesir, Türkiye
[b] Balikesir University, Faculty of Medicine, Department of Medical Pharmacology and Institute of Health Sciences, Department of Veterinary Pharmacology and Toxicology, Balikesir, Türkiye

ARTICLE INFO

Keywords:
Aflatoxins
Butter
HPLC
Milk
QuEChERS

ABSTRACT

Aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2 and AFTM1) are toxic and carcinogenic compounds produced by *Aspergillus species*. They can accumulate in animal-derived foods such as milk and dairy products, posing a serious threat to public health. In this study, sensitive high-performance liquid chromatography (HPLC) and a modified QuEChERS extraction method with simple process steps and using smaller sample and chemical volumes were developed and validated for the detection of aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2 and AFTM1) in cow milk and butter samples. Simple sample preparation was carried out, including only protein precipitation with acetonitrile, followed by phase separation induced by adding potassium chloride. HPLC separation was carried out at 60°C using a C18 analytical column (Zorbax Eclipse Plus, 250 mm × 4.6 mm, 5 μm). The mobile phase consisted of ultra-pure water (H_2O) with potassium bromide (4 M nitric acid, pH: 3.40) and acetonitrile:methanol (50:50), delivered in a gradient mode at a flow rate of 1 ml/min. The fluorescence detector (FLD) was set at an excitation wavelength of 360 nm and an emission wavelength of 435 nm. The elution of all five analytes was completed within 21 min. The method provides values for the linearity ($R^2 > 0.998$) and recovery rates (89.15 % to 95.14 % for milk and 50.81 % to 86.08 % for butter), the limits of detections (LOD, 0.004–0.008 ng/g for milk and butter), the limits of quantifications (LOQ, 0.012–0.025 ng/g for milk and butter), precision (RSD < 10 %), repeatability (RSD values 1.30–6.08 % for milk and 2.32–6.39 % for butter) and reproducibility (RSD values 5.03–11.09 % for milk and 6.38–11.99 % for butter). This developed and validated method offers an alternative option for the simultaneous analysis of multiple aflatoxin residues in milk and butter samples intended for human consumption, through its fewer processing steps, lower volume and use of smaller variety of chemicals. Unlike other methods in the literature, this technique uses very small sample amounts, such as 2 g. Furthermore, this method was applied to real milk (106 raw and 32 pasteurised milk) and 103 butter samples. While these findings demonstrate that the technique can be applied to the detection of aflatoxin residues in milk and butter, the limitations of the butter matrix must be considered.

1. Introduction

Mycotoxins are toxic compounds produced by the secondary metabolism of various types of moulds. A wide range of food and feed commodities can be affected by mycotoxin contamination. It is estimated that mycotoxins are present in some form in 25 % of the world's agricultural yield. This situation presents a significant risk to both human and animal health (Kuiper-Goodman, 1995;Fink-Gremmels., 1999).

Different fungal species, such as *Aspergillus flavus, Aspergillus parasiticus,* and *Aspergillus nomius,* produce aflatoxins, which are highly toxic secondary metabolites with teratogenic, carcinogenic, and mutagenic effects. Although numerous types of aflatoxins have been identified in feed and food products, the most significant ones include aflatoxin B1 (AFTB1), aflatoxin B2 (AFTB2), aflatoxin G1 (AFTG1), aflatoxin G2 (AFTG2), and the hydroxylated metabolites aflatoxin M1 (AFTM1) and aflatoxin M2 (AFTM2), which are derived from AFTB1 and AFTB2,

* Correspondence to: Balikesir University, Faculty of Medicine, Department of Medical Pharmacology and Institute of Health Sciences, Department of Veterinary Pharmacology and Toxicology, Balikesir 10145, Türkiye.
E-mail address: cgokbulut@balikesir.edu.tr (C. Gokbulut).

https://doi.org/10.1016/j.jfca.2025.108794
Received 18 July 2025; Received in revised form 3 December 2025; Accepted 15 December 2025
Available online 16 December 2025

respectively, in the liver of animals through cytochrome P450 enzymes (Kurtzman et al., 1987; Hussein, Brasel.,2001). Among these, AFTB1 is the most potent and hazardous, contributing to both acute and chronic aflatoxicosis. Although less toxic than AFTB1, AFTM1 is also classified as a human carcinogen due to its hepatotoxicity and its prevalence in milk Paniel et al.,2010).

In animals, mycotoxins can lead to acute poisoning, reduced weight gain, decreased productivity, immune system suppression, and increased susceptibility to infections, among other serious side effects. Additionally, several mycotoxins, such as aflatoxins, ochratoxins, and fumonisins, have been shown to have genotoxic effects that may contribute to the development of human tumours (Fink-Gremmels., 1999; Wang and Groopman, 1999).

Direct human exposure to aflatoxins can occur through consuming contaminated food, contact with infected foods via the skin, or inhaling contaminated air. Humans can also become indirectly exposed to aflatoxins by consuming animal products derived from livestock ingested contaminated feed (Turna and Wu, 2021). Notably, AFTM1 can be found in products such as milk, cheese, yogurt, milk powder, and butter, as it transfers into milk during the lactation of animals fed with AFTB1-contaminated feed (Govaris et al., 2001; Iha et al., 2013). The International Agency for Research on Cancer (International Agency for Research on Cancer. IARC., 1993), classifies AFTM1 as a Group 2B potential human carcinogen, while AFTB1 is classified as a Group 1 human carcinogen (Creppy, 2002; Moss, 2002). Consequently, the European Union (EU) has established maximum residue limits (MRLs) for milk and milk-based products, setting the limit for AFTM1 at 0.05 μg/kg in drinking milk, milk powder, and processed milk products (Commission Regulation European Commission. EC., 2006). The limit for all aflatoxins combined (AFTB1, AFTB2, AFTG1 and AFTG2) in foods intended for human consumption and feed for dairy animals is set at 20 μg/kg and AFTM1 as 0.5 μg/kg (Food and Drug Administration. FDA., 2000; 2019). In some European countries, the AFTM1 MRL for butter are accepted at 0.02 μg/kg (Manetta et al., 2005). Effective analytical methods that are specific, sensitive and cost-effective are essential to enforce these limits and reduce the diseases related to aflatoxin exposure (Zheng et al., 2006).

Various analytical methods are used to detect aflatoxins in milk and milk-based products. These methods include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), ion mobility spectrometry (IMS), Fourier-transform near-infrared (FT-NIR) spectrometry, high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography (GC), and liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS) (Aiko and Mehta, 2015; Ammida et al., 2004; Goto et al., 1988; Korde et al., 2003; Pirestani et al., 2011; Sheibani et al., 2008; Sherma, 2000; Stroka and Anklam, 2002). Among these methods, HPLC with fluorescence detection is the most widely used chromatographic technique for routine aflatoxin analysis, particularly after post-column bromination with a KOBRA CELL®. This is due to its high selectivity, sensitivity, capacity for simultaneous analysis of different mycotoxins, ability to analyse small quantities and versatility in various matrices (Huang et al., 2014; Shephard, 2009; Sørensen and Elbæk, 2005; Zhang et al., 2013). Before using these techniques for analysis, extraction and clean-up steps are necessary due to the high protein and fat content in milk and milk-based products (Shephard, 2009). Various extraction methods are employed, including solid-phase extraction (SPE), liquid phase extraction (LPE), and LLE followed by an immunoaffinity column (IAC) clean-up step or SPE (Campone et al., 2013; Chen et al., 2005; Hashemi and Taherimaslak, 2014). Both LPE and SPE have their drawbacks, such as high solvent consumption, limitations in the number of samples that can be processed simultaneously using the SPE manifold, and the expensive cartridges used in IAC methods (Hussain, 2011; Womack et al., 2016).

The QuEChERS, an acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe, was initially developed as a sample preparation technique for the detection of biocidal residues in fruits and vegetables but has since been successfully adapted for the residue analysis of veterinary drugs and mycotoxins in edible animal by-products and animal feeds (Anastassiades et al., 2003; Frenich et al., 2011; Lesueur et al., 2008). Recently, this method has been increasingly applied to animal-derived food products intended for human consumption for the detection of mycotoxins (Seo et al., 2021). QuEChERS enables the removal of matrix interferences such as fat, protein, and water in a single step using small volumes of acetonitrile and offers a rapid and straightforward extraction process with high analyte recovery rates, making it a favourable alternative to conventional extraction methods (Cao et al., 2018; Xu et al., 2019).

Milk and dairy products are generally analyzed for the presence of AFTM1 [Kim et al., 2000; Rodriguez Velasco et al., 2003; Behfar et al., 2012; Wang et al., 2012; Chavarría et al., 2015], and the presence of other aflatoxins is not taken into account. Additionally, previous aflatoxin analysis methods that utilized the OuEChERS-HPLC technique typically involved larger sample volumes, ranging from 5 to 10 g. As a result, the process steps were more complex and required significantly greater amounts of chemicals and solvents (8–35 ml), particularly during the extraction phases (Karaseva et al., 2014; Sartori et al., 2015; Michlig et al., 2016; Hamed et al., 2019; Morais et al., 2024). In this study, we aimed to develop and optimize an HPLC method with fewer processing steps, utilizing as few chemical species and volumes as possible, for the simultaneous determination of five aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2 and AFTM1) in cow milk and butter, employing straightforward sample preparation with a small sample size (2 g). Furthermore, this method was applied and evaluated in the detection of aflatoxins in milk and butter intended for human consumption.

2. Materials and methods

2.1. Reagents and solvents

The pure analytical standard of aflatoxin mixture (AFTB1, AFTB2, AFTG1 and AFTG2) solution (purity: ≥ 98 %, CAS Number: 75–05–8) was obtained from Sigma-Aldrich, Germany. Analytical standard of aflatoxin AFTM1 (purity: ≥ 98 %, CAS Number: 6795–23–9) was obtained from Lab® Instruments, Italy. HPLC grade acetonitrile (purity: ≥ 99.9 %, CAS Number: 75–05–8, 34851) and methanol (purity: ≥ 99.9 %, CAS Number: 67–56–1, 34860), potassium bromide (purity: ≥ 99.0 %, CAS Number: 7758–02–3), potassium chloride (purity: ≥ 99.0 %, CAS Number: 7447–40–7) and nitric acid 65 % (CAS Number: 7697–37–2, 07006) were purchased from Sigma-Aldrich (Germany). The Millipore Simplicity® water purification system (USA) provided ultrapure water for the preparation of mobile phase and analytical procedures.

2.2. Instrumentation

Chromatographic analyses were performed using an Agilent 1260 HPLC system (Agilent Technologies, Germany), which consists of a binary high-pressure gradient system for analysing aflatoxin 5 mix (AFTB1, AFTB2, AFTG1, AFTG2 and AFTM1). An Agilent binary pump (G1312B) delivered the mobile phase to the analytical column. The sample was injected using an Agilent autosampler (G1367E) connected to an injection valve (Rheodyne®, USA) with a 100-μl variable loop. A fluorescence detector (G1321B) and KOBRA CELL® (K01, R-Biopharm, Rhone LTD, Scotland) were used to achieve detection, in compliance with data acquisition ChemStation Software. The ChemStation® Software (C.01.08, Agilent, Germany) controlled the operations and functions of the entire HPLC system.

All evaporations following sample extraction were performed at 50 °C using a vacuum evaporator (Maxi-Dry Plus, Hettich, Germany). A vortex mixer (622, Isolab, Germany), a centrifuge (Hettich Rotina® 380 R, Germany) and an ultrasonic bath (Elmasonic® S40-H, Elma, Germany) were used for the extraction procedure.

2.3. Chromatographic conditions

A Zorbax Eclipse Plus C18 analytical column (5 μm, 250 mm × 4.6 mm; Agilent, Germany) with a Nucleosil C18 guard column (Phenomenex, UK), kept 60 °C in a column oven (G1316A), was used to separate aflatoxin 5 mix (AFTB1, AFTB2, AFTG1, AFTG2, and AFTM1). The mobile phase consisted of ultra-pure water (H2O): potassium bromide (4 M nitric acid, pH: 3.40) (A) and acetonitrile: methanol (50:50) (B), which was delivered in a gradient fashion at a flow rate of 1 ml/min. The gradient program changed from 70:30 (A: B, 0 min) to 60:40 (A: B) after 15 min, and then to 62:38 (A: B) at 17 min. It was adjusted back to its initial rate at 17:03, and the analysis continued for up to 21 min to equilibrate the column, in preparation for the next injection. The excitation and emission wavelengths of 360 nm and 435 nm, respectively, were used by the fluorescence detector (FLD, G1321B, Agilent, Germany). The KOBRA CELL® was operated at a power of 100 μA. Analyses performed using the KOBRA CELL® include some revisions, in addition to the standard derivatization procedures in the user manual (R-Biopharm.,2022). The standard HPLC method, which targets the analysis of aflatoxins AFTB1, AFTB2, AFTG1 and AFTG2, was modified in this study to include five toxins, including AFTM1. Within these modifications, particular attention was paid to using a mobile phase gradient that minimized the amount of acetonitrile to protect the KOBRA CELL® membrane and ensure more accurate peak resolution. Moreover, acetonitrile:methanol (50:50, v/v) mixture as the organic phase compared to a single solvent in the mobile phase provides improved resolution, peak shape and reduced backpressure. Throughout the study, a constant volume of 100 μl was used for the injections. The milk and butter samples were analysed using chromatography, with the process taking 21 min for an analysis.

2.4. Preparation of standard solution

Stock analytical standard solutions (500 ng/ml M1, 100 μg/ml G1, G2, B1, B2) of aflatoxins were prepared in acetonitrile and stored in glass bottles at 4°C. These were diluted with acetonitrile to produce 1, 5, 10 and 20 ng/ml standard solutions for milk and butter samples. These solutions were used to spike aflatoxin-free cow's milk and butter at various levels in order to generate standard curves and determine extraction recovery rates.

2.5. Sample preparations and extraction

Cow milk and butter samples (400 g) were collected from a dairy market in Balıkesir. Before the analytical procedure, each milk sample was vortexed for 5 min for homogenization and this process was repeated before collecting 2 g of samples. The butter samples were then left at room temperature (15 min) and then placed in a 50°C oven for 5 min to homogenize into liquid fat. Then, as with the milk samples, they were vortexed for 5 min. This process was repeated before collecting 2 g of samples. The samples were prepared using a modified salt-assisted liquid-liquid phase extraction procedure based on the method by Anastassiades et al. (2003). In this process, 2 g of the milk and butter samples (both blank and collected) were spiked with 50–100 μl of aflatoxin 5 mix standard solution to achieve the following final concentrations: 0.025, 0.05, 0.1, 0.5, and 1 ng/g. Subsequently, acetonitrile was added (4 ml for milk and 5 ml for butter) to the spiked and collected milk and butter samples for deproteinization and vortexed for 1 min. Next, 0.4 g of potassium chloride was added to the mixture and it was vortexed for a further minute. The samples were then subjected to a 5-minute centrifugation process at 5000 rpm. The upper organic phase was then transferred to a 10 ml glass tube and evaporated in a vacuum concentrator at 50 °C. The dried residue was then dissolved in 200 μl of the mobile phase and vortexed for 15 s. Finally, 100 μl of the resulting solution was injected into the chromatographic system for analysis.

2.6. Validation of analytical technique

The proposed analytical method was validated in accordance with EU requirements (Commission Decision 2002/657/EC). The validation of the method was carried out using spiked milk and butter samples, with the following parameters being taken into consideration: linearity, response and range, accuracy and precision (repeatability and between-day reproducibility), selectivity, sensitivity and decision limit (CCα) and detection capability (CCβ).

To calculate the signal suppression in the matrix-induced HPLC-FLD response, the matrix effect (ME) (%) was determined by comparing the slope of the calibration curve obtained from standard solutions with the slope of the calibration curve obtained by injecting matrix-matched solutions spiked at the same concentration levels.

2.7. Application to real milk and butter samples

We assessed the reliability of the developed analytical technique for the quantification of aflatoxin 5 mix (AFTB1, AFTB2, AFTG1, AFTG2, and AFTM1) by analysing the aflatoxins in cow milk and butter. The method was used to check for residues of any aflatoxin in raw and pasteurised milk and butter. A total of 106 raw and pasteurised cow milk (32 and 103, respectively) and butter samples (103) were collected (approximately 400 g each) from various dairy markets in Balıkesir, Türkiye, between 2023 and 2024.

2.8. Statistical analysis

Statistical analysis of the data obtained in the study was conducted using IBM SPSS Statistics for Windows, Version 30.0 (IBM Corp., Armonk, N.Y., USA). One-Way Analysis of Variance (One-Way ANOVA) was used to compare the recovery rates of five aflatoxins (AFTM1, AFTG2, AFTG1, AFTB2 and AFTB1) in milk and butter. Furthermore, a paired sample *t*-test was performed to compare milk and butter recovery and precision values for each aflatoxin. The level of significance was set at $p < 0.05$ in all statistical analyses. Data are presented as mean ± standard deviation (Mean ± SD).

3. Results and discussion

Typical HPLC chromatograms showing the results of analysis for five types of aflatoxins in spiked milk and butter samples are shown in Figs. 1

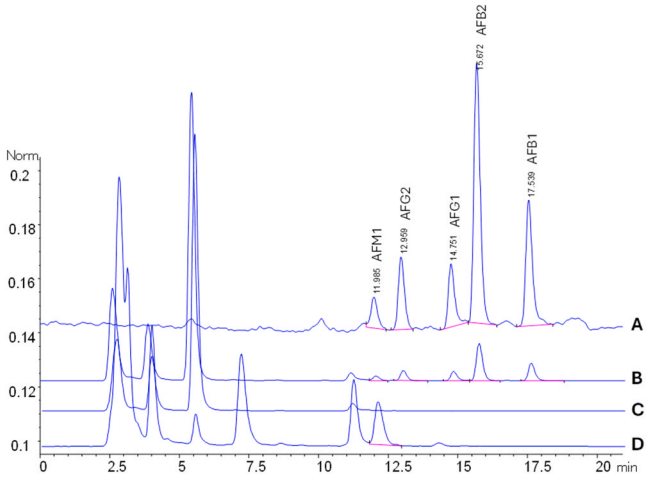

Fig. 1. Overlay chromatograms of analytical standard mixture (**A**, 0.5 ng/g; retention time for AFTM1 (11.98 min.), AFTG2 (12.92 min.), AFTG1 (14.75 min.), AFTB2 (15.67 min.) and AFTB1 (17.53 min.), spiked (**B**, 0.5 ng/g), blank (**C**) and AFTM1 residue positive (**D**, 6.2 ng/g) milk samples processed by the suggested method.

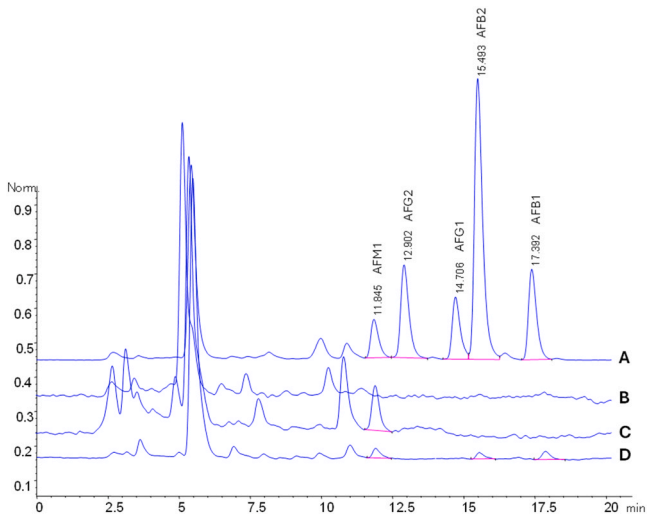

Fig. 2. Overlay chromatograms of spiked butter sample with analytical standard mixture (**A**, 1.0 ng/g; retention time for respectively AFTM1 (11.84 min.), AFTG2 (12.90 min.), AFTG1 (14.70 min.), AFTB2 (15.49 min.) and AFTB1 (17.39 min.), blank (**B**) and AFTM1 residue (**C**, 0.25 ng/g) and AFTM1, AFTB2 and AFTB1 residues positive (**D**, lower than the LOQ) butter samples processed by the suggested method.

and 2, respectively. When the blank milk and butter sample was analysed, no impurities were observed at the elution times of the analyte peaks. The separation of the mycotoxins was achieved within a 21-minute analysis time following gradient elution. The method for detecting aflatoxins in milk and butter samples, which involves a post-column derivatisation step, was developed using an HPLC-fluorescence detector.

Various analytical techniques such as ELISA, FT-NIR spectrometry, TLC, HPLC, GC and LC-MS/MS have been used for the detection of aflatoxins in milk and dairy products (Aiko and Mehta, 2015; Ammida et al., 2004; Goto et al., 1988; Korde et al., 2003; Pirestani et al., 2011; Sheibani et al., 2008; Sherma, 2000; Stroka and Anklam, 2002). Among these, HPLC combined with fluorescence detection after post-column derivatization using KOBRA CELL® is the preferred method due to its high specificity, sensitivity, and suitability for the analysis of multiple mycotoxins in various matrices (Huang et al., 2014; Shephard, 2009; Sørensen and Elbæk, 2005; Zhang et al., 2013). In recent years, the QuEChERS extraction procedure has been widely applied and used for the extraction of veterinary drugs, pesticides, and especially aflatoxins from various food matrices (Cao et al., 2018; Xu et al., 2019). Although the proposed analytical method, combined with modified QuEChERS extraction and post-column derivatization using KOBRA CELL®, does not yield as high recoveries in butter samples as in milk samples, it is considered to have limited sensitivity for the determination of aflatoxins due to its low LOD and LOQ values.

In a study using the HPLC-SPE method for the determination of aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2, and AFTM1) in milk, recovery rates were reported between 88.30 % and 98.10 % in contaminated milk samples at concentrations of 2 μg/kg and 20 μg/kg (Herzallah, 2009). In our study, similar recovery rates were obtained for milk samples at lower concentrations of 0.025 ng/g and 1 ng/g, ranging from 89.15 to 95.14 %. In addition, although the LOD values for milk samples in our study varied between 0.004 and 0.008 ng/g, the LOD value in this study (Herzallah, 2009) was stated as 0.05 μg/kg. In light of these findings, our QuEChERS-based HPLC method is considered more practical, considering the high volumes of chemicals used for conditioning the SPE cartridges (tetrahydrofuran, acetic acid, hexane, methylene chloride, etc.) and the similar recovery rates achieved at lower concentrations. Additionally, although the 21-minute analysis time in our study is longer than the 16-minute time of the mentioned study, this difference is considered tolerable due to the long extraction procedures of the SPE method. In another study (Shuib and Saad, 2022), AFTB1, AFTB2, AFTM1, and AFTM2 were determined using HPLC with in-syringe dispersive micro-solid phase extraction and recovery rates were reported between 73 % and 109.6 % using high volumes (4 ml) of solvents such as acetone, as in SPE. The concentrations studied varied between 0.05 ng/ml and 0.5 ng/ml, while LOD and LOQ values were reported as 0.003–0.005 ng/ml and 0.01–0.02 ng/ml, respectively. While the LOD and LOQ values in our study were similar (0.004–0.008 ng/g, 0.012–0.023 ng/g), the recovery rates were lower despite working with larger volumes. A UHPLC-MS and QuEChERS study was conducted to determine aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2, and AFTM1) in milk. In this study, recovery rates ranged from 72 % to 121 % for samples in the concentration range of 0.05–0.2 μg/kg, with LOD values of 0.005–0.014 μg/kg and LOQ values of 0.017–0.048 μg/kg (Sartori et al., 2015). Although their method used tandem mass spectrometry (MS/MS), the LOQ values in this study were higher than our method, and the recovery rates were lower. Similar results to this study were reported for the analysis of AFTB1, AFTB2, AFTG1 and AFTG2 using the HPLC-FLD-coupled IAC method (Sahin et al., 2016) and for the analysis of AFTM1 and AFTB1 using tandem mass spectrometry (MS/MS) (Yang et al., 2025). Various studies have used various methods for the analysis of aflatoxin M1 (AFTM1) in milk. A UHPLC-MS/MS and QuEChERS study by Michlig et al. (2016) achieved recovery rates between 70 % and 90 % for samples in the concentration range of 0.25–10 μg/L. The LOD and LOQ values in this study were reported as 0.002 μg/L and 0.007 μg/L, respectively. Furthermore, Mulunda and Mike (2014) combined the HPLC-FLD-KOBRA CELL method with SPE and IAC to study concentrations of 5–20 ng/ml, achieving recoveries of 67.3–98.9 % and a LOD of 0.01 ng/ml. Campone et al. (2013) used UHPLC-MS/MS and LLE methods in the concentration range of 5–500 ng/kg and reported recovery rates between 61.3 % and 75.3 %. Our study demonstrates higher recovery rates for milk samples compared to most of these studies that only analyzed AFTM1. ELISA is a method commonly used for the analysis of AFTM1 in milk and milk products. However, some studies in the literature point to its limitations. For example, in a study by Tarannum et al. (2020), despite high recovery rates of 93–119 %, a concentration range of 25–500 ng/kg was used, yielding a relatively high LOD of 18 ng/kg. Similarly, in another ELISA study by Yi et al. (2025), a high LOD of 0.051 ng/ml and linearity range 0.168–0.679 ng/ml was determined, despite recovery rates of 95–111 %. This suggests that the ELISA method does not always achieve the desired sensitivity. Furthermore, Zheng et al. (2006) emphasized that the antibodies used in ELISA are prone to false-positive results in oily matrices, making the method unsuitable for multiplex aflatoxin analysis.

There are several studies in the literature using different approaches for the analysis of aflatoxin M1 (AFTM1) in butter. Sakuma et al. (2011) achieved high recovery rates of 90–92 % at concentrations of 0.1–0.5 μg/kg using HPLC-FLD and IAC methods, but their LOD and LOQ values (0.04 μg/kg and 0.12 μg/kg, respectively) were higher than those in our study. In our study, LOD values for AFTM1, AFTG1, AFTG2, AFTB1, and AFTB2 were found to range from 0.005 to 0.008 ng/g, and LOQ values ranged from 0.016 to 0.025 ng/g. Our recovery rates for all aflatoxins ranged from 50.81 to 86.08 %. These lower recovery rates may be due to the use of smaller sample volumes or the use of acetonitrile instead of more common chemicals such as hexane or chloroform. While recovery rates were not as high as those for milk samples, the LOD and LOQ values in our study were lower than those reported in the literature for both ELISA and similar HPLC-FLD methods. This suggests that the developed method could be a potential alternative analysis option with higher sensitivity in challenging matrices such as butter. It is also believed that the use of KOBRA CELL® may contribute to this sensitivity.

3.1. Linearity and range

The linearity of the standards was established by examining five molecules at varying levels that spanned the entire analytical range. Furthermore, method linearity was verified through the construction and evaluation of calibration curves derived from six replicates (n = 6) of blank milk and butter samples fortified with five mycotoxins concentration levels (0.025, 0.05, 0.1, 0.5, and 1 ng/g). The relationship between peak area and aflatoxin concentration was assessed using the least-squares linear regression approach, with the slope, correlation coefficients (r), and relative standard deviations (RSD) calculated accordingly. The linear working range was determined via linear regression analysis, and the degree of linearity was expressed by the coefficient of determination (r^2). Calibration plots demonstrated linearity over the tested concentration intervals for all analytes (Fig. S1 and Fig. S2). Correlation coefficients exceeded 0.99 for each analyte, as shown in Table 1. These findings confirm that the method exhibits robust linearity and reliable correlation across the calibration standards of all target aflatoxins.

3.2. Accuracy and precision

The accuracy of the method was determined through a recovery study, which was conducted by spiking all analytical standards (AFTM1, AFTMG2, AFTMG1, AFTB2, AFTB1) into milk samples at four different concentrations (0.083, 0.163, 0.470, and 0.780 ng/g) and butter samples (0.080, 0.400, 0.750, and 0.910 ng/g). Following this process, the mean percentage recoveries and standard deviations were calculated. The extraction efficiency (recovery) of the five analytes was evaluated by comparing the peak areas obtained from fortified milk and butter samples with those obtained from the injection of analytical standards after post-column derivatisation procedure.

The precision of the extraction and chromatographic analysis procedures, both intra-assay (within-day) and inter-assay (between-day), was evaluated by analysing blank milk and butter samples spiked with five different concentrations (0.025, 0.05, 0.1, 0.5 and 1 ng/g) in four replicates, on the same day and across four separate days (Table 1). The recovery rates of the extraction technique were calculated by contrasting the results obtained from the fortified milk and butter specimens. The assessment of the method precision involved the use of inter-day reproducibility and intra-day repeatability, with the presentation of the results as relative standard deviation (RSD) in Table 1. Fig. S2 shows the chromatograms acquired from the analysis of the samples fortified with five aflatoxin compounds, as well as those from blank milk and butter samples.

The results related to the precision and accuracy are presented in Table 2. The mean recovery rates for aflatoxins ranged from 89.15 % to 95.14 % for milk and from 50.81 to 86.08 % for butter, while the coefficients of variation ranged from 1.30 to 6.08 for milk and from 2.88 to 6.39 for butter (Table 1). These recovery results fall within the acceptable ranges established by Commission Decision 2002/657/EC (Table 1). Milk recovery rates for all aflatoxins (AFTM1, AFTG2, AFTG1, AFTB2, AFTB1) were significantly higher than butter recovery rates ($p < 0.05$ for all groups). Therefore, milk recovery appears to be more efficient than butter recovery. Precision values did not show any significant difference in milk and butter samples for each aflatoxin ($p > 0.05$).

Table 2
Precision and accuracy data (n = 3 for each added concentration) were used to determine the aflatoxins (AFTM1, AFTB1, AFTB2, AFTG1 and AFTG2) in milk and butter.

Products	Aflatoxins	Added (ng/g)	Measured ± SD (ng/g)	Precision (RSD%)	Accuracy (%)
Milk	AFTM1	0.083	0.080 ± 0.004	5.35	97.10
		0.163	0.164 ± 0.008	4.68	101.20
		0.470	0.480 ± 0.036	7.51	102.13
		0.780	0.792 ± 0.022	2.84	101.48
	AFTG2	0.083	0.085 ± 0.008	8.93	102.62
		0.163	0.164 ± 0.004	2.52	101.06
		0.470	0.452 ± 0.023	5.09	96.20
		0.780	0.777 ± 0.018	2.37	99.60
	AFTG1	0.083	0.082 ± 0.003	3.45	98.98
		0.163	0.160 ± 0.005	2.94	98.65
		0.470	0.479 ± 0.024	5.09	101.85
		0.780	0.765 ± 0.022	2.81	98.07
	AFTB2	0.083	0.084 ± 0.004	4.94	101.82
		0.163	0.162 ± 0.004	2.47	99.99
		0.470	0.466 ± 0.015	3.31	99.20
		0.780	0.769 ± 0.012	1.51	98.63
	AFTB1	0.083	0.079 ± 0.003	4.22	96.08
		0.163	0.162 ± 0.002	1.38	99.69
		0.470	0.458 ± 0.021	4.66	97.54
		0.780	0.802 ± 0.025	3.16	102.76
Butter	AFTM1	0.080	0.081 ± 0.005	6.25	102.00
		0.400	0.432 ± 0.008	1.77	107.92
		0.750	0.750 ± 0.050	5.56	100.00
		0.910	0.900 ± 0.050	5.56	98.90
	AFTG2	0.080	0.082 ± 0.004	5.39	102.72
		0.400	0.387 ± 0.017	4.33	96.67
		0.750	0.725 ± 0.058	5.56	96.67
		0.910	0.918 ± 0.044	4.82	100.92
	AFTG1	0.080	0.083 ± 0.006	7.57	104.17
		0.400	0.399 ± 0.024	5.97	99.86
		0.750	0.730 ± 0.066	5.56	97.34
		0.910	0.888 ± 0.036	4.03	97.63
	AFTB2	0.080	0.081 ± 0.004	5.09	101.05
		0.400	0.416 ± 0.011	2.62	104.02
		0.750	0.735 ± 0.081	5.56	97.96
		0.910	0.920 ± 0.040	4.32	101.11
	AFTB1	0.080	0.085 ± 0.005	5.58	106.84
		0.400	0.397 ± 0.013	3.33	99.21
		0.750	0.763 ± 0.103	5.56	101.75
		0.910	0.916 ± 0.023	2.50	100.63

Table 1
The regression analysis data and the recoveries (%), inter-day and intra-day variations, LODs and LOQs of the developed method for determining the aflatoxins (AFTM1, AFTB1, AFTB2, AFTG1 and AFTG2) in milk and butter.

Products	Drugs	Spiked Conc. (ng/g)	Recovery (%) ± SD	Intra-day (RSD) (n = 20)	Inter-day (RSD) (n = 16)	Slope (ng^{-1})	Intercept	r^2	LOD (ng/g)	LOQ (ng/g)
Milk	AFTM1	0.000, 0.025, 0.050, 0.100,	95.14 ± 1.23	1.30	5.03	0.260	0.004	0.9996	0.007	0.022
	AFTG2	0.500 and	89.15 ± 2.78	3.12	11.09	0.139	-0.002	1.0000	0.007	0.020
	AFTG1	1.000	93.57 ± 2.95	3.15	5.57	0.141	0.003	0.9995	0.008	0.023
	AFTB2		92.26 ± 4.58	4.96	6.26	0.037	-0.002	0.9999	0.004	0.012
	AFTB1		90.49 ± 5.50	6.08	5.54	0.063	0.001	0.9999	0.005	0.014
Butter	AFTM1		86.08 ± 2.48	2.88	11.99	0.467	-0.009	0.9948	0.008	0.024
	AFTG2		76.94 ± 3.33	4.33	6.38	0.204	-0.010	0.9992	0.007	0.021
	AFTG1		64.42 ± 3.49	5.41	10.46	0.278	-0.009	0.9992	0.008	0.025
	AFTB2		60.14 ± 1.40	2.32	7.11	0.067	-0.016	0.9929	0.005	0.016
	AFTB1		50.81 ± 3.25	6.39	9.19	0.217	-0.020	0.9963	0.006	0.018

Recovery rates for each aflatoxin in butter samples were not as high as those in milk. Furthermore, inter-day variation values in butter samples were relatively higher than in milk samples. In this case, the matrix effect is thought to play an important role in the analytical results. Values above 100 % indicate matrix enhancement, while values below this value indicate matrix suppression (Morais et al., 2024). As a result of the analyses, it was determined that signal suppression was the dominant effect for the majority of the aflatoxins examined in butter. The matrix effect was observed in a wide range, ranging from −24–3 % for AFTM1; −35 % to −20 % for AFTG2; −50 % to −44 % for AFTG1; −58 % to −52 % for AFTB2; and −64 % to −55 % for AFTB1. Matrix suppression was particularly above 50 % for AFTB2, AFTB1 and AFTG1. This suggests that the butter matrix strongly suppresses the analytical signal of these aflatoxins, and that each aflatoxin responds differently to the matrix effect. When examining the milk matrix, the observed matrix effect remains lower compared to butter. Matrix effect values ranged from 2 % to −19 % for AFM1, AFG2, AFG1, AFB2, and AFB1. This finding suggests that the milk matrix exerts a more minimal effect on the analytical signal than the severe suppression caused by the high fat content of butter.

Butter is a challenging matrix due to its composition. High fat content, especially in analytical instruments, can reduce detector sensitivity, leading to unexpected signal increases and analyte losses (Liu et al., 2014; Stroka and Maragos, 2016; Iqbal, 2021; Schincaglia et al., 2023; Lin et al., 2025). Therefore, careful interpretation of the data is recommended. The findings indicate that this method can be successfully applied to milk matrices and offers a potential tool for challenging matrices such as butter. However, the butter results should be interpreted considering the method's limitations in these matrices. To improve the performance of our method in challenging matrices like butter, instead of adding additional steps to complicate the sample preparation process, exploring different solvent options for more effective extraction of aflatoxins from the butter matrix could be considered a potential avenue. This approach is believed to help improve analytical performance while maintaining the overall simplicity of the method. In addition to using alternative solvents, different adsorbents such as octadecyl silica (C18) and primary secondary amine (PSA) can also be considered as alternatives for purification. It has been reported that this process can provide satisfactory recovery results due to the reduced matrix effect (Sun et al., 2016). C18 has been reported to be suitable for removing interfering substances in matrices such as lipids, sterols, proteins, and carotenoids (Lin et al., 2025), while PSA is also suitable for removing interfering substances, especially in matrices with high sugar content (Sadighara et al., 2024). Therefore, the adsorbents to be used should be selected according to the composition of the sample to be extracted, and in some cases, more than one adsorbent type, such as PSA and C18, can be applied simultaneously (Michlig et al., 2016; Rodríguez-Carrasco et al., 2018).

3.3. Selectivity and sensitivity

The selectivity of the technique was tested by analysing blank milk and butter samples, as well as samples fortified with five different concentrations of aflatoxins, both below and above the Maximum Residue Limit (MRL). Selectivity was assessed by examining the presence of any impurity peaks at the retention times of the target aflatoxins in the corresponding blank samples. As a result, the method was found to be specific for each compound based on the reference analytical standards. When the blank milk and butter samples were analysed, no impurity peaks were observed at the elution times of the analytes; therefore, the compounds were analysed without any interference (Fig. 1 and Fig. 2, respectively).

The limits of detection (LOD) and quantification (LOQ) for each analyte were determined by HPLC analysis following the spiking of aflatoxin-free milk and butter samples with a mixture of analytical standards. Baseline noise was assessed at the elution times of the target peaks. LOD and LOQ values were calculated according to the guidelines proposed by Harris (2007), using the following equations: LOD = 3 × Standard Deviation / Slope, and LOQ = 10 × Standard Deviation / Slope. The LOQ values for aflatoxins ranged from 0.012 to 0.023 ng/g in milk samples and from 0.021 to 0.025 ng/g in butter samples (Table 1). The results show that the method can be used to detect and measure the presence of aflatoxins in milk and butter, even at levels below the maximum residue limits (MRLs) set by the European Union.

3.4. Decision limit and detection capability

Maximum limits for AFTM1 in milk and milk-based products in international regulations vary between 0.01 ppb and 0.5 ppb and total aflatoxins (AFTB1, AFTB2, AFTG1, AFTG2) in foods vary between 4 ppb and 20 ppb according to various countries, as presented in Table 3.

The decision limit (CCα) and detection capability (CCβ) were evaluated in accordance with the European Union requirements as set out in European Commission., 2021). CCα is defined as the concentration level at which it can be concluded that a sample is non-compliant, with a probability of a false positive result (α). For compounds without established Maximum Residue Limits (MRLs), α is accepted as 1 %, and CCα is considered the detection limit of the method (Rezende et al., 2012). To determine CCα, 20 blank milk and butter samples were fortified at the method's LOQ level for compounds with MRLs, and at the permitted concentration levels for regulated aflatoxins. CCβ is defined as the lowest concentration at which a compound in milk and butter samples can be reliably detected, with a maximum probability of 5 % for a false negative result (β). This value is calculated by adding 1.64 times the standard deviation of samples fortified at the CCα level to the CCα value itself. Therefore, the CCβ value plays a crucial role in protecting consumers from risky foods by minimizing the risk of products contaminated above the MRL reaching consumers (Cherif et al., 2015). For CCβ evaluation, milk and butter samples were spiked at the CCα level. The parameters related to CCα and CCβ are presented in Table 4. The obtained results demonstrate that the proposed method is appropriate and reliable for the determination of aflatoxins in butter and milk products.

3.5. Application to real samples

The developed method was evaluated against the legal limits set by the European Union (EU).

The results of the study indicated that AFTM1 (Fig. 1) was detected in 44.8 % of milk samples (95 % confidence interval: 36.5 %-53.1 %), with 28 % of samples found above (95 % confidence interval: 20.5 %-35.4 %) (0.06–9.4 ng/g) the permissible limit of the EU. AFTM1 was detected in 9.7 % of butter samples (95 % confidence interval: 4.0 %-15.4 %), with 4.5 % of butter samples found above (95 % confidence interval: 0.4 %-8.5 %) (0.07–0.36 ng/g) the recommended limit of the EU. Moreover, multiple aflatoxins (AFTM1, AFTB2 and AFTB1) (Fig. 2) were detected in 1.94 % of butter samples (95 % confidence interval: 0–4.6 %), but none of these samples were found above the recommended limit of the EU. Confidence intervals for milk samples are wider than for butter, suggesting that variability in the milk sample may be greater.

Several studies investigating the presence of AFTM1 in milk and milk-based products have been conducted in different regions of Türkiye. In a study investigating AFM1 levels in UHT milk samples from the Central Anatolia region of Türkiye, a total of 129 UHT whole milk samples were analyzed, and the mean value was determined to be 108.17 ng/L. AFM1 was reported to have a high incidence, with 75 (58.1 %) milk samples being contaminated. Although 68 (53 %) milk samples were below the limit, the remaining 61 (47 %) samples were reported to be significantly above the limit permitted by the EU (Unusan, 2006). In a study investigating the presence of AFTM1 in 27 milk samples from Ankara province, AFTM1 was reported in 59.3 % of the samples. However, only one sample exceeded the legal limit set by

Table 3
Maximum permissible limits of aflatoxin AFTM1 for milk, milk-based products and total AFT (B1,B2,G1,G2) for foods.

Country/ Organization	Maximum permissible limit of M1 (ng/g)	Maximum permissible limit of total aflatoxin (B1, B2, G1, G2) (foods) (ng/g)	References
European Union	0.05 (milk and milk-based products)	4–15	Commission Regulation European Commission. EC., 2006.
Food and Drug Administration	0.5 (milk and milk-based products), 0.025 (infant milk products)	20	Food and Drug Administration. FDA., 2000;2019.
Austria	0.05, 0.01 (pasteurized, infant milk), 0.02 (butter)	-	Iqbal et al., 2015.
Bulgaria	0.5 (milk), 0.1 (powdered milk)	-	
Switzerland	0.05 (milk), 0.025 (milk whey and products), 0.02 (butter)	-	
Iran	0.5 (milk)	-	
Türkiye	0.05 (milk and milk-based products)	4–15	Turkish Food Codex. TFC., 2011.
China	0.5 (milk), 0.2 (pasteurized)	-	Global Agricultural Information Network. GAIN., 2019.

Table 4
The decision limit (CCα) and detection capability (CCβ) values of the method were calculated at the MRL (n = 20) of aflatoxin M1 (AFTM1) in milk and butter.

Product	Toxin	MRL (ng/g) (FDA)	MRL (ng/g) (EU Codex)	Added (ng/g)	Measured (ng/g)	SD	Error α (1.64 x SD)	ME (%)	CCα (ng/g)	CCβ (ng/g)
Milk	M1	0.5	0.05	0.053	0.052	0.003	0.0049	from −17 to −2	0.055	0.060
	B1	-	-		0.053	0.004	0.0067	from −17 to −11	0.057	0.063
	B2	-	-		0.054	0.004	0.0073	from −19 to −14	0.057	0.065
	G1	-	-		0.056	0.004	0.0061	from −13 to −9	0.056	0.062
	G2	-	-		0.057	0.004	0.0065	from −13–2	0.057	0.063
Butter	M1	0.5	0.05	0.051	0.049	0.004	0.0066	from −24–3	0.057	0.061
	B1	-	-		0.0490	0.0140	0.0246	from −64 to −55	0.075	0.099
	B2	-	-		0.0492	0.0120	0.0148	from −58 to −52	0.065	0.080
	G1	-	-		0.0466	0.0110	0.0180	from −50 to −44	0.068	0.086
	G2	-	-		0.0489	0.0090	0.0197	from −35 to −20	0.070	0.089

MRL: Maximum residue level. ME: Matrix effect. CCα = C_{MRL} + 1.64SD20 representative MRL−spiked samples. CCβ = CCα + 1.64SD20 representative samples spiked at CCα level.

the EU (Gürbay et al., 2006). Another study conducted on butter in Ankara province reported that AFTM1 was detected in 25 of 27 butter samples (92.6 %). It was also reported that AFTM1 was detected in 7 of the butter samples (13.2 %) above the limits set by the EU and Turkish Food Codex (Aycicek et al., 2005). In Van province, 90 raw milk samples were examined, and AFTM1 was detected in 79 (87.77 %). 35 (44.30 %) of the positive samples were reported to be above the legal limit (0.05 ppb) set by the EU (Bakirci, 2001). In Sakarya province, it was reported that 61.5 % and 29.6 % of 26 milk and 27 butter samples, respectively, were contaminated with AFTM1, but the legal limit set by the EU was not exceeded (Öztürk Yılmaz and Altinci, 2018). In Çanakkale province, AFTM1 was detected in 107 (89.2 %) of 120 raw milk samples. It was reported that 4 samples (3.3 %) were above the legal limit determined by the Turkish Food Codex and the EU, while 13 samples (10.8 %) did not exceed this legal limit; it was found in trace amounts below the permitted limit (0.05 ppb) (Eker et al., 2019). In a meta-analysis examining the prevalence of AFTM1 in milk and dairy products in Middle Eastern countries (Iran, Jordan, Türkiye, Kuwait, Lebanon, Syria, Egypt, Cyprus and the United Arab Emirates), high AFTM1 contamination was reported in milk (87 %) and raw milk (73 %) (Arghavan et al., 2025). A study systematically examining the presence of AFTM1 in milk and dairy products from Mediterranean countries reported a prevalence of 40 %, regardless of milk type (Malissiova et al., 2024). A study investigating the presence of AFTM1 in raw milk from northwestern France (Boudra et al., 2007) reported detection in 3 out of 264 samples (0.026 ng/g or less), and in 68 out of 72 milk samples (94.4 %) from Catalonia, Spain (between 0.006 and 0.01 ng/g) (Cano-Sancho et al., 2010). In this study, the AFTM1 contamination rates detected in milk and butter samples (44.8 % and 9.7 %) were lower than in most provinces in Türkiye and Middle Eastern countries, but higher than in some European countries. In addition, the AFTM1 contamination rate detected in butter samples (9.7 %) remains quite low compared to other provinces in Türkiye.

Based on weighted average concentrations of AFTM1 in milk from five regions, intakes were 0.1 ng/day for the African diet, 0.6 ng/day for the Middle Eastern diet, 3.5 ng/day for the Latin American diet, 6.8 ng/day for the European diet, and 12 ng/day for the Far Eastern diet. When these intakes are expressed as nanograms of AFTM1 per kg body weight per day and assuming a body weight of 60 kg, intakes are 0.002 for the African diet, 0.10 for the Middle Eastern diet, 0.058 for the Latin American diet, 0.11 for the European diet, and 0.20 for the Far Eastern diet (JECFA, 2001). Although 28 % of the milk samples analysed in this study exceeded the EU limits (0.06–9.4 ng/g), to assess the worst-case scenario, the daily intake estimate (EDI) for a single milk sample with the highest contamination (9.4 ng/g) was calculated as follows. EDI based on population milk consumption was calculated using the formula: EDI (ng/kg body weight) = toxin (ng/kg) x milk consumption (kg/day)/body weight (kg) (Kuiper-Goodman., 1990). Considering the average daily milk consumption reported for the Middle East region (0.12 kg/day) and a body weight of 60 kg (JECFA, 2001), for a milk sample with the highest contamination (9.4 ng/g): EDI= (9.4 ng/g x 1000) x 0.12 kg/day /60 kg= 18.8 ng/kg/day. This high value, calculated for just one milk sample, exceeds the tolerable daily intake (TDI) value (0.2 ng/kg/day) (Kuiper-Goodman., 1990) by approximately 90 times, posing a risk. When the average value of milk samples contaminated with the same formula is evaluated as 4.5 ng/g, the EDI is calculated as 9 ng/kg/day. In this case, it exceeds the TDI value by 45 times, posing a risk. However, 4.5 % of butter samples were found to have levels above (0.07–0.36 ng/g) the EU recommended limit. However, the simultaneous detection of AFTM1, AFTB2, and AFTB1 in some butter samples can be considered a significant risk factor due to their potential cumulative toxic effects. A strong association has been

reported between AFTB1 exposure and the incidence of hepatocellular carcinoma (HCC) (Hussain et al., 2007). Oxidative stress has also been reported to play a significant role in carcinogenesis and DNA damage, and AFTB1 has been reported to induce apoptosis through various mechanisms by inducing reactive oxygen species and oxidative stress (Liu and Wang, 2016). Furthermore, when simultaneous exposure to AFTM1 and AFTB1 was examined, high-dose AFTM1 exposure was reported to result in adenocarcinoma (Cullen et al., 1987). Because exposure to aflatoxins can be multiple, some studies have examined the effects of different aflatoxin mixtures. After pigs were fed a diet contaminated with a mixture of aflatoxins (AFTB1, AFTB2, AFTG1, and AFTG2) for 28 days, crusting and skin ulceration were reported on the nose, lips, and cheeks (Harvey et al., 1990). AFTB1 and AFTB2 administered orally to broilers for up to 42 days were reported to disrupt cell cycle progression and apoptosis, causing histopathological lesions of varying severity in the thymus and bursa fabricius, where T and B lymphocytes mature, respectively (Peng et al., 2017). Furthermore, it has been reported that aflatoxins can compromise macrophage function; in particular, co-exposure to AFB1, AFB2, AFM1, and AFM2 can create interactions that can significantly affect immunoreactivity (Bianco et al., 2012).

4. Comparison of analytical methods

Analytical methods and extraction procedures can vary significantly in terms of sensitivity, specificity, accuracy, limit of detection (LOD), cost, applicability, and analysis time. In this context, it is essential to compare the performance characteristics of each method to determine their suitability for detecting target compounds in complex food matrices. The studies conducted to develop and validate a chromatographic method for the detection of aflatoxin residues in milk and butter, along with the corresponding analytical performance parameters, are presented in Table 5. Such comparisons allow researchers to select the most appropriate method depending on the analytical objective, available instrumentation, and regulatory requirements.

Liquid-liquid extraction (LLE) is a well-established technique employed for isolating aflatoxins from milk and milk-based products (Pittet, 2005). This method utilizes appropriate solvents such as chloroform, acetonitrile, methanol or dichloromethane (DCM). Research has demonstrated that the use of chloroform in milk sample extractions

Table 5
Comparison of analytical methods and performance for quantification of the aflatoxins (AFTM1, AFTB1, AFTB2, AFTG1 and AFTG2) in milk and butter reported in the literature.

Aflatoxins	Type of Product	Analytical instrument and total run time	Sample preparation	Linearity range	LOD-LOQ	Recovery, % (SD)	Reference
B1, B2, G1, G2, M1	Cow, Sheep, Goat milk	HPLC-FLD-UV. / 18 min.	SPE	2, 5, 10, 15, 20 μg/kg	0.05 μg/kg-FLD (LOD)-0.1 μg/kg-UV (LOD)	88.32–98.10	Herzallah, 2009.
B1, B2, M1, M2	Market milk	HPLC-FLD. / 35 min.	In-syringe dispersive micro-SPE	0.05, 0.1, 0.5 ng/ml	0.003–0.005 ng/ml (LOD), 0.01–0.02 ng/ml (LOQ)	73.0–109.6	Shuib and Saad, 2022.
M1, M2, G1, G2, B1, B2,	Market milk	UHPLC-MS. / 2.60 min.	QuEChERS	0.05, 0.1, 0.2 μg/kg	0.005–0.014 μg/kg (LOD)- 0.017–0.048 μg/kg (LOQ)	72–121	Sartori et al., 2015.
B1, B2, G1, G2, M1	Cow milk	HPLC-FLD. / 15.3 min	IAC	0.3–25 μg/l	0.004–0.059 μg/kg (LOD)- 0.014–0.197 μg/kg (LOQ)	83.1–95.0	Sahin et al., 2016.
B1, M1	Market milk	HPLC-FLD./ 4–5 min.	QuEChERS	0.03–10 μg/kg	0.01–0.1 μg/kg (LOD)-0.03–0.3 μg/kg (LOQ)	50–75	Karaseva et al., 2014.
M1	Market milk	HPLC-FLD, ELISA. / -	SPE	5, 10, 50, 100, 500 pg/ml	10 pg/ml, HPLC, 2 pg/ml,ELISA (LOD)	103–120 HPLC, 88–106 ELISA	Kim et al., 2000.
M1	Cow milk	HPLC-FLD./ 5 min.	IAC	100, 1000 ng/l	10 ng/l (LOQ)	80.7 ± 97.9	Rodriguez Velasco et al., 2003.
M1	Market milk	HPLC-FLD. / 35 min.	SPE-IAC	0.020–0.050 μg/kg	0.006 μg/kg (LOD)-0.020 μg/kg (LOQ)	92.6 ± 2.1	Wang et al., 2012.
M1	Market milk	HPLC-FLD. / 7 min.	IAC	500 ng/l	14 ng/l (LOD)- 42 ng/l (LOQ)	97.2–109.7	Chavarría et al., 2015.
M1	Market milk	HPLC-FLD. / 9 min.	IAC	0.1, 0.5, 1 ppb	15.5 ng/l (LOD)- 50 ng/l (LOQ)	94–98	Behfar et al., 2012.
M1	Sheep milk	HPLC-MS. / 5 min.	IAC	1, 50, 200 ng/l	250 ng/l (LOD)-500 ng/l (LOQ)	68.31–90.06	Bognanno et al., 2006.
M1	Market milk	UHPLC-MS/MS. / 5 min.	Dispersive liquid-liquid microextraction	5, 25, 50, 500 ng/kg	0.6 ng/kg (LOD)-2.0 ng/kg (LOQ)	61.3–75.3	Campone et al., 2013.
M1	Market milk	Fluorescence spectrophotometer. / -	Dispersive liquid-liquid microextraction by SPE using magnetic nanoparticles	0.02–200 μg/l	13 ng/ml (LOD)	91.3–99.5	Amoli-Diva et al., 2015.
M1	Cow milk	UHPLC-MS/MS. / 7 min.	QuEChERS	0.25–10 μg/l	0.002 μg/l (LOD)-0.007 μg/l (LOQ)	70–90	Michlig et al., 2016.
M1	Plant-based milk	HPLC-FLD. / 23 min.	Dispersive liquid-liquid microextraction and QuEChERS	0.5,1, 2.5, 5, 10 μg/l	-	82–104	Hamed et al., 2019.
M1	Cow milk	HPLC-FLD-KOBRA cell./ 12.5 min.	IAC-SPE	5, 10, 20 ng/ml	0.01 ng/ml (LOD)	67.3–98.8	Mulunda and Mike, 2014.
M1	Butter	ELISA. / -	IAC	1–250 ng/kg	-	-	Tekinşen and Uçar, 2008.
M1	Butter	HPLC-FLD. / -	IAC	0.1–0.5 μg/kg	0.04 μg/kg (LOD)-0.12 μg/kg (LOQ)	90–92	Sakuma et al., 2011.
M1	Butter	HPLC-FLD. /-	IAC	-	1.10 ng/kg (LOD) – 3.02 ng/kg (LOQ)	104.01 ± 1.90	Öztürk Yılmaz and Altinci, 2018.

yields satisfactory recovery rates, ranging from 89 % to 99 % (Fallah, 2010b; Herzallah, 2009). Nevertheless, chloroform-based extraction demands significant amounts of solvent, which contributes to environmental challenges (Hussain, 2011). Alternatively, methanol and acetonitrile have gained increased preference in aflatoxin extraction due to their superior environmental compatibility and their compatibility with antibodies used in the subsequent cleanup processes involving IAC columns (Pisoschi et al., 2023). Recovery rates for AFTM1 extraction using methanol were reported to range between 77 % and 99 %, whereas ACN yielded recovery rates between 85 % and 97 % (Navas et al., 2005; Temamogullari and Kanici, 2014; Wang et al., 2010). Researchers (Wang et al., 2011) evaluated the efficiency of SPE and IAC columns for AFTM1 extraction. For raw milk samples containing 0.05 μg/kg of AFTM1, the average recovery rates were 96 % (RSD 2.1 %) with SPE and 92 % (RSD 4.7 %) with IAC columns, respectively. While IA extraction is a comparable method to others, it is relatively costly. As a result, the LLE technique is preferred for extracting aflatoxins from milk and milk-based products. Among the options, acetonitrile or methanol are regarded as the most effective organic solvents due to their satisfactory recovery rates and environmental compatibility. Furthermore, acetonitrile has been shown to yield the cleanest extracts, as it causes milk proteins to precipitate effectively (Wang et al., 2011). Therefore, the QuEChERS method, which predominantly utilizes acetonitrile, offers a faster, more practical, and environmentally friendly alternative compared to other extraction techniques, while also being more cost-effective than conventional often expensive methods.

Analytical techniques for quantifying aflatoxins include thin-layer chromatography (TLC) and liquid chromatography (LC). TLC has gained widespread acceptance because of its straightforward operation, capability for repeated detection and measurement, cost-efficiency, and the ability to process multiple samples on a single plate with minimal solvent consumption (Fuchs et al., 2011). Several studies have utilized TLC to examine AFTM1-contaminated milk and dairy products, achieving detection limits (LOD) as low as 0.01 μg/kg (Fallah, 2010a; Kafle et al., 2012). Nevertheless, with advancements in LC technology, the use of TLC for AFTM1 analysis has been largely phased out in most laboratories. In research conducted on milk samples, AFTM1 analysis was performed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) with electrospray ionization (ESI) following a liquid-liquid extraction process. The method demonstrated a quantification limit (LOQ) of 0.015 μg/kg (Biancardi et al., 2013). The key benefits of HPLC-MS/MS include the excellent separation efficiency of HPLC combined with the high sensitivity and specificity of MS. Nevertheless, the drawbacks of this technique involve the significant expense of the instrumentation and the substantial consumption of organic solvents. As a result, HPLC-MS/MS may not be a feasible option for small laboratories routine analytical applications. The primary detection technique for quantifying aflatoxins using HPLC is fluorescence detection (HPLC-FLD), which depends on the presence of fluorescence characteristics in the molecules. AFTM1, AFTB1, AFTB2, AFTG1, and AFTG2 exhibit inherent fluorescence, enabling their direct identification through HPLC-FLD. Furthermore, pairing HPLC-FLD with a KOBRA CELL® intensifies the fluorescence characteristics of B1 and G1, improving their visibility and suitability for analytical purposes. In addition, most studies in the literature do not clearly specify the total analysis time, which generally includes not only the chromatographic separation time but also all sample preparation processes. In our study, the peak time obtained with HPLC-FLD was 21 min. However, the time from extraction to instrument analysis was determined to be 4.30 h for approximately 30 milk or butter samples. A review of studies in the literature that analyzed multiple aflatoxins revealed that peak times for HPLC, UHPLC and MS analyses varied between 2.60 and 35 min (Herzallah, 2009; Karaseva et al., 2014; Sartori et al., 2015; Sahin et al., 2016; Shuib and Saad, 2022; Morais et al., 2024). The simplicity and ease of extraction procedures up to the analysis stage is also an important factor affecting the total analysis time. Methods such as SPE, LLE or IAC are multi-stage and relatively more costly than the QuEChERS method. It has also been stated that the amount of solvent used in the extraction phase can vary between 10 and 50 ml, depending on the amount of sample used in immunoaffinity-based procedures (Şenyuva and Gilbert, 2010; AlFaris et al., 2020). In this study, the modified QuEChERS and HPLC-FLD method is considered to offer a simpler, easier, less solvent use (4–5 ml) and relatively lower-cost approach for the sample preparation stage, due to the use of fewer chemicals. The method ability to be applied to multiple samples in parallel can reduce time losses in laboratory workflows. This increased efficiency suggests that the method could be a favourable choice for practical applications.

Although the ELISA (Enzyme-Linked Immunosorbent Assay) method is a fast and user-friendly technique for aflatoxin analysis, it has several limitations. The antibodies used may sometimes cross-react with compounds of similar structure, reducing the reliability of the results and leading to false positives. Furthermore, other substances present in food or feed samples can interfere with the reaction and affect the results. For example, in milk or fatty matrices, matrix effects may result in inaccurate findings (Zheng et al., 2006; Turner et al., 2009). Additionally, ELISA cannot achieve the low limit of detection (LOD) provided by advanced detection techniques such as HPLC-FLD or HPLC-MS/MS. These disadvantages highlight the limitations of ELISA, particularly in terms of accuracy, sensitivity, and matrix effects. Therefore, for critical analyses such as aflatoxins, more advanced methods should be preferred over ELISA.

5. Limitations

The primary limitation of this study is the limited sample size and that the samples were collected only from the Balikesir region of Türkiye. This may limit the representativeness of the results across Türkiye. Moreover, seasonal variations can have a considerable impact on the levels of aflatoxin contamination in milk and dairy products. Some studies in the literature have indicated that when assessing the seasonal presence of aflatoxin in milk and milk-based products, contamination levels detected during the winter season were significantly higher than those observed in the summer season (Tajkarimi et al., 2008; Fallah, 2010a; Iqbal et al., 2013; Tomašević et al., 2015). Therefore, future studies are recommended to adopt a broader and more stratified sampling strategy and investigate variability across different seasons and geographic regions.

Regarding the analytical methodology, another significant limitation of this study is the lower recovery rates in butter samples compared to those in milk. Furthermore, the lack of comprehensive measurement uncertainty calculations necessary to fully support the validity of the method is also considered a limitation of this study.

6. Conclusion

The combination of QuEChERS-based extraction and KOBRA CELL®-assisted high-performance liquid chromatography (HPLC-FLD) with fluorescence detection offers a potential analytical approach for the determination of five aflatoxins in milk and butter. QuEChERS simplifies sample preparation by reducing extraction time and solvent consumption and is relatively compatible with complex matrices such as milk and butter. In particular, when combined with HPLC-FLD with post-column bromination using KOBRA CELL®, the method allows the measurement of multiple aflatoxins (AFTM1, AFTB1, AFTB2, AFTG1, and AFTG2). The fluorescence enhancement provided by KOBRA CELL® significantly increases the detectability of AFTB1 and AFTG1, which have lower fluorescence sensitivity without derivatization. This integrated approach could offer a more stable and practical alternative to techniques such as ELISA and LC-MS/MS used in aflatoxin analysis. Unlike ELISA, which suffers from matrix interference and cross-reactivity issues, this method allows accurate analysis at regulatory limit levels (ng/g) while maintaining operational simplicity. In contrast to the high cost and solvent

density of LC-MS/MS, it reduces extraction steps, enabling the use of fewer volumes and fewer variety of chemicals. These advantages of the modified QuEChERS procedure suggest that the method may have the potential to be integrated with higher-sensitivity detectors, such as LC-MS/MS, in the future. However, since recovery rates in butter samples are not as high as in milk samples, this requires consideration of method limitations in the butter matrix. Therefore, further studies on reducing the matrix effect and increasing recovery in butter are recommended.

CRediT authorship contribution statement

Gokbulut Cengiz: Writing – review & editing, Validation, Supervision, Software, Project administration, Methodology, Conceptualization. **Busra Aslan Akyol:** Writing – original draft, Methodology, Investigation.

Funding Declaration

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.108794.

Data availability

Data will be made available on request.

References

Aiko, V., Mehta, A., 2015. Occurrence, detection and detoxification of mycotoxins. J. Biosci. 40, 943–954. https://doi.org/10.1007/s12038-015-9569-6.

AlFaris, N.A., Wabaidur, S.M., Alothman, Z.A., Altamimi, J.Z., Aldayel, T.S., 2020. Fast and efficient immunoaffinity column cleanup and liquid chromatography–tandem mass spectrometry method for the quantitative analysis of aflatoxins in baby food and feeds. J. Sep. Sci. 43, 2079–2087. https://doi.org/10.1002/jssc.201901307.

Ammida, N.H., Micheli, L., Palleschi, G., 2004. Electrochemical immunosensor for determination of aflatoxin B1 in barley. Anal. Chim. Acta 520, 159–164. https://doi.org/10.1016/j.aca.2004.04.024.

Amoli-Diva, M., Taherimaslak, Z., Allahyari, M., Pourghazi, K., Manafi, M.H., 2015. Application of DLLME coupled with VA-MNP-SPE for determination of aflatoxin M1 in milk samples. Talanta 134, 98–104. https://doi.org/10.1016/j.talanta.2014.11.007.

Anastassiades, M., Lehotay, S.J., Štajnbaher, D., Schenck, F.J., 2003. Fast and easy multiresidue method using acetonitrile extraction/partitioning and dispersive-SPE for pesticides in produce. J. AOAC Int. 86, 412–431. https://doi.org/10.1093/jaoac/86.2.412.

Arghavan, B., Kordkatuli, K., Mardani, H., Jafari, A., 2025. A comprehensive systematic review and meta-analysis on the prevalence of Aflatoxin M1 in dairy products in selected Middle East Countries. Vet. Med. Sci. 11 (1), e70204. https://doi.org/10.1002/vms3.70204.

Aycicek, H., Aksoy, A., Saygi, S., 2005. Determination of aflatoxin levels in some dairy and food products which consumed in Ankara, Turkey. Food Control 16 (3), 263–266. https://doi.org/10.1016/j.foodcont.2004.03.004.

Bakirci, I., 2001. A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. Food Control 12, 47–51. https://doi.org/10.1016/S0956-7135(00)00020-7.

Behfar, A., Khorasgani, Z.N., Alemzadeh, Z., Goudarzi, M., Ebrahimi, R., Tarhani, N., 2012. Determination of aflatoxin M1 levels in pasteurized milk in Ahvaz City using HPLC. Jundishapur J. Nat. Pharm. Prod. 7, 80.

Biancardi, A., Piro, R., Dall'asta, C., Galaverna, G., 2013. LC–MS/MS method for determination of aflatoxin M1 in milk. Food Addit. Contam. Part A 30, 381–388. https://doi.org/10.1080/19440049.2012.743041.

Bianco, G., Russo, R., Marzocco, S., Velotto, S., Autore, G., Severino, L., 2012. Modulation of macrophage activity by aflatoxins B1 and B2 and their metabolites aflatoxins M1 and M2. Toxicon 59, 644–650. https://doi.org/10.1016/j.toxicon.2012.02.010.

Bognanno, M., La Fauci, L., Ritieni, A., Tafuri, A., De Lorenzo, A., Micari, P., 2006. Survey of aflatoxin M1 in ovine milk by HPLC and confirmation by MS. Mol. Nutr. Food Res. 50, 300–305. https://doi.org/10.1002/mnfr.200500224.

Boudra, H., Barnouin, J., Dragacci, S., Morgavi, D.P., 2007. Aflatoxin M1 and ochratoxin A in raw bulk milk from French dairy herds. J. Dairy Sci. 90, 3197–3201. https://doi.org/10.3168/jds.2006-565.

Campone, L., Piccinelli, A.L., Celano, R., Russo, M., Rastrelli, L., 2013. Rapid analysis of aflatoxin M1 in milk using DLLME–UHPLC–MS/MS. Anal. Bioanal. Chem. 405, 8645–8652. https://doi.org/10.1007/s00216-013-7277-3.

Cano-Sancho, G., Marin, S., Ramos, A.J., Peris-Vicente, J., Sanchis, V., 2010. Occurrence of aflatoxin M1 and exposure assessment in Catalonia (Spain). Rev. Iberoam. Micol. 27, 130–135. https://doi.org/10.1016/j.riam.2010.05.003.

Cao, X., Li, X., Li, J., Niu, Y., Shi, L., Fang, Z., 2018. Determination of mycotoxins in biological matrices and animal-derived foods by HPLC–MS/MS. J. Chromatogr. B. 1073, 191–200. https://doi.org/10.1016/j.jchromb.2017.10.006.

Chavarría, G., Granados-Chinchilla, F., Alfaro-Cascante, M., Molina, A., 2015. Detection of aflatoxin M1 in dairy products from Costa Rica using enzyme-assisted extraction and HPLC. Food Addit. Contam. Part B 8, 128–135. https://doi.org/10.1080/19393210.2015.1015176.

Chen, C.Y., Li, W.J., Peng, K.Y., 2005. Determination of aflatoxin M1 in milk and milk powder by SPE–LC–MS/MS. J. Agric. Food Chem. 53, 8474–8480. https://doi.org/10.1021/jf052142o.

Cherif, K.T., Peris-Vicente, J., Carda-Broch, S., Esteve-Romero, J., 2015. Analysis of fluoroquinolones in honey using micellar LC and validation per 2002/657/EC. Anal. Methods 7, 6165–6172. https://doi.org/10.1039/c5ay01241d.

Commission Regulation European Commission. (EC). (2006) No 1881/2006: EU Directive 2002/32 and EU Recommendation 2006/576/EC. Retrieved from ⟨https://eur-lex.europa.eu/eli/reg/2006/1881/oj/eng⟩. Accessed November 29, 2025.

Creppy, E.E., 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol. Lett. 127, 19–28. https://doi.org/10.1016/S0378-4274(01)00479-9.

Cullen, J.M., Ruebner, B.H., Hsieh, L.S., Hyde, D.M., Hsieh, D.P., 1987. Carcinogenicity of dietary aflatoxin M1 in male Fischer rats compared to aflatoxin B1. Cancer Res. 47, 1913–1917.

Eker, F.Y., Muratoglu, K., Eser, A.G., 2019. Detection of aflatoxin M1 in milk and milk products in Turkey. Environ. Monit. Assess. 191, 523. https://doi.org/10.1007/s10661-019-7668-9.

European Commission, 2021. Commission implementing regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing decisions 2002/657/EC and 98/179/EC. Off. J. In: Eur. Union, 180, pp. 84–109. ⟨https://eur-lex.europa.eu/eli/reg_impl/2021/808/oj/eng/⟩. Accessed November 29, 2025.

Fallah, A.A., 2010a. Aflatoxin M1 contamination in dairy products marketed in Iran during winter and summer. Food Control 21, 1478–1481. https://doi.org/10.1016/j.foodcont.2010.04.017.

Fallah, A.A., 2010b. Assessment of aflatoxin M1 contamination in pasteurized and UHT milk marketed in central part of Iran. Food Chem. Toxicol. 48, 988–991. https://doi.org/10.1016/j.fct.2010.01.014.

Fink-Gremmels, J., 1999. Mycotoxins: their implications for human and animal health. Vet. Q. 21, 115–120. https://doi.org/10.1080/01652176.1999.9695005.

Food and Drug Administration. (FDA). (2000). Guidance for industry: Action levels for poisonous or deleterious substances in human food and animal feed. Retrieved from ⟨https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-action-levels-poisonous-or-deleterious-substances-human-food-and-animal-feed#afla⟩. Accessed November 29, 2025.

Food and Drug Administration. (FDA). (2019). FDA Sec. 683.100 Action Levels for Aflatoxins in Animal Food, Compliance Policy Guide 03.2019. Retrieved from ⟨https://www.fda.gov/media/121202/download⟩. Accessed November 29, 2025.

Frenich, A.G., Romero-González, R., Gómez-Pérez, M.L., Vidal, J.L.M., 2011. Multi-mycotoxin analysis in eggs using QuEChERS-based extraction and UHPLC–MS/MS. J. Chromatogr. A. 1218, 4349–4356. https://doi.org/10.1016/j.chroma.2011.05.005.

Fuchs, B., Süß, R., Teuber, K., Eibisch, M., Schiller, J., 2011. Lipid analysis by thin-layer chromatography—a review. J. Chromatogr. A. 1218, 2754–2774. https://doi.org/10.1016/j.chroma.2010.11.066.

Global Agricultural Information Network. (GAIN). (2019). China notifies standard for maximum levels of mycotoxins in foods. Retrieved from ⟨https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=China%20Notifies%20Standard%20for%20Maximum%20Levels%20of%20Mycotoxins%20in%20Foods%20_Beijing_China%20-%20Peoples%20Republic%20of_6-26-2019⟩. Accessed November 29, 2025.

Goto, T., Matsui, M., Kitsuwa, T., 1988. Determination of aflatoxins by capillary column gas chromatography. J. Chromatogr. 447, 410–414. https://doi.org/10.1016/S0021-9673(01)91503-1.

Govaris, A., Roussi, V., Koidis, P.A., Botsoglou, N.A., 2001. Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. Food Addit. Contam. 18, 437–443. https://doi.org/10.1080/02652030120550.

Gürbay, A., Aydın, S., Girgin, Engin, A.B., Şahin, G., 2006. Assessment of aflatoxin M1 levels in milk in Ankara, Turkey. Food Control 17, 1–4. https://doi.org/10.1016/j.foodcont.2004.07.008.

Hamed, A.M., Abdel-Hamid, M., Gámiz-Gracia, L., García-Campaña, A.M., Arroyo-Manzanares, N., 2019. Determination of aflatoxins in plant-based milk and dairy products by DLLME and HPLC-FLD. Anal. Lett. 52, 363–372. https://doi.org/10.1080/00032719.2018.1467434.

Harris, D.C. (2007). Quantitative Chemical Analysis (7th ed.). W.H. Freeman and Company (Chapter 25).
Harvey, R.B., Kubena, L.F., Corrier, D.E., Huff, W.E., Rottinghaus, G.E., 1990. Cutaneous ulceration and necrosis in pigs fed aflatoxin-and T-2 toxin-contaminated diets. J. Vet. Diagn. Invest 2, 227–229. https://doi.org/10.1177/104063879000200314.
Hashemi, M., Taherimaslak, Z., 2014. Determination of aflatoxin M1 in liquid milk using HPLC-FLD after magnetic SPE. RSC Adv. 4, 33497–33506. https://doi.org/10.1039/c4ra04254a.
Herzallah, S.M., 2009. Determination of aflatoxins in eggs, milk, meat and meat products using HPLC-FLD and UV. Food Chem. 114, 1141–1146. https://doi.org/10.1016/j.foodchem.2008.10.077.
Huang, L.C., Zheng, N., Zheng, B.Q., Wen, F., Cheng, J.B., Han, R.W., 2014. Simultaneous determination of AFTM1, OTA, ZEN and α-ZEL in milk by UHPLC–MS/MS. Food Chem. 146, 242–249. https://doi.org/10.1016/j.foodchem.2013.09.047.
Hussain, I., 2011. Aflatoxin measurement and analysis. In: Irineo, T.P. (Ed.), Aflatoxins–detection, Measurement and Control. InTech Open, pp. 129–146.
Hussain, S.P., Schwank, J., Staib, F., Wang, X.W., Harris, C.C., 2007. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 26, 2166–2176. https://doi.org/10.1038/sj.onc.1210279.
Hussein, H.S., Brasel, J.M., 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicol 167, 101–134. https://doi.org/10.1016/S0300-483X(01)00471-1.
Iha, M.H., Barbosa, C.B., Okada, I.A., Trucksess, M.W., 2013. Aflatoxin M1 in milk and distribution and stability during yoghurt and cheese production and storage. Food Control 29, 1–6. https://doi.org/10.1016/j.foodcont.2012.05.058.
International Agency for Research on Cancer. (IARC). (1993). Monographs on the evaluation of carcinogenic risks to humans, Some Naurally Occurring Substances: Food items and Constituents, Heterocyclic amines and mycotoxins, Volume 56. Retrieved from IARC Publications Website - Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Accessed November 29, 2025.
Iqbal, S.Z., 2021. Mycotoxins in food, recent development in food analysis and future challenges; a review. Curr. Opin. Food Sci. 42, 237–247. https://doi.org/10.1016/j.cofs.2021.07.003.
Iqbal, S.Z., Asi, M.R., Jinap, S.J.F.C., 2013. Variation of aflatoxin M1 contamination in milk and milk products collected during winter and summer seasons. Food Control 34, 714–718. https://doi.org/10.1016/j.foodcont.2013.06.009.
Iqbal, S.Z., Jinap, S., Pirouz, A.A., Faizal, A.A., 2015. Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: a review. Trends Food Sci. Technol. 46, 110–119. https://doi.org/10.1016/j.tifs.2015.08.005.
JECFA. (2001). Safety Evaluation of Certain Mycotoxins in Food Prepared by the 56th meeting of the JECFA (Joint FAO/WHO Expert Committee on Food Additives) – FAO Food and Nutrition Paper 74/WHO Foods Additives Series 47. Retrieved from ⟨https://openknowledge.fao.org/server/api/core/bitstreams/2b67f5b0-1abe-4980-a49d-3c31097a32dc/content⟩. Accessed November 29, 2025.
Kafle, D., Sedai, K.P., Rai, B.P., Pokharel, B.B., 2012. Study on the level of aflatoxin M1 contamination in raw and processed milk marketed in Kathmandu Valley. J. Food Sci. Technol. Nepal 7, 52–56. https://doi.org/10.3126/jfstn.v7i0.10598.
Karaseva, N.M., Amelin, V.G., Tret'yakov, A.V., 2014. QuEChERS coupled to dispersive liquid-liquid microextraction for the determination of aflatoxins B1 and M1 in dairy foods by HPLC. J. Anal. Chem. 69, 461–466. https://doi.org/10.1134/S1061934814030071.
Kim, E.K., Shon, D.H., Ryu, D., Park, J.W., Hwang, H.J., Kim, Y.B., 2000. Occurrence of aflatoxin M1 in Korean dairy products determined by ELISA and HPLC. Food Addit. Contam. 17, 59–64. https://doi.org/10.1080/026520300283595.
Korde, A., Pandey, U., Banerjee, S., Sarma, H.D., Hajare, S., Venkatesh, M., Pillai, M.R.A., 2003. Development of a radioimmunoassay procedure for aflatoxin B1 measurement. J. Agric. Food Chem. 51, 843–846. https://doi.org/10.1021/jf025570s.
Kuiper-Goodman, T., 1990. Uncertainties in the risk assessment of three mycotoxins: aflatoxin, ochratoxin, and zearalenone. Can. J. Physiol. Pharmacol. 68, 1017–1024. https://doi.org/10.1139/y90-155.
Kuiper-Goodman, T., 1995. Mycotoxins: risk assessment and legislation. Toxicol. Lett. 82, 853–859. https://doi.org/10.1016/0378-4274(95)03599-0.
Kurtzman, C.P., Horn, B.W., Hesseltine, C.W., 1987. Aspergillus nomius, a new aflatoxin-producing species related to Aspergillus flavus and Aspergillus tamarii. Antonie Leeuwenhoek 53, 147–158. https://doi.org/10.1007/BF00393843.
Lesueur, C., Knittl, P., Gartner, M., Mentler, A., Fuerhacker, M., 2008. Analysis of 140 pesticides from conventional farming foodstuff samples after extraction with the modified QuEChERS method. Food Control 19, 906–914. https://doi.org/10.1016/j.foodcont.2007.09.002.
Lin, R., Yuan, J., Feng, H., Liu, Y., Fan, Y., Tan, Y., Tang, Z., 2025. Comprehensive overview: QuEChERS methods for mycotoxin determination in different matrices. Food Addit. Contam. Part A 130. https://doi.org/10.1080/19440049.2025.2572012.
Liu, Y., Han, S., Lu, M., Wang, P., Han, J., Wang, J., 2014. Modified QuEChERS method combined with ultra-high performance liquid chromatography tandem mass spectrometry for the simultaneous determination of 26 mycotoxins in sesame butter. J. Chromatogr. B 970, 68–76. https://doi.org/10.1016/j.jchromb.2014.06.033.
Liu, Y., Wang, W., 2016. Aflatoxin B1 impairs mitochondrial functions, activates ROS generation, induces apoptosis and involves Nrf2 signal pathway in primary broiler hepatocytes. Anim. Sci. J. 87, 1490–1500. https://doi.org/10.1111/asj.12550.
Malissiova, E., Tsinopoulou, G., Gerovasileiou, E.S., Meleti, E., Soultani, G., Koureas, M., Manouras, A., 2024. A 20-year data review on the occurrence of aflatoxin M1 in Milk and dairy products in Mediterranean countries—current situation and exposure risks. Dairy 5, 491–514. https://doi.org/10.3390/dairy5030038.
Manetta, A.C., Di Giuseppe, L., Giammarco, M., Fusaro, I., Simonella, A., Gramenzi, A., Formigoni, A., 2005. HPLC with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M1 in milk and cheese. J. Chromatogr. A. 1083, 219–222. https://doi.org/10.1016/j.chroma.2005.06.039.
Michlig, N., Repetti, M.R., Chiericatti, C., García, S.R., Gaggiotti, M., Basílico, J.C., Beldoménico, H.R., 2016. Multiclass compatible sample preparation for UHPLC–MS/MS determination of aflatoxin M1 in raw milk. Chromatographia 79, 1091–1100. https://doi.org/10.1007/s10337-015-2972-1.
Morais, D.N., Massarolo, K.C., Ardohain, E.N., Lima, J.F., Ferreira, F.D., Drunkler, D.A., 2024. Method for determination of multi-mycotoxins in milk: QuEChERS extraction modified followed by HPLC-FL analysis. Food Anal. Methods 17, 47–60. https://doi.org/10.1007/s12161-023-02550-0.
Moss, M.O., 2002. Risk assessment for aflatoxins in foodstuffs. Int. Biodeterior. Biodegrad. 50, 137–142. https://doi.org/10.1016/S0964-8305(02)00078-1.
Mulunda, M., Mike, D., 2014. Occurrence of aflatoxin M1 from rural subsistence and commercial farms from selected areas of South Africa. Food Control 39, 92–96. https://doi.org/10.1016/j.foodcont.2013.11.011.
Navas, S.A., Sabino, M., Rodriguez-Amaya, D.B., 2005. Aflatoxin M1 and ochratoxin A in a human milk bank in the city of Sao Paulo, Brazil. Food Addit. Contam. 22, 457–462. https://doi.org/10.1080/02652030500110550.
Öztürk Yılmaz, S., Altinci, A., 2018. Incidence of aflatoxin M1 contamination in milk, white cheese, kashar and butter from Sakarya, Turkey. Food Sci. Technol. 39 (. 1), 190–194. https://doi.org/10.1590/fst.40817.
Paniel, N., Radoi, A., Marty, J.L., 2010. Development of an electrochemical biosensor for the detection of aflatoxin M1 in milk. Sensors 10, 9439–9448. https://doi.org/10.3390/s101009439.
Peng, X., Bai, S., Ding, X., Zhang, K., 2017. Pathological impairment, cell cycle arrest and apoptosis of thymus and bursa of fabricius induced by aflatoxin-contaminated corn in Broilers. Int. J. Environ. Res. Public Health 14, 77. https://doi.org/10.3390/ijerph14010077.
Pirestani, A., Tabatabaei, S.N., Fazeli, M.H., Antikchi, M., Baabaei, M., 2011. Comparison of HPLC and ELISA for determination of aflatoxin concentration in the milk and feeds of dairy cattle. J. Res. Agric. Sci. 7, 71–78.
Pisoschi, A.M., Iordache, F., Stanca, L., Petcu, A.I., Purdoiu, L., Geicu, O.I., Serban, A.I., 2023. Comprehensive overview and critical perspective on the analytical techniques applied to aflatoxin determination – a review paper. Microchem. J. 191, 108770. https://doi.org/10.1016/j.microc.2023.108770.
Pittet, A., 2005. Modern methods and trends in mycotoxin analysis. Mitt. Lebensm. Hyg. 96, 424–444.
R-Biopharm. (2022). Retrieved from ⟨https://food.r-biopharm.com/wp-content/uploads/k01_kobra-cell_ifu_v25_2022-07.pdf⟩. Accessed November 29, 2025.
Rezende, C.P., Souza, L.F., Almeida, M.P., Dias, P.G., Diniz, M.H., Garcia, J.C., 2012. Optimisation and validation of a quantitative and confirmatory method for residues of macrolide antibiotics and lincomycin in kidney by LC-MS. Food Addit. Contam. Part A 29, 587–595. https://doi.org/10.1080/19440049.2011.652196.
Rodriguez Velasco, M.L., Calonge Delso, M.M., Ordonez Escudero, D., 2003. ELISA and HPLC determination of the occurrence of aflatoxin M1 in raw cow's milk. Food Addit. Contam. 20, 276–280. https://doi.org/10.1080/0265203021000045208.
Rodríguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G., Mañes, J., Ritieni, A., 2018. Simultaneous determination of AFB1 and AFM1 in milk samples by ultra high performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry. Beverages 4, 43. https://doi.org/10.3390/beverages4020043.
Sadighara, P., Basaran, B., Afshar, A., Nazmara, S., 2024. Optimization of clean-up in QuEChERS method for extraction of mycotoxins in food samples: a systematic review. Microchem. J. 197, 109711. https://doi.org/10.1016/j.microc.2023.109711.
Sahin, H.Z., Celik, M., Kotay, S., Kabak, B., 2016. Aflatoxins in dairy cow feed, raw milk and milk products from Turkey. Food Addit. Contam. Part B 9, 152–158. https://doi.org/10.1080/19393210.2016.1152599.
Sakuma, H., Kamata, Y., Sugita-Konishi, Y., Kawakami, H., 2011. Method for determination of aflatoxin M1 in cheese and butter by HPLC using an immunoaffinity column. Food Hyg. Saf. Sci. 52, 220–225. https://doi.org/10.3358/shokueishi.52.220.
Sartori, A.V., Swensson de Mattos, J., de Moraes, M.H.P., da Nóbrega, A.W., 2015. Determination of aflatoxins M1, M2, B1, B2, G1, G2 and ochratoxin A in UHT and powdered milk by modified QuEChERS method and UHPLC-MS/MS. Food Anal. Methods 8, 2321–2330. https://doi.org/10.1007/s12161-015-0128-4.
Schincaglia, A., Aspromonte, J., Franchina, F.A., Chenet, T., Pasti, L., Cavazzini, A., Beccaria, M., 2023. Current developments of analytical methodologies for aflatoxins' determination in food during the last decade (2013–2022), with a particular focus on nuts and nut products. Foods 12 (3), 527. https://doi.org/10.3390/foods12030527.
Şenyuva, H.Z., Gilbert, J., 2010. Immunoaffinity column clean-up techniques in food analysis: a review. J. Chromatogr. B 878, 115–132. https://doi.org/10.1016/j.jchromb.2009.05.042.
Seo, H., Jang, S., Jo, H., Kim, H., Lee, S., Yun, H., Cho, H., 2021. Optimization of the QuEChERS-based analytical method for investigation of 11 mycotoxin residues in feed ingredients and compound feeds. Toxins 13, 767. https://doi.org/10.3390/toxins13110767.
Sheibani, A., Tabrizchi, M., Ghaziaskar, H.S., 2008. Determination of aflatoxins B1 and B2 using ion mobility spectrometry. Talanta. https://doi.org/10.1016/j.talanta.2007.11.006.
Shephard, G.S., 2009. Aflatoxin analysis at the beginning of the twenty-first century. Anal. Bioanal. Chem. 395, 1215–1224. https://doi.org/10.1007/s00216-009-2857-y.
Sherma, J., 2000. Thin-layer chromatography in food and agricultural analysis. J. Chromatogr. A. 880, 129–147. https://doi.org/10.1016/S0021-9673(99)01132-2.

Shuib, N.S., Saad, B., 2022. In-syringe dispersive micro-solid phase extraction method for the HPLC-fluorescence determination of aflatoxins in milk. Food Control 132, 108510. https://doi.org/10.1016/j.foodcont.2021.108510.

Sørensen, L.K., Elbæk, T.H., 2005. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. J. Chromatogr. B. 820, 183–196. https://doi.org/10.1016/j.jchromb.2005.03.020.

Stroka, J., Anklam, E., 2002. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. TrAC. Trends Anal. Chem. 21, 90–95. https://doi.org/10.1016/S0165-9936(01)00133-9.

Stroka, J., Maragos, C.M., 2016. Challenges in the analysis of multiple mycotoxins. World Mycotoxin J. 9, 847–862. https://doi.org/10.3920/WMJ2016.2038.

Sun, J., Li, W., Zhang, Y., Hu, X., Wu, L., Wang, B., 2016. QuEChERS purification combined with ultrahigh-performance liquid chromatography tandem mass spectrometry for simultaneous quantification of 25 mycotoxins in cereals. Toxins 8, 375. https://doi.org/10.3390/toxins8120375.

Tajkarimi, M., Aliabadi-Sh, F., Nejad, A.S., Poursoltani, H., Motallebi, A.A., Mahdavi, H., 2008. Aflatoxin M1 contamination in winter and summer milk in 14 states in Iran. Food Control 19, 1033–1036. https://doi.org/10.1016/j.foodcont.2007.10.011.

Tarannum, N., Nipa, M.N., Das, S., Parveen, S., 2020. Aflatoxin M1 detection by ELISA in raw and processed milk in Bangladesh. Toxicol. Rep. 7, 1339–1343. https://doi.org/10.1016/j.toxrep.2020.09.012.

Tekinşen, K.K., Uçar, G., 2008. Aflatoxin M1 levels in butter and cream cheese consumed in Turkey. Food Sci. Technol. 39 (. 1), 27–30. https://doi.org/10.1016/j.foodcont.2007.01.003.

Temamogullari, F., Kanici, A., 2014. Aflatoxin M1 in dairy products sold in Şanlıurfa, Turkey. J. Dairy. Sci. 97, 162–165. https://doi.org/10.3168/jds.2012-6184.

Tomašević, I., Petrović, J., Jovetić, M., Raičević, S., Milojević, M., Miočinović, J., 2015. Two year survey on the occurrence and seasonal variation of aflatoxin M1 in milk and milk products in Serbia. Food Control 56, 64–70. https://doi.org/10.1016/j.foodcont.2015.03.017.

Turkish Food Codex. (TFC). (2011). Turkish Food Codex Contaminants Regulations. Retrieved from ⟨https://www.resmigazete.gov.tr/eskiler/2011/12/20111229m3-8.htm⟩. Accessed November 29, 2025.

Turna, N.S., Wu, F., 2021. Aflatoxin M1 in milk: a global occurrence, intake, & exposure assessment. Trends Food Sci. Technol. 110, 183–192. https://doi.org/10.1016/j.tifs.2021.01.093.

Turner, N.W., Subrahmanyam, S., Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: a review. Anal. Chim. Acta 632, 168–180. https://doi.org/10.1016/j.aca.2008.11.010.

Unusan, N., 2006. Occurrence of aflatoxin M1 in UHT milk in Turkey. Food Chem. Toxicol. 44, 1897–1900. https://doi.org/10.1016/j.fct.2006.06.010.

Wang, J.S., Groopman, J.D., 1999. DNA damage by mycotoxins. Mutat. Res. 424, 167–181. https://doi.org/10.1016/S0027-5107(99)00017-2.

Wang, Y., Liu, X., Xiao, C., Wang, Z., Wang, J., Xiao, H., Yue, T., 2012. HPLC determination of aflatoxin M1 in liquid milk and milk powder using solid phase extraction on OASIS HLB. Food Control 28, 131–134. https://doi.org/10.1016/j.foodcont.2012.04.037.

Wang, H., Zhou, X.J., Liu, Y.Q., Guo, Q.L., 2010. Determination of aflatoxin M1 in milk by triple quadrupole liquid chromatography-tandem mass spectrometry. Food Addit. Contam. 27, 1261–1265. https://doi.org/10.1080/19440049.2010.487501.

Wang, H., Zhou, X.J., Liu, Y.Q., Guo, Q.L., 2011. Simultaneous determination of chloramphenicol and aflatoxin M1 residues in milk by triple quadrupole liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. 59, 3532–3538. https://doi.org/10.1021/jf2006062.

Womack, E.D., Sparks, D.L., Brown, A.E., 2016. Aflatoxin M1 in milk and milk products: a short review. World Mycotoxin J. 9, 305–315. https://doi.org/10.3920/WMJ2014.1867.

Xu, X., Xu, X., Han, M., Qiu, S., Hou, X., 2019. Development of a modified QuEChERS method based on magnetic multiwalled carbon nanotubes for the simultaneous determination of veterinary drugs, pesticides and mycotoxins in eggs by UPLC-MS/MS. Food Chem. 276, 419–426. https://doi.org/10.1016/j.foodchem.2018.10.051.

Yang, X., Liu, B., Zhang, L., Wang, X., Xie, J., Liang, J., 2025. Spatial distribution and dietary risk assessment of aflatoxins in raw milk and dairy feedstuff samples from different climate zones in China. Toxins 17, 41. https://doi.org/10.3390/toxins17010041.

Yi, L., Liu, H., Liu, Y., Aiyisi, He, Ming, J., Jirimutu, L., 2025. Nanobody-based indirect competitive ELISA for the detection of aflatoxin M1 in dairy products. Sci. Rep. 15, 785. https://doi.org/10.1038/s41598-024-83869-4.

Zhang, K., Wong, J.W., Hayward, D.G., Vaclavikova, M., Liao, C.D., Trucksess, M.W., 2013. Determination of mycotoxins in milk-based products and infant formula using stable isotope dilution assay and liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 61, 6265–6273. https://doi.org/10.1021/jf4018838.

Zheng, M.Z., Richard, J.L., Binder, J., 2006. A review of rapid methods for the analysis of mycotoxins. Mycopathologia 161, 261–273. https://doi.org/10.1007/s11046-006-0215-6.