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Development and validation of HPLC-FLD method for aflatoxin M_1 determination in milk and dairy products

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Abstract: Milk and dairy products are the most consumed foods in human diet and their safety is in the attention centre of control authorities. Aflatoxin M_1 (AFM_1) is a dangerous toxin that can occur in milk and dairy products as a metabolite formed from aflatoxin B_1 contained in contaminated animal feed. Therefore, the aim of this study was to develop and validate a reliable method for the determination of AFM_1 content in milk and dairy products based on HPLC with fluorescence detection employing immunoaffinity columns (IAC) pre-treatment. Optimal chromatographic separation of AFM_1 was achieved using a water/acetonitrile mixture (80/20, v/v) as a mobile phase, column with C_{18} stationary phase maintained at 25 °C, and fluorescence detection at excitation wavelengths of 360 nm and emission of 440 nm. Efficacy of AFM_1 extraction from the samples was found to be influenced by the elution agent composition. The best results were obtained using 1.25 mL of acetonitrile/methanol (3/2, v/v) and 1.25 mL of water. Validation parameters of the proposed method met the criteria set by the European legislation with the limits of detection and quantification at 0.002 and 0.007 µg/kg, respectively. Also, suitability of the method was confirmed by its application for AFM_1 determination in certified reference material. Finally, the method was applied for AFM_1 determination in 25 milk and dairy products collected in Slovakia; the AFM_1 content was below the limit of quantification. It was concluded that the method is suitable for AFM_1 content monitoring in milk and dairy products.

Keywords: aflatoxin M_1, HPLC, immunoaffinity chromatography, milk, dairy products, validation, optimisation, food safety

Introduction

Aflatoxins are low molecular weight secondary metabolites mainly generated by fungal species of *Aspergillus flavus*, *Aspergillus parasiticus*, or *Aspergillus nominus*. These fungi produce more than 20 toxic metabolites, however, in terms of food safety, the most relevant are aflatoxin B_1 and B_2 (AFB_1, AFB_2), aflatoxin G_1 and G_2 (AFG_1, AFG_2), and aflatoxin M_1 (AFM_1) (Buzás et al., 2023). Aflatoxins were discovered in England in 1960, when a severe outbreak of turkey *'X'* disease resulted in the death of more than 100,000 turkeys (Bellio et al., 2016; Pickova et al., 2021). Aflatoxins are chemical substances without any smell or taste, they are fluorescent in ultraviolet light and withstand temperatures above 320 °C without decomposing (Maggira et al., 2021).

According to the US Food and Drug Administration (FDA), AFB_1 is one of the most dangerous unavoidable food contaminants with serious adverse effects such as acute illness and death, liver cancer, nutritional interference, or immunologic suppression. AFM_1 is metabolised in liver from AFB_1 after the consumption of contaminated food/feed and then excreted into milk of lactating animals (Murphy et al., 2006; Šimko and Kolarič, 2022). Aflatoxins have been classified into group 1 (human carcinogens) by the International Agency for Research on Cancer (IARC) (Silva et al., 2023). Therefore, some countries have limited the maximum acceptable limits for AFM_1 in milk: 0.5 µg/kg in the USA and 0.05 µg/kg in the EU for adult food and 0.025 µg/kg for infant food (Šimko and Kolarič, 2022).

According to Pickova et al. (2021) approximately 4.5 billion people have been chronically exposed to aflatoxins through contaminated food. The latest risk assessment of aflatoxins in food published by the European Food and Safety Authority (EFSA) panel (EFSA CONTAM Panel, 2020) revealed that grains and grain-based products make the largest contribution to AFB_1 exposure, while liquid milk and fermented milk products are the main contributors to the mean exposure of AFM_1. In milk, AFM_1 binds strongly to casein, and higher levels of AFM_1 can be expected in dairy products high in protein, such as cheese. Furthermore, traditional milk manufacturing processes, such as pasteurisation or sterilisation, do not destroy its molecule (Silva et al., 2023). The contamination with aflatoxins occurs mainly after harvesting and during inappropriate management of transport and storage, e.g., at high humidity (>65 %) and temperature (Azam et al., 2021). In the past, aflatoxin outbreaks were mainly the problem of tropical and subtropical areas. Until 2004, aflatoxin

contamination in the EU was confined only in imported foods. However, in the last 15 years, several hot and dry seasons have led to severe infections with *Aspergillus flavus* in maize in several countries in Europe, including Italy, Romania, Serbia, and Spain. Regarding the recent climate change problems, in the +2 °C scenario, increased probability in aflatoxin risk in many areas throughout Europe is expected (Moretti et al., 2019).

Thus, a wide range of analytical methods have been developed for aflatoxins analysis, with the most applied methods being liquid chromatography (LC) combined with fluorescence detection (FLD) or mass spectrometry (MS); for the determination of AFM_1 content, the most common methods include enzyme-linked immunosorbent assays (ELISA) or LC with FLD or MS detection (EFSA CONTAM Panel, 2020). In general, chromatographic methods are mainly used to confirm the results obtained from rapid screening tests or for precise quantitative determination of aflatoxins content. Immunochemical methods are used for rapid screening of aflatoxins in various sample matrices (Bellio et al., 2016). High-performance liquid chromatography (HPLC) with FLD detection using eventual post-column derivatisation is a reference method and currently the most often applied method for qualitative and quantitative determination of AFM_1 content in milk (Maggira et al., 2021). The methodology of AFM_1 analysis in milk has been improved by the application of immunoaffinity column technology (IAC), which provides a combination of extraction and clean-up stages of the analysis. Previously, liquid-liquid extraction or solid phase extraction (SPE) followed by a silica gel column, or another purification step were used (Dragacci et al., 2001). Wang et al. (2012) presented a simple and inexpensive method using SPE for the determination of AFM_1 in liquid milk and milk powder and achieved recoveries between 85.4 and 96.9 %. Moreover, a comparison of SPE and IAC showed that both clean-up methods provided consistent results.

Therefore, considering recent observations, the objective of this study was to validate a rapid and reliable method for quantitative determination of AFM_1 content in milk and dairy products purchased in the Slovak market using HPLC-FLD detection with IAC sample clean-up extraction. During the study, optimal IAC and HPLC conditions were developed and tested employing the analysis of a certified reference material. Finally, obtained results were compared with the rapid screening method MilkSafe™ AflaM_1 applied in the screening of AFM_1 in raw milk in a Slovak dairy company (RAJO s.r.o.).

Material and Methods

Samples and Chemicals

For the determination of AFM_1 content, 25 milk samples were tested: 11 samples of raw milk were obtained from RAJO s.r.o. (Bratislava, Slovakia), and were collected from five different farms in Slovakia (Bukovec, Búč, Sevita, Prašník, and Uherec) during April 2023. Moreover, other four samples of raw milk were purchased from local markets during the summer of 2022. Then, ten samples of milk and dairy products from local markets were examined (UHT homogenised milk with the fat content of 0.5 %, 1.5 %, and 3.5 %; milk drink "Actimel"; Greek yogurt; milk drink "Brejky"; mini creamers; milk dessert "Pribináček"; cooking cream with the fat content of 10 %; curd cheese with the fat content of 0.5 %). Certified reference material (ERM-BD284, whole milk powder) was obtained from the European Commission Joint Research Centre (Belgium). AFM_1 analytical standard solution in acetonitrile (0.5 µg/mL) with the purity of ≥ 99,9 % was obtained from Sigma-Aldrich (USA). Acetonitrile and methanol were of HPLC grade and were purchased from Fisher Chemical (UK) and Lab-Scan Analytical Sciences (Poland).

Optimisation of HPLC conditions

First, HPLC conditions were tested using AFM_1 solution in water/acetonitrile mixture (80/20, v/v) with the concentration of 0.5 µg/kg. The HPLC system (Agilent Technologies 1260 Infinity system, Santa Clara, CA, USA) was equipped with a vacuum degasser, quarterly pump, autosampler, and FLD detector. A Zorbax Eclipse Plus C_{18} column (2.1 × 150 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA) with the guard column Zorbax SB-C_{18} (4.6 × 12.5 mm, 5 µm particle size, Agilent, Santa Clara, CA, USA) were used as the stationary phase. As the mobile phase, three liquids (acetonitrile, methanol, water) were tested in various compositions: 1. water/acetonitrile/methanol (67/25/8, v/v/v); 2. water/acetonitrile/methanol (80/15/5, v/v/v); 3. water/acetonitrile (50/50, v/v); 4. water/acetonitrile (70/30, v/v); 5. water/acetonitrile (80/20, v/v); 6. acetonitrile/methanol (50/50, v/v); 7. water/methanol (80/20, v/v). The separation was carried out in the temperature range of 5—40 °C. Optimisation of FLD detection was made using various excitation wavelengths (260—360 nm) and emission wavelengths (400—460 nm). Injection volume was 50 µL. Flow rate of the mobile phase was set to 0.5 mL/min. The results were recorded using the OpenLab CDS software, ChemStation Edition for LC, and LC/MS systems (product version A.01.08.108).

Optimisation of IAC purification

Extraction of AFM_1 from milk matrix was performed using an IAC column (Vicam, AFM_1) according to the modified procedure of Mendonça and Venâncio (2005) with the variables being the type of elution solvent and volume as follows: A. 1.25 mL of water/acetonitrile 7/3 (v/v) + 1.25 mL of deionised water; B. 3 mL of methanol/acetonitrile 7/3 (v/v); C. 2 mL of acetonitrile/methanol 3/2 + 0.5 mL of deionised water; D. 2 mL of acetonitrile/methanol 3/2 + 2 mL of deionised water; E. 1.25 mL of water/methanol 17/3 (v/v) + 1.25 mL of deionised water; F. 1.25 mL of acetonitrile/methanol 3/2 + 1.25 mL of deionised water. Optimisation measurements were carried out in skim milk matrix enriched with AFM_1 standard to reach the final concentration of 0.1 µg/kg. Then, 50 mL of the tested sample were transferred to the column with a syringe and passed at the slow steady flow rate of 2—3 mL/min in vacuum manifold. The column was then washed twice with 10 mL of deionised water. Elution of the analyte was performed with different solvents and volumes (A—F) and collected in glass cuvettes each, which were vortexed and injected into HPLC.

In-house method validation

The optimised method was validated according to previously published methodology (Kolarič and Šimko, 2020) and the Eurachem guide for The fitness for purpose of analytical methods (Magnusson and Örnemark, 2014) using a certified reference material (ERM-BD284, whole milk powder). The milk reference sample was prepared according to the procedure reported by the manufacturer: 10 g of the sample were weighed in a beaker, 50 mL of pre-warmed water were added at 50 °C, and the mixture was mixed until a homogeneous mixture was obtained. The solution was cooled to 20 °C and quantitatively transferred to a 100 mL volumetric flask. The reference value of AFM_1 was reported at 0.44 µg/kg.

Linearity was recorded using the correlation coefficient (R^2) of the calibration curve calculated by FLD signal plotting against a known amount of AFM_1 standards at 0.003, 0.005, 0.008, 0.01, 0.015, 0.02, 0.03, 0.05, 0.075, and 0.1 ng. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as 3 and 10 times the standard deviation of the blanks divided by the slope of the calibration curve, respectively. Accuracy of the method was studied as a recovery test in two approaches: 1. standard addition at three concentrations (0.10, 0.30, and 0.60 µg/kg), and 2. comparison of measured concentration with the reference one. Accuracy of the HPLC-FLD method was also compared to that of the screening method used in Rajo s.r.o. For that purpose, raw milk was spiked with four different AFM_1 standard concentrations to obtain AFM_1 concentration equal to 0.03, 0.05, 0.08 or 0.10 µg/kg and analysed by HPLC-FLD detection and MilkSafe™ $AflaM_1$ (Chr. Hansen Holding, Denmark). Precision of the detection was determined by comparing repeatability and intermediate precision. Repeatability was recorded by analysing four replicates of the samples on the same day, and intermediate precision was then evaluated on three different days. Precision was calculated from standard deviation (SD) and relative standard deviation (RSD). The Horwitz ratio (HorRat) was also calculated as the ratio of the observed RSD to the corresponding predicted relative standard deviation calculated from the Horwitz equation, $PRSD_R$ (%) = $2C^{-0.15}$, where C is the concentration expressed as mass fraction (Horwitz and Albert, 2006).

Determination of AFM_1 content in milk and dairy products

The analysis of AFM_1 content in 25 milk and dairy products was performed applying the developed method of HPLC-FLD and IAC extraction described earlier in this study. All high fat samples were first defatted by centrifugation (Hettich Zentrifugen, Tuttlingen, Germany) at 1800 g for 15 min and the upper layer was removed before the extraction.

Statistical analysis

Statistical evaluation was performed using Microsoft Excel 365 (version 2012). All results are expressed as mean ± standard deviation or as a percentage and were repeated at least twice. Data were subjected to a one-way analysis of variance (ANOVA) with the Tukey comparison test, the values were considered significantly different at $p < 0.05$.

Results and discussion

Optimisation of HPLC conditions

In general, HPLC is the most used method for AFM_1 content determination in milk. The main parameters influencing the effectivity and efficiency of liquid chromatography are: mobile phase composition, stationary phase type, or column particle size (Albuquerque et al., 2016). Regarding the characteristics of the AFM_1 molecule, the mobile phase is mostly polar, while the stationary phase is silica, alumina, or polysiloxanes with the most common hydrophobic octadecyl (C_{18}) group (Pisoschi et al., 2023). A comparison of different AFM_1 detection methods, showed that ELISA, HPLC-FLD and simultaneous use of ELISA and HPLC-FLD were used in 55 (76.4 %), 10 (13.9 %),

3 (4.2 %) and 4 (5.5 %) studies, respectively (Massahi et al., 2023).

In this work, three types of liquids were tested as mobile phase components (acetonitrile, methanol, and water). Manetta et al. (2005) used a mobile phase composed of four various liquids: acetic acid/acetonitrile/2-propanol/water (2/10/10/78, v/v/v/v). However, most current methods use the mixtures of methanol, water, and acetonitrile (Vaz et al., 2020). The obtained results relating mobile phase composition can be seen in Fig. 1. The first two mobile phases were the mixtures of all solvents tested. It was found that the resolution of AFM_1 cannot be properly achieved. Chavarría et al. (2015) used ternary mobile phase of water/methanol/acetonitrile (65/25/10, v/v/v) and achieved satisfactory elution of AFM_1 in milk, cheese, and sour cream samples, similarly to Bellio et al. (2016), who used water/methanol/acetonitrile (65/20/15, v/v/v). However, according to Dragacci et al. (2001), most participants in the collaborative trial used the recommended mobile phase (water/acetonitrile 75/25 (v/v)) and only two participants selected a ternary mobile phase. In this study, better resolution of AFM_1 was observed using a binary mobile phase composed of water and acetonitrile. In the equimolar mixture of water and acetonitrile, the elution time of AFM_1 was too short (1.9 min). The best results were obtained with the water to acetonitrile ratio of 70/30 and 80/20 (v/v), respectively. The peaks were sufficiently shaped with appropriate elution time (4.6 min). Finally, it was noticed that the mobile phase composed of water and methanol (80/20, v/v) or acetonitrile and methanol (50/50, v/v) was not suitable for the elution of AFM_1 from IAC extracts. The same suitable composition of the mobile phase (water/acetonitrile 80/20 (v/v)) was used by Jakšić et al. (2021) in the optimisation of HPLC method for AFM_1 detection in cheese. The ratio 75/25 (v/v) of these solvents was applied in the study by Masrouri et al. (2021). According to Shuib et al. (2017), the most common mobile phase used in the determination of AFM_1 by chemical derivatisation is water/methanol/acetonitrile (70/20/10, v/v/v). In their study, acetonitrile was substituted by methanol in different ratios (water/methanol 70/30, 65/35, 60/40, v/v). Among these methanolic mixtures, 65/35 (v/v) produced sharper peaks and improved resolution. Additionally, the retention time for AFM_1 was increased to 9 min compared to 6 min with a mixture of water/methanol/acetonitrile (70/20/10, v/v/v). Chiavaro et al. (2001) reported that the water/methanol mixture (58/42, v/v) resulted in higher response than the acetonitrile/water mixture. However, in both studies, the AFM_1 molecule was derivatised prior to FLD detection. According to Shephard (2009), binary systems of water and methanol resulted in broad HPLC peaks and long chromatographic run times, which was also confirmed in this work. Based on the elution strength, water/acetonitrile mobile phase seems to provide the highest elution strength. The elution strength of water/methanol mixture was lower thus the elution time of AFM_1 was higher. On the contrary, acetonitrile/methanol mixture resulted in higher elution strength than water/acetonitrile, thus the retention of AFM_1 on the stationary phase was weakened and elution time was too low. Similarly, to the ternary mobile phase of water/methanol/acetonitrile, as methanol has lower polarity than acetonitrile, the elution strength of such mixtures was weakened and AFM_1 resolution was not appropriate (Ramis-Ramos et al., 2017).

The second part of the optimisation process of HPLC-FLD detection of AFM_1 was dedicated to monitoring the influence of column temperature and FLD wavelength on the retention time and the area of AFM_1 peak. The results are summarised in Table 1. Temperature of the column statistically affected the area and the elution time of AFM_1. The results for the optimal temperature of 25 °C and other temperatures were statistically different at $p < 0.05$. This is in accordance with the theory of molecular spectroscopy, when intensification of vibration and relaxation processes in the molecules at elevated temperatures compete with fluorescence, thus causing decreased fluorescence output, while at lowered temperatures, the fluorescence output is affected negatively by the lack of energy needed for excitation. In other studies, the results are variable, Jakšić et al. (2021) maintained the column temperature at 30 °C, Bellio et al. (2016) at 35 °C, and Shuib et al. (2017) at 40 °C. However, no study was found to apply temperatures below 20 °C. Aflatoxins are colourless to pale yellow crystals and they fluoresce in ultraviolet light: blue for AFB_1 and AFB_2, green for AFG_1 and AFG_2, and blue violet for AFM_1 (EFSA CONTAM Panel, 2020). The presence of a chromophore in the molecule supports the detection of AFM_1 through fluorescence (Iqbal et al., 2015). Therefore, FLD is the most widely used detection technique for AFM_1 determination, as it provides detection limits at the level of ng/kg, which is recommended according to the Commission Regulation (EC) No. 401/2006 laying down the sampling and analysis methods for official control of mycotoxin levels in foodstuffs. Optimal excitation and emission wavelengths of 360 and 450 nm, respectively, are used in most methods (Pisoschi et al., 2023), which was also confirmed in this study, where the highest signal of the AFM_1 peak was observed

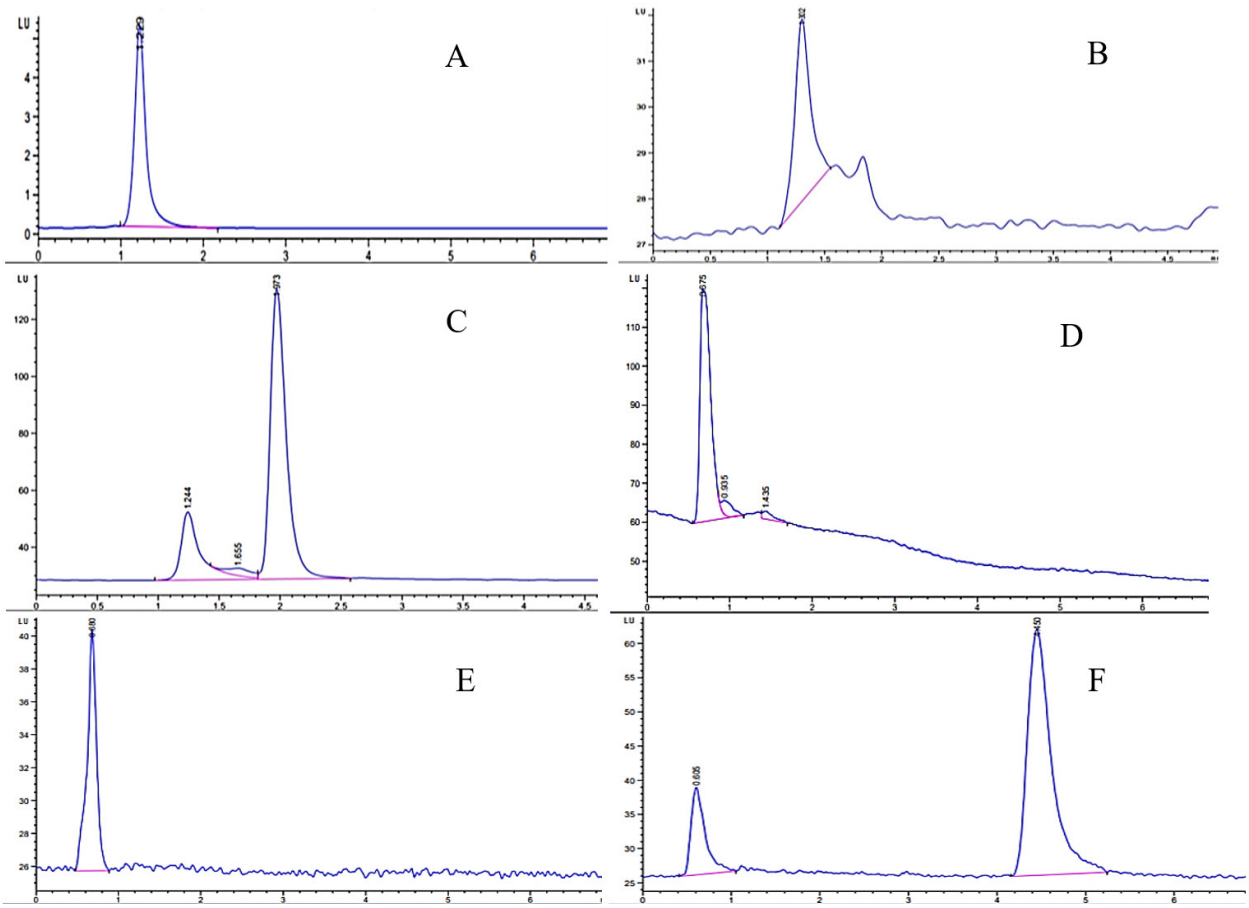

Fig. 1. Selected chromatograms of HPLC-FLD analysis of AFM_1 standard solution (0.5 µg/kg) using various compositions of mobile phase: A — water/acetonitrile/methanol (67/25/8, v/v/v); B — water/acetonitrile/methanol (80/15/5, v/v/v); C — water/acetonitrile (50/50, v/v); D — acetonitrile/methanol (50/50, v/v); E — water/methanol (80/20, v/v); F — water/acetonitrile (80/20, v/v).

at the excitation and emission wavelengths of 360 and 440 nm, respectively. Statistically significant differences were observed at $p < 0.05$ in comparison to the values obtained at other wavelengths.

Therefore, it can be concluded that optimal HPLC-FLD conditions for the analytical standard AFM_1 are: 1. mobile phase — water/acetonitrile (80/20, v/v), 2. — stationary phase — column with C_{18}, 3. — column temperature –25 °C, 4. FLD setting — excitation at 360 nm and emission at 440 nm.

Optimisation of IAC purification

Methods for mycotoxins extraction from milk include techniques such as liquid-liquid extraction (LLE), SPE, or LLE followed by a SPE cleaning step (Aguilera-Luiz et al., 2011). Commonly used purification procedures for the extraction of AFM_1 from milk matrix are IAC or multifunctional one-step cleaning columns, which are very efficient and easy to use providing high selectivity. However, they are only for disposable use due to denaturation of antibodies during the elution step and their costs are high (Vaz et al., 2020). The principle of IAC is the immobilisation of analyte in an antibody (polyclonal or monoclonal) on a gel packed into a small plastic column. The column is initially conditioned with a buffer solution, mostly phosphate buffered saline, and the crude sample extract is applied slowly to the column. After the extract is loaded, the gel is washed and finally the analyte is eluted from the IAC column by breaking the antibody-antigen bond. Liquid matrices are frequently defatted and/or filtrated before passing directly through the IAC because as extraction and purification in an IAC proceed simultaneously. In case of solid matrices (e.g., cheese), solvent extraction is required, which is usually carried out in a blender or by shaking. Elution solvents need to be aqueous to be compatible with the IAC, so methanol/water and acetonitrile/water combinations are mostly applied (Şenyuva and Gilbert, 2010).

In this work, it was found that the type of elution solvent significantly affects the recovery of AFM_1 from the milk matrix (Table 2). The combination of acetonitrile and methanol without the addition of water (solvent B) was not suitable for proper

Tab. 1. Influence of column temperature and FLD wavelengths on retention time and area of AFM_1 peak.

Column temperature [°C]	Average area [LU][a]	Elution time [min][a]
5	325.4 ±7.2*	6.3 ±0.0*
15	333.9 ±12.0*	5.4 ±0.1*
25	426.7 ±17.3	4.6 ±0.0*
40	358.0 ±14.8*	3.8 ±0.1*
Excitation wavelength [nm][b]		
260	285.0 ±16.5*	–
300	66.2 ±5.0*	–
320	237.5 ±1.2*	–
360	806.8 ±13.4	–
Emission wavelength [nm][c]		
400	12.2 ±1.8*	–
420	305.8 ±98.9*	–
440	816.3 ±0.7	–
460	700.8 ±18.2*	–

[a]Values are expressed as mean ± standard deviation, n = 3.

[b]Values are determined at emission wavelength of 440 nm.

[c]Values are determined at excitation wavelength of 360 nm.

*Values are significantly different at $p < 0.05$.

elution of AFM_1 as the peak was not separated and interference from other substances occurred. According to the Commission Regulation (EC) No. 401/2006, the recovery of AFM_1 in concentrations above 0.05 µg/kg should reach values between 70 and 110 %. This value was obtained only with one elution solvent, 1.25 mL of acetonitrile/methanol (3/2, v/v) and 1.25 mL of water (solvent F). Moreover, it was noticed that the volume of solvents also played an important role. In case of solvents C and D, the same composition as in F was used, however, different volumes caused a decrease in the recovery to 35.2 % and 60.2 %, respectively.

Khalil et al. (2013) tested various amounts of acetonitrile and methanol and suggested that all solvents showed recovery above 70 %, but the best recoveries were obtained from the mixture of acetonitrile/methanol (3/2, v/v). The AOAC official method 2000.08 of AFM_1 content determination in liquid milk uses elution with acetonitrile alone (4 mL) with the recovery of not less than 80 % (Dragacci et al., 2001). Jakšić et al. (2021) tested two types of IAC columns for the treatment of cheese matrix (AlfaStar™ M1R, Protealmmun IAC AFM_1), and the recovery of both types reached satisfactory efficiency using acetonitrile as the elution agent. Some studies also showed the application of other methods. For example, Aguilera-Luiz et al. (2011) evaluated the efficiency of SPE or QuEChERS methods, finding that the latter procedure was not suitable for AFM_1 extraction at very low levels, while SPE allowed the extraction without increasing the amount of impurities in the extract. The analytes were eluted with 5 mL of pure methanol. Wang et al. (2012) studied clean-up efficiency of OASIS HLB and IAC cartridges for AFM_1 elution from liquid milk and milk powder. Optimal results of the elution were observed with 5 mL of methanol and 5 mL of acetonitrile. Using OASIS HLB, the recovery reached 92.6 %. Compared to IAC, impurity peaks were obtained using OASIS HLB, however, the cleaning effect was sufficient for qualitative and quantitative determination of AFM_1.

In-house method validation

After optimisation of the HPLC and IAC conditions, the method was validated for linearity, accuracy, and precision using certified reference material in milk powder form provided by the European Commission Joint Research Centre. The results are summarised in Table 3. Validation criteria for AFM_1 content determination milk are set in the Commission Regulation (EC) No. 401/2006.

Linearity of the method was calculated from the calibration curve, the results were linear in all concentration ranges of 0.003 to 0.1 ng of AFM_1 in the calibration solution, R^2 was 0.99. From the calibration curve parameters (slope and intercept), LOD

Tab. 2. Influence of elution agent composition on AFM_1 recovery.

Agent	The percentage of AFM_1 recovery [%][a]
A	51.4 ±8.8*
B	–
C	35.2 ±2.1*
D	60.2 ±1.6*
E	11.4 ±5.6*
F	90.5 ±3.6

[a]Values are expressed as mean ± standard deviation, n = 2.

*Values are significant different at $p < 0.05$. Composition of agent: A — 1.25 mL of water/acetonitrile (7/3, v/v) + 1.25 mL of water; B — 3 mL of methanol/acetonitrile (7/3, v/v); C — 2 mL of methanol/acetonitrile (3/2, v/v) + 0.5 mL of water; D — 2 mL of methanol/acetonitrile (3/2, v/v) + 2 mL of water; E — 1.25 mL of water/methanol (17/3, v/v) + 1.25 mL of water; F — 1.25 mL of methanol/acetonitrile (3/2, v/v) + + 1.25 mL of water.

and LOQ values were calculated. Regarding the analysis of AFM_1 content in milk samples in the EU, it is important to reach LOQ of at least 0.05 µg/kg as it is the maximal acceptable level of AFM_1 in milk according to the Commission Regulation (EC) No. 1881/2006. In this work, both the LOD and the LOQ values were significantly below this limit, 0.002 and 0.007 µg/kg, respectively. These results are also similar to other studies, which used the HPLC-FLD method for AFM_1 content determination in milk. According to EFSA CONTAM Panel (2020), the reported LOQs for AFM_1 are typically between 0.0007 and 0.014 µg/kg. Similar conditions for the analysis of AFM_1 in raw milk were used by Patyal et al. (2020) and their method exhibited LOD and LOQ at 0.004 and 0.01 µg/kg, respectively. In addition, Bellio et al. (2016) showed the same LOD (0.002 µg/kg) in milk purchased in Northern Italy. Chen et al. (2005) reached lower LOD for AFM_1 in whole milk (0.0006 µg/kg) using liquid chromatography coupled with tandem mass spectrometry. Comparison of ELISA, HPLC-FLD and HPLC-MS/MS methods showed that all methods are suitable for the determination of AFM_1 in milk samples with almost the same LOQ ranging from 0.004 to 0.008 µg/kg (Kos et al., 2016).

Another important parameter that describes the suitability of the method for the given purpose is its accuracy, which was monitored in two steps as AFM_1 recovery. First, AFM_1 concentration obtained by the

Tab. 3. Validation parameters of proposed HPLC-FLD method for AFM_1 determination in milk.

Method linearity				
Slope	16925			
Intercept	0.3132			
Correlation coefficient (R^2)	0.99			
LOD [µg/kg]	0.002			
LOQ [µg/kg]	0.007			
Method recovery[a]				
Certified reference material	Reference value [µg/kg]	Determined value [µg/kg]	Recovery [%]	
ERM-BD 284	0.44 ±0.06	0.38 ±0.01	87.3 ±0.6	
Spiked milk sample [µg/kg]	Determined value [µg/kg]	Recovery [%]		
0	> LOQ	–		
0.10	0.10 ±0.01	103.7 ±9.0		
0.30	0.24 ±0.01	86.2 ±4.1		
0.60	0.55 ±0.01	91.7 ±1.4		
Method precision[b]				
Reference material	Repeatability	RSD [%]	Intermediate precision [RSD%]	Horrat
ERM-BD 284	Day 1	3.1	4.2	0.12
	Day 2	4.7		0.18
	Day 3	3.8		0.15

[a]Values are expressed as mean ± standard deviation, n = 3.

[b]Values are expressed as mean ± standard deviation, n = 4.

LOD — limit of detection; LOQ — limit of quantification.

analysis of certified reference material was compared with the manufacturer's data, it was shown to be 87.3 %. Subsequently, accuracy was evaluated in milk spiked with three various amounts of AFM_1 standard solution. In this case, the recoveries ranged from 86.2 to 103.7 %. It is notable that all values are acceptable according to the Commission Regulation (EC) No. 401/2006. Maggira et al. (2021) published that the HPLC-FLD method for the determination of AFM_1 content in raw milk exhibited recoveries between 90—109 %. Moreover, they found that HPLC showed slightly better accuracy for the target compound than ELISA. The mean recovery of the HPLC method for AFM_1 determination in spiked milk and cheese samples was found to be 90 % for milk and 76 % for cheese (Manetta et al., 2005). In Shuib et al. (2017), recovery values for milk and milk products ranged from 85.2 to 107.0 %. The proposed method was also compared with the screening method for AFM_1 content determination in raw milk used by the Slovak dairy company Rajo s.r.o. This company applied the MilkSafe™ instrument (Chr. Hansen Holding, Denmark), which is suitable for rapid determination of antibiotics and aflatoxins presence in milk. Four different spiking concentrations of AFM_1 were tested as requested by the provider (CLIMAX, s.r.o., Slovakia); the results are shown in Fig. 2. Both methods showed excellent recovery value ranging from 82.9 to 103.6 % for HPLC and 92.3 to 103.6 % for MilkSafe™.

Finally, precision of the proposed method was examined as the RSD of values obtained in one day (repeatability) and on three different days (intermediate precision). In addition, the HorRat parameter was calculated. The results are shown in Table 3. The repeatability RSD ranged from 3.1 to 4.7 %, while the intermediate precision RSD was 4.2 %. HorRat ranged from 0.12 to 0.18. According to official requirements, reproducibility of a method is approved when HorRat is below or equal to 2 (Horwitz and Albert, 2006; Ribeiro and Brandäo, 2017). Very low HorRat values (0.31—0.42) were also confirmed by Dragacci et al. (2001) in a collaborative study for the determination of AFM_1 in liquid milk. Relatively the same values of repeatability were reported by Maggira et al. (2021) (RSD < 3.6 %) in the determination of AFM_1 in raw milk using the HPLC-FLD method. In contrast, higher values were reported by Kos et al. (2016), ranging from 9.5 to 11.3 %. Manetta et al. (2005) published a HPLC method with postcolumn derivatisation and FLD detection for AFM_1 determination in milk and cheese and found the RSD precision ranging from 1.7 to 2.6 % for milk and 3.5 to 6.5 % for cheese.

Determination of AFM_1 content in milk and dairy products

Milk and dairy products are predominant subject of AFM_1 contamination. According to the worldwide systematic review and meta-analysis (Mollayusefian et al., 2021), total AFM_1 content in raw and pasteurised milk was found to be 0.06 µg/kg and 0.09 µg/kg, respectively. Additionally, the highest content was found in raw buffalo milk and pasteurised cow milk, indicating that the pasteurisation process cannot reduce the concentration of aflatoxin to an acceptable level (Mollayusefian et al., 2021). According to recent risk assessment of aflatoxins in food (EFSA CONTAM Panel, 2020), the highest estimated mean lower bound (LB) and upper bound (UB) exposure to AFM_1 in infants was up to 0.001 ng/kg and 0.002 µg/kg bw per day, respectively. The main contributors to overall AFM_1 mean exposure are liquid milk and fermented milk products throughout all age groups. In Hriciková et al. (2023), positive findings for AFM_1 in milk in Slovakia were presented (32 samples were tested

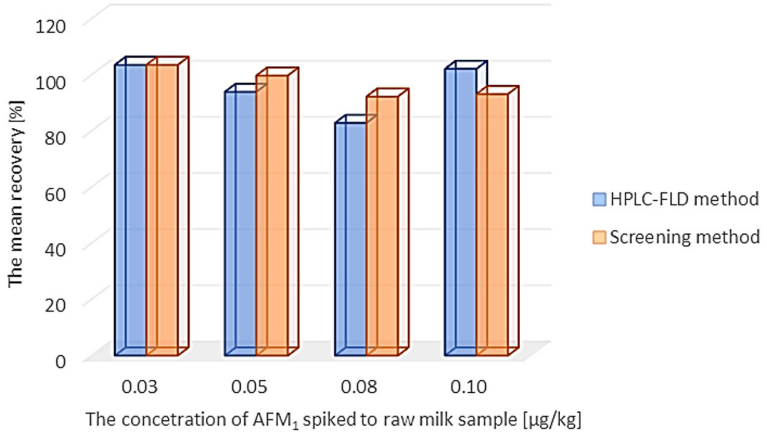

Fig. 2. Comparison of AFM_1 recovery using our proposed HPLC-FLD method and MilkSafe™ AFM_1 screening method applied in RAJO s.r.o.

Tab. 4. Determination of AFM_1 content in milk samples collected in Slovakia.

Sample	AFM_1 content [µg/kg]	Sample	AFM_1 content [µg/kg]
Raw milk (1)	>LOQ	UHT pasteurized skimmed milk (fat content 0.5 %)	>LOQ
Raw milk (2)	>LOQ	UHT pasteurized milk (fat content 1.5 %)	>LOQ
Raw milk (3)	>LOQ	UHT pasteurized milk (fat content 3.5 %)	>LOQ
Raw milk (4)	>LOQ	Curd cheese (fat content 0.5 %)	>LOQ
Raw milk (5)	>LOQ	Greek yogurt	>LOQ
Raw milk (6)	>LOQ	Mini creamers	>LOQ
Raw milk (7)	>LOQ	Cooking cream (fat content 33 %)	>LOQ
Raw milk (8)	>LOQ	Milk dessert "Pribináček"	>LOQ
Raw milk (9)	>LOQ	Milk drink "Brejky"	>LOQ
Raw milk (10)	>LOQ	Milk drink "Actimel"	>LOQ
Raw milk (11)	>LOQ		
Raw milk (12)	>LOQ		
Raw milk (13)	>LOQ		
Raw milk (14)	>LOQ		
Raw milk (15)	>LOQ		

n = 2; LOQ = 0.007 µg/kg.

positive and 8 negative). In this study, 15 raw milk samples collected in Slovakia were tested for AFM_1 concentration. The AFM_1 content was found to be below LOQ in all cases (Table 4). Furthermore, other ten dairy products were purchased randomly in Slovak markets and the AFM_1 content was also found below the LOQ. In a one-year survey (September 2021 to November 2022) on AFM_1 detection in raw and drinking milk in Hungary (Buzás et al., 2023), 11.9 % of raw milk samples and 0.5 % of drinking milk samples contained AFM_1 above the EU limit (0.05 µg/kg). Furthermore, the highest mean AFM_1 level was measured during fall and lower in spring and summer. According to Bellio et al. (2016), only 0.5 % of total milk samples collected in northern Italy during a three-year period (2012—2014) were found to be non-compliant with the EU regulation. Analysis of 109 samples collected from the Greek market (52 samples of infant/toddler milk and formulas, 32 pasteurised milk, and 25 samples of feta cheese) revealed that AFM_1 was present in 60 % of infant/toddler formulas, 69 % of pasteurised milk samples, and 28 % of cheese samples; however, no sample exceeded the maximum tolerable limit (Malissiova et al., 2022). AFM_1 levels in Europe are increasing due to climate changes; however, generally they are still lower than in other regions, such as African or South Asian countries. The lower content of AFM_1 in European milk and dairy products could also be attributed to modern processing technology, especially strict regulations, hygiene, adoption of food safety systems, continuous monitoring of aflatoxins, and advanced analytical techniques (Mollayusefian et al., 2021).

Conclusion

In this study, the HPLC-FLD method was developed, optimised, and validated for the determination of AFM_1 content in milk. First, HPLC conditions were tested for the analytical standard AFM_1 solution, and the best AFM_1 chromatographic separation was obtained at the following conditions: 1. mobile phase composed of water/acetonitrile mixture (80/20, v/v); 2. column with stationary phase C_{18}; 3. column temperature of 25 °C; and 4. FLD wavelengths at excitation at 360 nm and emission at 440 nm. IAC extraction of AFM_1 from the spiked milk sample was optimised for the elution agent. The best recovery was obtained using 1.25 mL of methanol/acetonitrile (3/2, v/v) + 1.25 mL of water. The method was validated according to the Commission Regulation (EC) No. 401/2006 regulations, and it was observed that all parameters meet the criteria set by the Regu-

lation including LOD and LOQ which were 0.002 and 0.007 µg/kg, respectively. Precision of the proposed method was confirmed with the data declared by the reference material provider, and significant similarity was found with the method used in daily screening of AFM_1 concentration at the dairy factory. Finally, AFM_1 was determined in 25 milk and dairy products collected in Slovakia; however, the concentration in all samples was below LOQ.

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